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Curauá genetic diversity in germplasm banks and natural populations

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***Ananas comosus* var. erectifolius (L. B. Sm.) Coppens & F. Leal, popularly known as curauá, is a bromeliaceae, found in the Amazonian flora. It is of great commercial interest, mostly for the automobile industry. Despite the curauá's potential, little has been done to conserve its germplasm. For this, it's necessary to know its genetic variability. An efficient way of knowing it is to use molecular markers because they are polymorphic and not influenced by the environment. Thus, this work is aimed to evaluate the genetic diversity in curauá's access of different germplasm banks and its natural populations, using simple sequence repeat (SSR) and random amplified polymorphic DNA (RAPD) molecular markers. The similarity between the cultivars was calculated based on the Dice coefficient. From the similarity analysis, the cultivars grouping dendrogram was constructed using the unweighted pair group mean average method (UPGMA). High genetic similarity was observed between the individuals of each group and most of the variability found was between the groups. The low variability found within the groups is due to the way in which *A. comosus* var. *erectifolius* has been multiplied, through asexual reproduction in plantation areas. The data suggest that the conservation strategies of this species should focus on the largest possible number of collections in different geographic regions to increase the variability of the banks.**

Key words: *Ananas comosus* var. *erectifolius,* conservation, amazon plants, biodegradable products, genetic similarity.

INTRODUCTION

Ananas comosus var. erectifolius (L. B. Sm.) Coppens & F. Leal, known as curauá, is a fibrous plant belonging to the Bromeliaceae family occurring in the Amazon Forest (Ledo, 1967; Morais et al., 2016). The high-quality curauá fiber is in use in the automobile industry, where its value has already been recognized.

The value of this fiber in the fabrication of auto parts has made it commercially viable for companies to invest

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in larger-scale production. Moreover, curauá fiber has great potential in the production of cellulose nanofibers because of its high cellulose content. These nanofibers can be produced using different methods, given varying structures which includes nanowhiskers, nanofibers and/or nanofibrillated cellulose (Souza et al., 2015).

In recent years, genetic erosion of the genus *Ananas* has become increasingly severe, due to the cultivation of only a few varieties and the anthropization of their areas of native occurrence (Silva et al., 2016). This requires intensified efforts to preserve this germplasm, mainly to *A. comosus* var. *erectifolius*, considering that in the literature there is little information on its genetic variability and because the specie is subject to genetic erosion. This is due to the reduction of its cultivation by the aboriginal, owing to the replacement of its fiber by synthetic fibers in the utensils manufactured.

There are currently curauá germplasm banks located in several institutions, which vary in size and level of characterization. The maintenance of these collections can be optimized by better understanding the genetic variability contained in them, for example, duplicates can be identified and eliminated and collection enrichment decisions can be better targeted. According to the curauá potential economic and its risk of genetic erosion, this work aims to evaluate the genetic diversity between the accessions of curauá (*A. comosus* var. *erectifolius*) kept in the germplasm bank of Faculdade de Ciências Agronômicas de Botucatu (Unesp), Embrapa Amazônia Oriental and two cultivated regions (Arapiuns and Tapajós river).

MATERIALS AND METHODS

Plant material

For the plant material, 90 accessions of Faculdade de Ciências Agronômicas de Botucatu (Unesp) germplasm bank (60 of purple curauá - FCA_{PC} and 30 of white curauá - FCA_{WC}) and 26 accessions of the Embrapa Amazônia Oriental active germplasm bank, were evaluated; of which there are six white curauá from Bragança (B_{WC}), four white curauá from Ponta de Pedra (PP_{WC}), four purple curauá from Ponta de Pedra (PP_{PC}), six white curauá from Marilda (M_{WC}) and six purple curauá micropropagated of Embrapa (E_{PC}). We also evaluated 30 purple curauá individuals from Arapiuns river region (A_{PC}), and 15 purple curauá individuals of the Tapajós river region (T_{PC}).

Obtaining and analyzing SRH data

Genomic DNA was extracted from young leaf tissue. The amount and quality of DNA were estimated by comparisons with lambda DNA of known concentrations on 1% agarose gel, stained with ethidium bromide. The gels were visualized under UV light. Genomic libraries were developed from the protocol of enrichment for microsatellites developed by Billotte et al. (1999).

