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MOLECULAR CHARACTERIZATION OF ELITE LINES OF PAPAYA (*Carica papaya* L.) VIA SSR MARKERS

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Abstract: This study aimed to analyze the genetic variability and to estimate diversity parameters of 23 elite papaya lines based on microsatellite molecular markers. The plant material was composed of elite lines, 18 from the 'Formosa' and five from the 'Solo' group. The expected heterozygosity (H_E), observed heterozygosity (H_O), and the coefficient of inbreeding (f) for each genotype were estimated. The weighted index obtained the dissimilarity matrix, and the estimated genetic distance was presented graphically through the cluster analysis by the Ward method, as well as by the principal coordinates analysis (PCoA). The genotypes were also analyzed for genetic structure, using the Bayesian clustering method. Genetic variability was observed among the analyzed genotypes, mainly among the lines from the Formosa group. As for H_O, four elite lines from the 'Solo' group had values equal to zero. However, the elite lines from the 'Formosa' group showed higher segregation in the loci with values ranging from 0.05 to 0.14. About f, from the five lines in the 'Solo' group, four exhibited maximum fixation indexes for the analyzed loci, with the variation observed from 0.90 to 1.0, while the lines from the 'Formosa' group had a variation from 0.61 to 1.00. These results indicate the need for greater care in the process of obtaining inbreeding seeds to avoid pollen contamination, as well as the need to advance generations of self-fertilization with the lines from the 'Formosa' group to increase the level of inbreeding and ensure greater stability to hybrids that are developed from them.

Keywords: *Carica papaya* L., germplasm bank, SSR markers.

Introduction

The *Caricaceae* family is divided into six genera, the *Carica* genus being represented only by the species *Carica papaya* (Badillo, 2000). Papaya cultivars are widely planted in tropical and subtropical regions, is an essential part of the economy for many countries. Brazil appears in this economic scenario as the second-largest producer and third-largest exporter of papaya fruit (FAOSTAT, 2018).

Despite the great importance of papaya for agriculture and the various cultivars developed, a small number of these are still used in commercial crops, limiting the expansion of the crop and contributing to it is vulnerability to diseases (Oliveira et al., 2010). Therefore, a continuous effort to develop new, more attractive cultivars with excellent production, fruit quality, and resistance to diseases is essential. An alternative for the development of cultivars that combine all these characteristics is the exploration of variability present in the germplasm banks.

The genotypes belonging to the papaya germplasm collection maintained by the Universidade Estadual do Norte Fluminense (UENF) in partnership with the company Caliman Agrícola SA have been widely used in hybridization programs, allowing to create new allelic combinations for desirable characteristics, such as fruit yield, disease resistance, lower deformation, and higher fruit quality (Marin et al., 2006; Ide et al., 2009; Cardoso et al., 2017). Also, these genetic materials available in the UENF/Caliman germplasm bank have been characterized in terms of morpho-agronomic attributes (Barbosa et al., 2011; Quintal et al., 2012) and disease resistance (Vivas et al., 2010; Vivas et al., 2015). These efforts have greatly contributed to the progress of the genetic improvement of papaya in Brazil over the past two decades (Pereira et al., 2019a), which can be confirmed by the number of new cultivars developed and registered recently (Pereira et al., 2019a,b,c).

However, there is a lack of comprehensive molecular characterization of the accessions of this bank, especially of the elite lines that make up the working collection of this germplasm bank aim to understand better and make effective use of the available genetic variability.

Molecular marker analyzes provide an important alternative approach to characterize genetic diversity, population structure, and genetic relationships among elite materials within a given germplasm collection (Wu et al., 2016). Among the different classes of molecular markers available, microsatellites, or SSR (Simple Sequence Repeat) are preferred for applications in genetic studies in plant breeding due to their codominant nature, a high degree of polymerphism, and high reproducibility. Collevatti et al. (1999) point out that the codominant nature makes polymorphisms based on microsatellite regions more robust for estimating genetic diversity parameters in populations or groups of genotypes.

In papaya crop, this class of molecular marker has been used in phylogeographic and genetic structuring studies (Hasibuzzamanet al., 2020), analysis of genetic diversity and molecular characterization (Oliveira et al., 2010; Ramos et al., 2014; Pirovani et al., 2018), construction of a genetic map (Chen et al., 2007), identification of QTL (Blas et al., 2012), selection assisted by markers for the development of pure lines (Oliveira et al., 2012), among others.