Plasmid extraction was performed by transferring 10 µL of a solution containing positive clones from ELISA plates to a Deep plate (Axygen) containing 1 mL of Circle Grow medium (4%) 0+30 µL of ampicillin (100 µg/mL) per well, being incubated for 22 h at 37°C in a stirrer at 250 rpm. The adhesive was changed and the material centrifuged for 6 min at 3000 rpm. The supernatant was discarded and 240 µL of GTE (Glucose-Tris-EDTA) was added to each sample. Plates were sealed and suspended in the vortex for 2 min and centrifuged for 6 min at 4000 rpm. The supernatant was again discarded and 60 µL of the material was transferred to 96-well PCR plates containing 5 µL of RNase (10 mg/mL). Additionally, 60 µL of 0.2 M NaOH – 1% SDS was added to each sample and then the plates were sealed and mixed 10 times by inversion. After incubation for 10 min at ambient temperature, the material was centrifuged at 1000 rpm.

Subsequently, 60 µL of 3M KOC was added to each clone and the plate was sealed and mixed 10 times by inversion. The adhesive was removed and the plate incubated in an oven at 90°C for 30 min. After cooling the plate on ice for 10 min, the material was centrifuged for 4 min at 4000 rpm at 20°C. The material was transferred to a filter plate (Axygen) and centrifuged for 6 min at 3000 rpm. Thereafter, 100 µL of isopropanol was added and the solution was mixed by inversion. The supernatant was discarded and 200 µL of 70% ice-cold ethanol was added. The solution was centrifuged for 5 min at 4000 rpm at 20°C, the supernatant was discarded, and the plate was inverted to dry for 60 min at ambient temperature. The material was resuspended in MilliQ water. Plasmid DNAs were confirmed by visualization on 1% agarose gel stained with ethidium bromide.

Sequencing reactions and conditions were described in the Big Dye Terminator Kit (Applied Biosystems, CA, USA), using the primer SP6 and an ABI Prism 3700 sequencer (Applied Biosystems) located at the Sylvio Moreira Center for Citrus Production in the Agronomic Institute of Campinas, Cordeirópolis, São Paulo State, Brazil. The obtained sequences were submitted to quality analysis and elimination of vector and adapter sequences using the software suite, Phred, Phrap, Consed, and Cross_Match (Laboratory of Phil Green; Genome Sciences Department, University of Washington; available on http://www.phrap.org/index.html).

Forward and reverse primers were designed using the software Primer 3, respecting the following criteria: Tm (annealing temperature) between 55 and 63 °C, maximum of 60% GC, fragment sizes between 150 and 350 bp, and at least 18 and at most 25 nucleotides without repetitive sequences. Synthesized primers were identified by the first letter of the genre in capital letters, followed by the first two letters of the species in lowercase letters and the number of the designed sequence.

The 19 pairs of synthesized primers were assessed using five purple curauá (*Ananas erectifolius*) individuals belonging to the UNESP Germplasm Bank. Amplification reactions were performed with 15 ng genomic DNA, 1 U Taq DNA polymerase (LGC Biotechnology), 1X PCR buffer (200 mM Tris pH 8.4, 500 mM KCl), 1.5 to 2.5 mM $MgCl_2$, 0.6 to 0.8 µL of Mix dNTPs (containing 2.5 mM of each dNTP), and 0.3 µM of each primer in a final volume of 10 µL.

Amplifications were performed in a PTC 100 thermocycler (MJ Research, Inc., Watertown, MA, USA). The programs followed the following conditions: 95°C for 5 min, 35 cycles of 94°C for 45 s, 60°C for 1 min, 72°C for 1 min and a final extension for 10 min at 72°C. Products received 5-µL loading buffer (95% formamide). After denaturation at 95°C for 10 min, they were immediately placed on ice and loaded with denaturing acrylamide gel (5%) preheated for 1 h. The process of separating the fragments by electrophoresis was performed with 1X TBE buffer for 2 h at 60 W. Fragments were visualized after staining with silver nitrate, which was performed according to the protocol described by Creste et al. (2001).

The pairs of primers with nonspecific band amplification were again tested at annealing temperatures between 61 to 65°C and at different $MgCl_2$ concentrations. In the absence of amplification of some genotypes, replications were performed to ensure no flaws in the reaction.

The transfer level of microsatellite loci was assessed by using 11 pairs of primers developed for *Ananas lucidus* in samples of *Ananas comosus* of the varieties Roxo-de-tefé (4 individuals) and Gomo-de-mel (4 individuals) from the germplasm bank of pineapples from the Agronomic Institute of Campinas (IAC/SP). Amplification reactions were performed according to those previously described for characterizing and detecting the polymorphism of *A. lucidus*.

In order to analyze the genetic diversity, the following parameters were estimated: average number of alleles/loci (A), percentage of polymorphic loci (P), observed average heterozygosity (H_o), and expected average heterozygosity (H_e). The average number of alleles per loci was obtained by the arithmetic mean of the total number of alleles divided by the total number of loci. Only the loci where the frequency of the most common allele did not exceed 95% were considered as polymorphic and took into account the percentage calculations.

The observed heterozygosity values were obtained by the average number of heterozygous genotypes in relation to the total genotypes of each locus. The expected heterozygosity was obtained by means of the average of H_e of all loci (estimate of multi-loci), calculated according to Nei (1978). Genetic similarity was estimated according to the Dice coefficient using the software NTSYSpc 2.01 (Rohlf, 2000). Based on the similarity analysis, a group dendrogram of cultivars was constructed using the UPGMA (Unweighted Pair Group Mean Average) method.

Obtaining and analyzing RAPD data

For RAPD (Random Amplification of Polymorphic DNA), 10 oligonucleotide primers were tested (OPG–03, OPG–17, OPJ–10, OPK–096, OPU–01, OPU–03, OPU–14, and OPZ–16 from the Operon Technologies, Inc.). The amplification test was performed using four individuals from each group (UNESP Germplasm Bank, Arapiuns River, Tapajós River, and Embrapa Germplasm Bank). The criteria for selection were the number of amplified loci, number of polymorphic loci, and amplified band intensity. The RAPD loci were analyzed for presence (1) or absence (0) of an allele in each of the individuals. The similarity between cultivars was calculated based on the Dice coefficient using the software NTSYSpc 2.01 (Rohlf, 2000). Based on the similarity analysis, a group dendrogram of cultivars was constructed using the UPGMA (Unweighted Pair Group Mean Average) method.

RESULTS

From the 46 sequences obtained from the sequencing of 131 clones from the enriched library for sequenced CT and GT, 22 of them contained SSR sequences formed by more than five replications (47.8%). Microsatellites with dinucleotide motifs accounted for more than half of those identified (69%), followed by trinucleotides (18%), and compounds (14%). From the assessed microsatellites, only two had CA sequences, one of them being a composite microsatellite formed by a replication (GA).

From the 22 sequences with microsatellites, primers were selected for 19, of which 11 (57.9%) allowed the amplification of clear bands and good repeatability, six (31.6%) amplified nonspecific bands, and two (10.5%) did not amplify any fragment. Table 1 shows the sequences of the 11 pairs of primers selected for the assessment of genetic variability in the germplasm of *A. comosus* var. *erectifolius*. Table 1 also shows the motifs, annealing temperatures, MgCl2 concentrations, and their respective allelic ranges.

Single fragments were considered as loci in homozygosity, whereas double fragments were considered as heterozygous. In the sample, 48 different alleles were observed for the set of 10 polymorphic loci and the number of alleles per locus ranged from three at the loci Alu 10, Alu 11, and Alu 12 to 10 at the locus Alu 05, with an average of 4.8 alleles per locus (Table 1).

Table 2 shows that the number of alleles/locus varied between 1.36 and 1.55 (~1.47), and polymorphic loci from 36.4 to 54.5% (~47.47%). The expected heterozygosity ranged from 0.181 to 0.272 (~0.237), while the observed went from 0.363 to 0.545 (~0.474). Also, the numbers of exclusive alleles were different among the nine accession groups, with the highest for white and purple curauá (six) from UNESP, and the lowest for individuals (three) from the rivers Arapiuns and Tapajós. Among the accessions from Embrapa germplasm bank, no exclusive alleles were observed. The genetic divergence (FST) observed among individuals composing the groups of *A. lucidus* was 0.5665, showing a genetic variability between groups of 56.6%.

A separation of individuals into two main groups was observed (Figure 1). The first group is subdivided into two subgroups, one of which includes the individuals of purple curauá from AGB–UNESP (FCA_{PC}) and individuals of purple curauá from Arapiuns River (A_{PC}), and the other group includes individuals of white (M_{WC}, PP_{WC}, and B_{WC}) and purple curauá (E_{PC} and PP_{PC}) from AGB–Embrapa.

In addition, the individuals of white curauá (FAC_{WC}) from AGB–UNESP and the individuals of purple curauá from the Tapajós River (T_{PC}) were included in the second group. The average distance between pairs of accession groups was 0.50 ± 0.24, with the highest value (0.82) between FCA_{WC} and the groups M_{WC}, PP_{WC}, and B_{WC}. However, the low genetic distances between accessions of purple (E_{PC} and PP_{PC}) and white curauá (M_{WC}, PP_{WC}, and B_{WC}) from Embrapa suggest a reduced variability between both individuals.