Given the above, this study aimed to characterize the genetic variability of 23 elite papaya lines from the UENF/Caliman Germplasm Bank based on SSR molecular markers.

Material and methods

Genetic material

Twenty-three elite papaya lines from the Germplasm Bank in vivo, belonging to the Universidade Estadual Norte Fluminense (UENF), in partnership with the Caliman Agrícola S.A. Company, located in Linhares, ES, were evaluated. Among the 23 elite papaya lines, 18 lines are from the 'Formosa' group, and five lines are from the 'Solo' group.

The JS-12 genotypes and Sunrise Solo 72/12 (SS-72/12) are widely used in the UENF breeding program as narrow genetic-base testers (Barros et al., 2017). The genotypes Waimanalo, Sekati, JS-12, Maradol, and São Mateus, have already been identified as possible carriers of alleles that tend to contribute to the reduction of phoma leaf spot in papaya hybrids (Vivas et al.,

2010). The elite lines 37/4, 39/6, 36/1, 41/2, 36/7, 42/1, 42/2, 36/2, 40/2, 41/7, 41/5, 36/5, 41/3 were developed based on the selection of superior genotypes in a segregating population obtained from the self-fertilization of the hybrid Tainung 01, which have superior agronomic traits, such as fruit quality and yield and resistance to black spot, phoma leaf spot and powdery mildew (Ide et al., 2009; Vivas et al., 2012a,b; Vivas et al., 2016).

DNA extraction

DNA extraction from young leaves was carried out following the CTAB method (Doyle and Doyle, 1990), with modifications suggested by Daher et al. (2002). Then, the samples were subjected to quantification on 0.8% agarose gel and diluted to a 10ng/µL working concentration, using the High DNA Mass Ladder marker (Invitrogen, USA). The gel was stained in a GelRed^{TM}/blue juice solution (1:1) and the images captured by the MiniBis Pro photo documentation system (Bio-Imaging Systems).

Molecular analysis via SSR

For the molecular characterization, 22 SSR primers (Table 1) developed by Eustice et al. (2008), were used, with genomic location determined by genetic mapping (Chen et al., 2007). SSR markers covering all papaya chromosomes were selected.

Table 1. Characteristics of the 22 pairs of microsatellite primers used in the analysis of the 23 genotypes of *C. papaya* from the UENF/Caliman Germplasm Bank.

SSR Locus*	Forward primer	Reverse primer	LG	AT (°C)
P8K39CC	CGTCAAGTTGTTGGGTTGGTC	TGACATCTCCGAAGAGCTGAGA	1	60
CPM1554C2	TTGACGAATTCAAACCCATGC	CACCTCGTGGCATCAAACAA	1	63
P6K1117CC	GAACAGGAGGGTTGCTGGTG	CATTCCAGCTACTCAGGCGG	1	63
P3K6912CC	TGAAGCCTCAGTGAATCCAAA	CCCATGGGAACACATCTATTG	2	60
P3K1850CC	TTTCTCCCACATGACCCACA	GGGGGTGCTTTGGAATCTTT	2	60
CPM1621CC	ATGGTAACCCAGCGTGAGGA	ACGCCAAATATTCCCAACCC	3	60
P3K3968A5	TGCGATCGAAAGGTTCTTGAG	TGGAAATGGCTGGTTTTGTCA	4	60
P3K1883CC	GGTTGAAACGTTAACGGCG	GGGTAGAGAGTCAATGGATTTTGC	4	60
P6K268CC	ATGCTTGAGGGACAACCCTT	AAAAGTATGCAGTCCCCAGTTG	4	63
P6K128CC	GCCGGCTCAGGAGGTTAAGA	CAATGACCAAACGCCACACA	4	63
ctg-365A5	TTCTTTCACCCGCTCCTCTG	AAACAACTCGGCCCAACTGA	5	60
P3K23CC	CGTAAAGGTCGGGTCAGCTA	TGGTCTTCACATGAAATGAGCTT	6	60
P3K6467CC	GGGGGACCATCTTCCTCTCT	CTTGGGTTGAGATGCTCTCCT	6	60
ctg-64CC	CATCCCGAACTACTCACATAAACA	TGCTTGCTGCTCACTTATGG	7	60
ctg-41S5	TTCATCGTCTCGCTGAAATTGA	CCAGTAGGCTCTCCAAATGGG	7	60
CPM727CC	ACTTTTGTGGTGCAAAAGGC	CCAATTGTTAACTGTGGAAGG	8	63
ctg-138A0	CCCACTGAAAGCTTCCTGTAAA	CCACACAAAGAAGACGAAACAAA	8	60
CPM766CC	TACCAAGTTCAGCAAGCGGT	ATACTTTCTCCCCCTTCGGA	8	60
P3K1497CC	TGACGGTGAAAATTGCAACA	AAAAGGGGAGTCCAAATTGGTT	9	60
P3K7484C0	CGGTAGCGACTCATCGGACT	TTGACTCGCGAGGAAAGGAG	10	60
P3K149C0	TGGTGGATGTTGATGCATGTT	TCTGGTGGTCATGATGGTGG	11	63
P3K3510C0	GTAGCCGAACGCACAACACA	CGTGTAAAAGAAGCGGTAGATCG	12	63