From the ten primers tested by RAPD, seven provided clear amplification and good repeatability products. The selected primers were OPG–03, OPG–17, OPU–01, OPU–03, OPZ–03, OPZ–14, and OPZ–16, which amplified fragments ranging from 300 to 4000 bp. These primers allowed the amplification of 35 RAPD fragments, 22 of them being polymorphic. The number of loci per primers ranged from two (for the primers OPG–03 and OPZ–14) to five (for the primers OPU–01 and OPZ–03), with an overall average of 2.9 loci per primer (Table 3).

The percentage of polymorphic loci per group ranged from 3.5% for FCA_{PC} to 17.5% for PC, with an average of 11.2%. Among banks, variability levels between the germplasm of purple and white curauá are similar, except for the accessions of white curauá from AGB–UNESP,

Table 1. Pairs of microsatellite primers developed for *Ananas comosus* var. *erectifolius* motif types, forward and reverse primers sequences, expected fragment sizes, observed allele amplitudes, annealing temperatures and magnesium chloride concentrations used for each primer pair.

Locus	Motif	Sequence of pairs of primers	Size of expected fragments	Allele range (bp)	Number of alleles/loci	Tm (°C)	$MgCl_2$ (mM)
Alu 01	$(AGA)_{10}$	F-5'TATTTGGCCATTTCACCCTC3' R-5'GATCCTCCACAAAGCTCCAA3'	193	197-209	4	60	1.5
Alu 03	$(CA)_{10}$	F-5'TGTTAGATTTGGGCCGTTTC3' R-5'GGCATCCCCATATCTTAGCA3'	260	260-280	4	60	1.5
Alu 04	$(CT)_{10}$	F-5'TAATTTGGTAGCACGGAGGC3' R-5'TCCCTTCATCCAAAAAGTCG3'	156	156	1	60	1.5
Alu 05	$(CT)_{19}$	F-5'TGATGGGAAATAGCTGAGCC3' R-5'AAAAACGAGCACAATCCCAC3'	266	240-280	10	60	1.5
Alu 08	$(TC)_{10}$	F-5'TTTCCTTTCCGCACAATTTC3' R-5'GTCGTGTGTGGAAACCACTG3'	219	220-250	5	60	1.5
Alu 09	$(AAG)_{10}$	F-5'ATGTAATTGACCCACCCCAA3' R-5'TTTCTATGCGGACTGAACCC3'	208	200 -230	4	60	1.5
Alu 10	$(TC)_{10}$	F-5'GGGTTCAGTCCGCATAGAAA3' R-5'CCAGTCCTGCAGTGACAATC3'	238	223-250	3	65	1.5
Alu 11	$(TC)_{16}$ $(AAG)_8$	F-5'ACAGCTTGCGAGAAACAAGG3' R-5'CAAGTTTTGCGACACCAATG3'	237	230-250	3	65	1.5
Alu 12	$(TC)_{16}$ $(AAG)_8$	F-5'GCCCTCCATTTCCACCTAAC3' R-5'GGTGGTATTGGTCGCTGTCT3'	169	169 -290	3	65	1.5
Alu 15	$(GA)_{13}$	F-5'AGGATACTCGATCTCCCGCT3' R-5'TCACCTGCAAAGGGAATAGG3'	203	260-300	5	65	1.5
Alu 17	$(CT)_{13}$ $(CA)_{10}$	F-5'GGAGCCATCTAATTGTTCCA3' R-5'ATATGCGACCAAGCACAACA3'	248	240-290	7	65	1.5

which present a higher percentage of polymorphic loci (12.3%) than those of purple curauá (3.5%), as shown in Table 4.

Two groups were formed, one consisting exclusively of purple curauá plants and the other of white curauá. Within groups of purple curauá, only A_{PC} and T_{PC} presented 100% similarity. The relationships between species were different from those established with microsatellites, which did not allow a clear distinction between purple and white curauá (Figure 2).