LG: Linkage group; AT: Annealing temperature (°C)

The amplification reactions for the 22 SSR primers were performed with a final volume of 15µL, following the same procedure described by Ramos et al. (2011a,b), with variation in annealing temperature between 60ºC and 63ºC, according to each primer (Table 1). The amplification products were separated on a Metaphor 4% agarose gel, stained with the GelRed^{TM}/blue juice solution (1:1), and visualized under Ultra Violet light through the Mini Document Pro photo documentation system (Bio-Imaging Systems).

Data analysis

The data obtained from the amplification of SSR primers were converted into a numerical code for each allele per locus. The numerical matrix was developed by assigning values from 1 to the maximum number of alleles in the locus, as described below: for a locus that has three alleles, we have the representation 11, 22 and 33 for the homozygous forms (A1A1, A2A2, and A3A3) and 12, 13 and 23 for heterozygotes (A1A2, A1A3 and A2A3) as described by Ramos

et al. (2011a,b). From the numerical matrix, the genetic dissimilarity matrix was generated based on the Weighted index, with the aid of the GENES software (Cruz, 2013). The cluster analysis was performed using Ward's Hierarchical Agglomerative Clustering method, and the definition of the optimal number of groups was performed by the Cluster package of the R software (R Core Team, 2018).

For the graphic dispersion in the two-dimensional plane based on the Principal Coordinates Analysis (PCoA) method, the GenAlex 6 software was used (Peakall and Smouse, 2006). The observed heterozygosity (H_O), expected heterozygosity (H_E), and the coefficient of inbreeding (f) were also estimated with the aid of the PowerMarker software version 3.25 (Liu and Muse, 2005).

The Bayesian grouping method was used through the Structure 2.3.1 software (Pritchard et al., 2000), to analyze the genetic structure of the population, where the number of groups (K) was determined using the ΔK method (Evanno et al., 2005). The estimate of the optimal K was obtained using the admixture model and independent allele frequencies with ten runs for each K value, which ranged from 1 to 5, using "Burn-In Period = 1,000", followed by an extension of 50,000 repetitions during the analysis.

Results

A total of 56 alleles were generated, with the number of alleles per locus ranging from two to four, with an average of 2.55 alleles per locus.

All 23 genotypes studied showed observed heterozygosity (H_O) below the expected heterozygosis (H_E). Some elite lines in the 'Formosa' group showed values ranging from 0.05 to 0.14, with only one elite line in the 'Solo' group showing a value of 0.05 (Table 2). From the coefficients of inbreeding (f) found in five elite lines from the 'Solo' group, four lines exhibited maximum fixation indexes for the analyzed loci, at the same time, the elite lines from the 'Formosa' group showed a variation from 0.61 to 1.00. However, the overall mean of the genotypes was 0.93, considered high.

By grouping analysis of the 23 genotypes (Figure 1), three distinct groups were formed. Group I formed by all genotypes of the 'Solo' group; group II formed by the Sekati and JS-12-4 genotypes and the new lines 37/4, 39/6, 42/1, 41/2, 41/7, 41/3, 40/2, 42/2, 41/5; group III was formed by the lines developed from the self-fertilization of the hybrid Tainung 01 (36/1, 36/7, 36/2, 36/5) and by the genotypes Waimanalo, Maradol, and JS-12-N.

Table 2. Descriptive analysis of 23 *C. papaya* genotypes from the UENF/Caliman Germplasm Bank, values of observed heterozygosity (H_O), expected heterozygosis (H_E), and coefficient of inbreeding (f).