DISCUSSION

Crossing and molecular data of the seven species of the genus Ananas indicate their intercompatibility and fertility for hybrids. There is also molecular evidence on the close relationship between species of the genus Ananas in studies conducted by Ruas et al. (2001), which is considered to be phylogenetically close. Thus, the probability of transfer of primers between them is high. However, this study demonstrates that none of them has similarity to sequences of other species of Ananas, making the set of pairs of primers presented in this study an extra and new set for the assessment of *A. comosus* var. *erectifolius* and other species of Ananas. From the 22 sequences containing microsatellites, 19 pairs of primers (86%) were designed and the other three had an insufficient number of repetitions or the flanker sequences were very small, precluding the selection of primers meeting the established parameters.

Sequences formed by dinucleotides were the most frequent because of the use of CT and GT probes. Microsatellites with AG/CT and GA/TC motifs were more frequent than those with CA/GT. Oliveira et al. (2004) verified the higher frequency of GT and CT motifs for

Table 2. Diversity indices for the nine *Ananas comosus* var. *erectifolius* groups, where n: access sampled number; NA: total of alleles; P: polymorphic loci percentage; A: average number of alleles / lozenges; He: expected heterozygosity and Ho: observed heterozygosity, using 10 microsatellite loci.

Diversity indices	Population									Mean
	AGB–UNESP		Arapiuns/ Tapajós		AGB–Embrapa Amazônia Oriental					
	FCA_{PC}	FCA_{WC}	A_{PC}	T_{PC}	B_{WC}	PP_{WC}	PP_{PC}	M_{WC}	E_{PC}	
N	60	30	1	1	8	4	4	8	8	18.55
NA	15	16	15	15	16	16	14	16	14	15.2
P	45.5	54.5	45.5	45.5	54.5	54.5	36.4	54.5	36.4	47.47
A	1.45 −0.52	1.55 −0.52	1.45 −0.52	1.45 −0.52	1.55 −0.52	1.55 −0.52	1.36 −0.5	1.55 −0.52	1.36 −0.5	1.47 -
H_e	0.227 −0.261	0.272 −0.261	0.227 −0.261	0.227 −0.261	0.272 −0.261	0.272 −0.261	0.181 −0.252	0.272 −0.261	0.181 −0.252	0.237
H_o	0.454 −0.522	0.545 −0.522	0.454 −0.522	0.454 −0.522	0.545 −0.522	0.545 −0.522	0.363 −0.304	0.545 −0.522	0.363 −0.304	0.474 -

Note: The amount of expected heterozygosis was estimated according to Nei (1978); number in parentheses is the standard deviation.

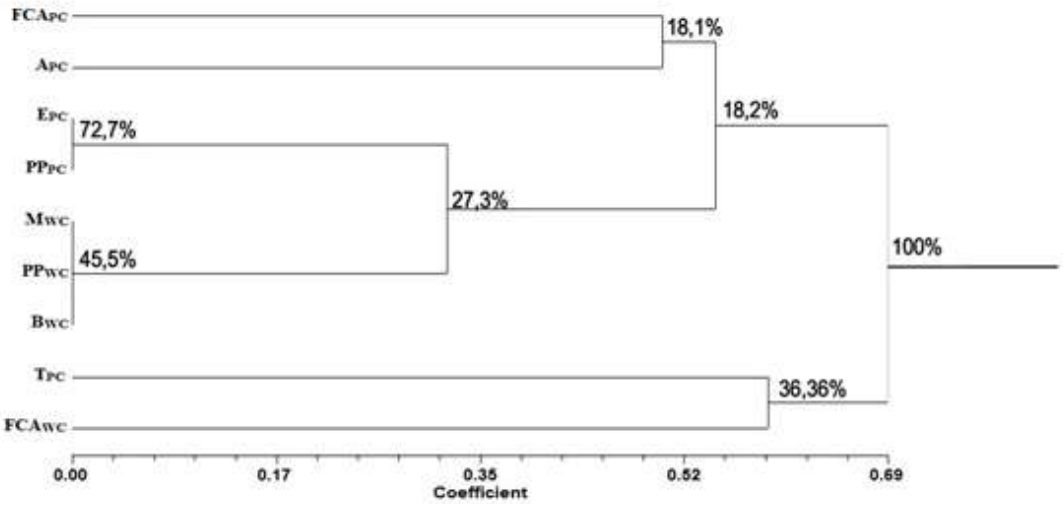

Figure 1. Dendogram showing the relationships among the nine access groups of *Ananas comosus* var. *erectifolius* evaluated. The distances between accesses were obtained by the UPGMA grouping criterion, based on the Dice coefficient Data from 10 microsatellite loci.

Table 3. Primers selected sequence for *Ananas comosus* var. *erectifolius* and number of loci obtained.