Genotypes	Group	H_O	H_E	ƒ
Maradol	'Formosa'	0.00	0.66	1.00
Sekati	'Formosa'	0.00	0.60	1.00
JS-12 - N	'Formosa'	0.05	0.65	0.93
JS-12 - 4	'Formosa'	0.19	0.59	0.61
SS-72/12	'Solo'	0.00	0.46	1.00
Golden	'Solo'	0.00	0.44	1.00
19' genitor TN	'Solo'	0.05	0.49	0.90
São Mateus	'Solo'	0.00	0.55	1.00
Waimanalo	'Formosa'	0.00	0.68	1.00
SS 783	'Solo'	0.00	0.59	1.00
37/4	'Formosa'	0.00	0.60	1.00
39/6	'Formosa'	0.00	0.66	1.00
36/1	'Formosa'	0.00	0.51	1.00
41/2	'Formosa'	0.14	0.65	0.78
36/7	'Formosa'	0.05	0.60	0.92
42/1	'Formosa'	0.00	0.66	1.00
42/2	'Formosa'	0.09	0.65	0.86
36/2	'Formosa'	0.00	0.59	1.00
40/2	'Formosa'	0.05	0.66	0.93
41/7	'Formosa'	0.10	0.64	0.85
41/5	'Formosa'	0.14	0.64	0.79
36/5	'Formosa'	0.10	0.61	0.85
41/3	'Formosa'	0.00	0.65	1.00
Mean		**0.04**	**0.60**	**0.93**

The genotypes were also analyzed from the graphic dispersion based on the Principal Coordinates Analysis (PCoA), as shown in Figure 2. The first two coordinates together explained 42.03% of the total variation of the data, with 22.21% of this variation explained by coordinate 1 and 19.82% explained by coordinate 2. The quadrants I and II brought together only genotypes of the 'Formosa' group, grouping most of the genotypes analyzed, while in quadrants III and IV, all genotypes of the 'Solo' group and seven representatives of the 'Formosa' group are found.

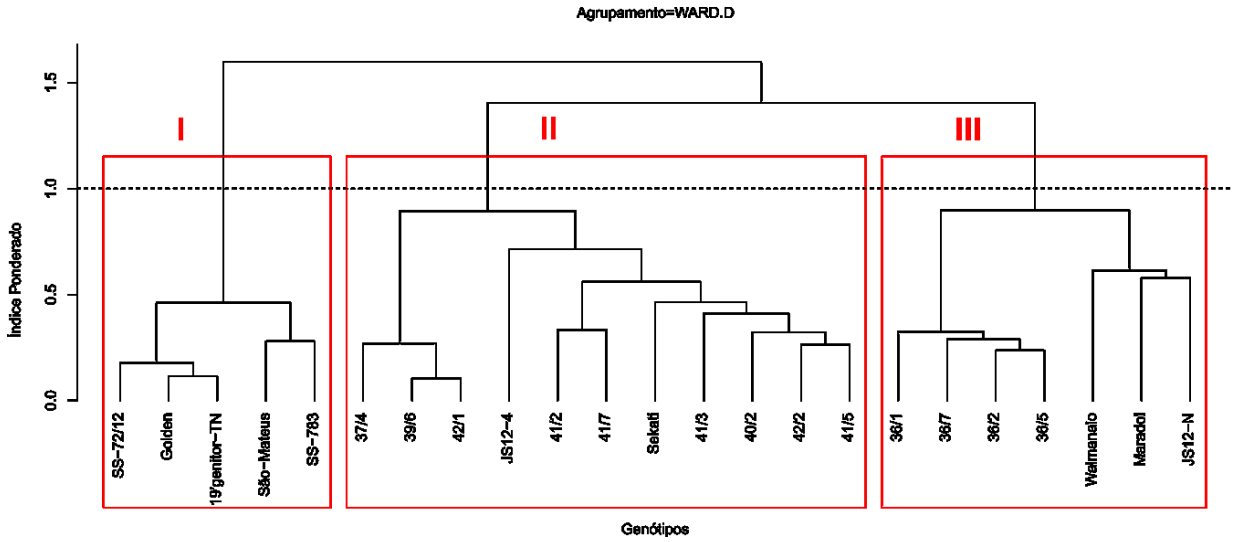

Figure 1. Dendrogram of genetic dissimilarity among 23 genotypes of *C. papaya*, from the UENF/Caliman Germplasm Bank, obtained by the Ward hierarchical method using the weighted index dissimilarity matrix (Cophenetic Correlation Coefficient = 0.81).

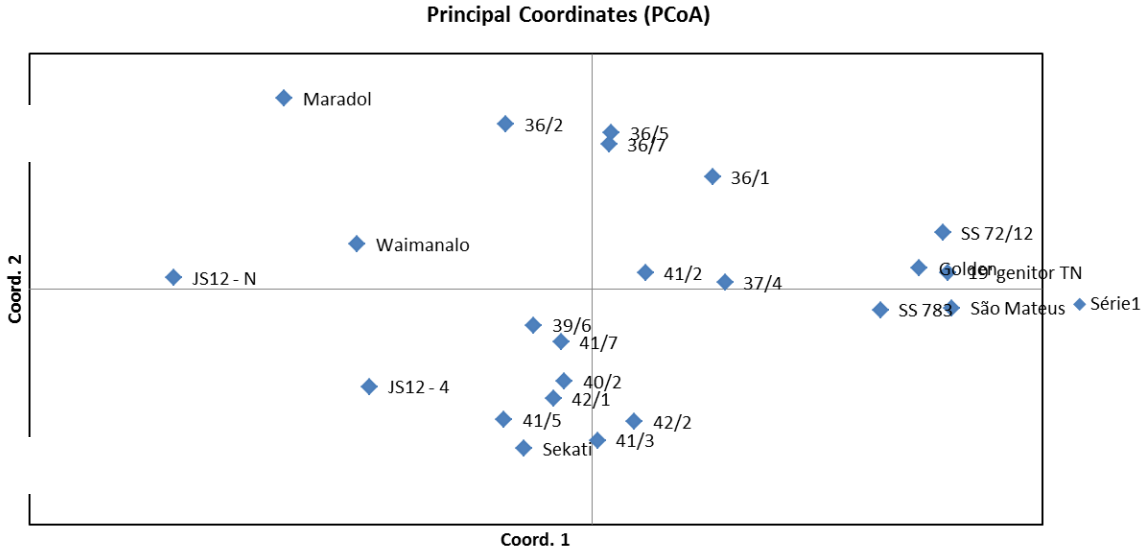

Figure 2. Principal Coordinates Analysis considering 23 genotypes of *C. papaya*, from the UENF/Caliman Germplasm Bank, based on the distance matrix obtained by analyzing the 22 microsatellite markers.

Despite the genotypes of the 'Solo' group being present in two quadrants, the dispersion of these genotypes is restricted to a small area of the graph, showing less genetic variability concerning the genotypes from the 'Formosa' group.

The optimal number of groups (K) estimated via delta K analysis was equal to three, as shown in Figure 3. Based on the value of delta K, the 23 genotypes analyzed were distributed in three subpopulations, one of which was composed of the genotypes of the 'Solo' group and two of the genotypes of the 'Formosa' group (Figure 4). This result is similar to the grouping obtained by Ward's hierarchical method, with reservations for genotypes 41/2 and JS-12/N.

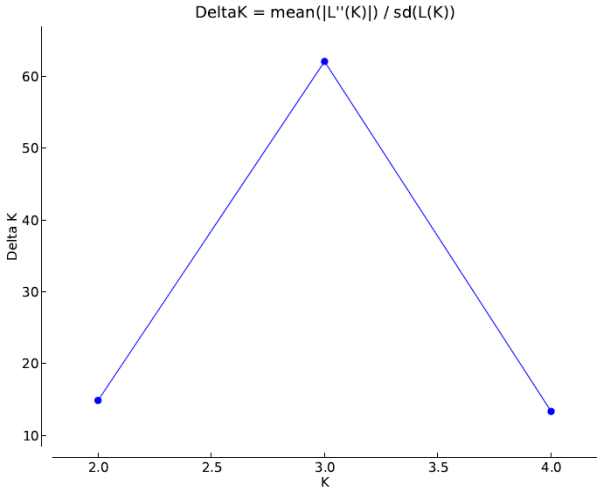

Figure 3. Number of groups by the ΔK method, based on the rate of change in the logarithmic probability of data by analyzing the 22 microsatellite markers.

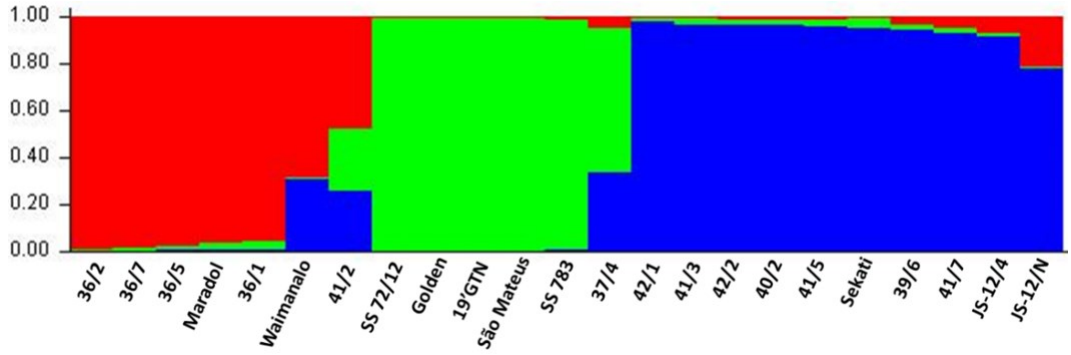

Figure 4. Analysis of the genetic structure of the 23 genotypes of *C. papaya* from the UENF/Caliman Germplasm Bank (K = 3).

Discussion

The highest proportion of genetic variability and the lowest level of inbreeding observed in the eighteen lines from the 'Formosa' group concerning the five lines from the 'Solo' group (Table 2) may be related to the reproductive biology of this group, which has a high rate of allogamy, that is, a higher rate of cross-fertilization, as described by Damasceno Junior et al. (2009). According to these authors, the preferred reproduction mode of the hermaphrodite papaya is optional autogamous with cleistogamy, that is, the papaya can exhibit both the mode of reproduction by autogamy as well as that of allogamy. This optional allogamy in the 'Formosa' genotypes requires greater care in the process of obtaining inbreeding seeds to avoid contamination by pollen.

On the other hand, from the five lines of the 'Solo' group analyzed, four exhibited the observed heterozygosity (H_o) values equal to zero, probably due to the representatives of the 'Solo' group preferentially adopting the reproductive mode by autogamy and presenting low cross-fertilization rate (Damasceno Junior et al., 2009). According to Ingvarsson (2002), autogamy has effects on genetic variation between and within populations, increasing inbreeding and contributing to the low genetic variation among the genotypes of a population (Charlesworth, 2003), this effect was observed among individuals from the 'Solo' group, whose maximum inbreeding value was observed in most lines.

Regarding the grouping of genotypes (Figure 1), groups were formed according to the heterotic group, that is, group I was constituted by the five genotypes belonging to the 'Solo' group, and groups II and III were composed of genotypes belonging to the 'Formosa' group. This organization of germplasm in heterotic groups is favorable for the systematic and efficient exploration of heterosis, making these genotypes promising for desirable crosses and obtaining new commercial hybrids that meet the demands of the national and international market (Silva et al., 2017).

The grouping of genotypes resulting from the analysis of genetic structuring was similar to the other grouping methods (Ward and PCoA). It corroborated the high similarity between the genotypes from the 'Solo' group, whose loci analyzed did not show similarity with the lines from the 'Formosa group'. This separation of heterotic groups by genetic structuring analysis is explained by the distinctions between genotypes based on similar variations that occur in the genome, based on the best adjustment for the variation patterns of each individual through Bayesian analysis of the data (Porras-Hurtado et al., 2013).

The genotypes evaluated in this study belonging to the heterotic groups 'Formosa' and 'Solo', are potential materials to be used in future hybridization programs. However, the need to advance generations of self-fertilization with the lines from the 'Formosa' group is evident due to greater segregation and less fixation for the analyzed loci. This is because, in the process of developing hybrids, it is essential to use pure lines to avoid segregation in the F_1 generation.

The relevance of basic procedures such as the adequate choice of parent materials from germplasm for the optimal design of crosses, as well as the selection of the best progenies for new tests in segregating generations, stands out for

conducting a plant breeding program. Such procedures determine the potential of selection and are a crucial part of conventional breeding (He et al., 2017). According to Oliveira et al. (2010), hybrids resulting from the lines of the group 'Formosa' and 'Solo' have commercial importance for both the Brazilian and international markets. Therefore, knowledge of the level of diversity of the strains analyzed is an essential factor for the effective and conscious use of accesses in the Papaya Germplasm Bank of UENF/Caliman, in the production of high-performance hybrids in future breeding programs.

Conclusion

From five lines from the 'Solo' group, four showed Ho values equal to zero and maximum fixation indexes for the analyzed loci. On the other hand, the lines from the 'Formosa' group showed greater segregation in the loci and less fixation for the analyzed loci. These results point to the need to advance generations of self-fertilization with lines from the 'Formosa' group to increase the level of inbreeding and guarantee stability and uniformity of the hybrids developed from the crossing of such lines.

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