Primer	Sequence 5' – 3'	Number of loci	Number of polymorphic loci	Percentage of polymorphism
OPG–03	GAGCCCTCCA	6	2	33.3
OPG–17	ACGACCGACA	4	2	50
OPU–01	AGATGCAGCC	6	5	83.3
OPU–03	ACTGGGACTC	7	3	42.8
OPZ–03	CAGCACCGCA	7	5	71.4
OPZ–14	TCGGAGGTTC	2	2	100
OPZ–16	TCCCCATCAC	3	3	100
Total	-	35	22	-

Table 4. Percentage and number of polymorphic loci for the nine groups of *Ananas como*sus var. *erectifolius*.

Group	Curauá samples	Percentage of polymorphic loci (%)	Number of polymorphic loci
AGB–UNESP	FCA_{PC}	3.5	2
	FCA_{WC}	12.3	7
Arapiuns River	PC	17.5	10
Tapajós River	PC	12.3	7
AGB–Embrapa Amazônia Oriental	Bragança – WC	10.5	6
	Ponta de Pedra – WC	15.8	9
	Ponta de Pedra – PC	10.5	6
	Marila – WC	8.8	5
	Embrapa – micropropagated plant – PC	8.8	5
Average of groups	-	11.2	6.4

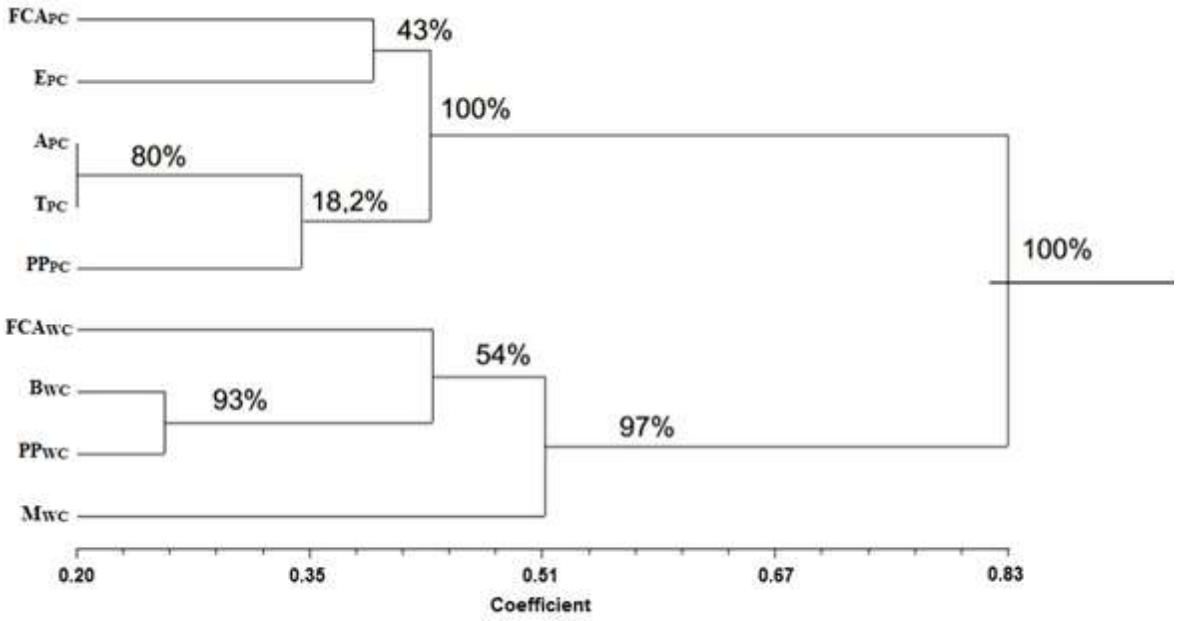

Figure 2. Relationship between the nine groups of *Ananas comosus* var. *erectifolius* individuals, defined by the UPGMA grouping criterion, based on the Dice coefficient.

Passiflora edulis when using the same enrichment protocol. In another study, GT/CA and GA/CT motifs occurred in a higher percentage for the genus Phaseolus (Campos et al., 2005). The frequency of microsatellite motifs is quite adjustable in plants. Once probes are used, the enrichment process are seen to have very similar annealing temperatures because they are composed of the same amounts of purines and pyrimidines, and if the possibility of technical problems with the GT probe is excluded, the data suggest the higher frequency of microsatellites with CT/GA motifs in curauá than GT/CA.

RAPD and SSR marker analyses showed a low genetic variability within each of the nine analyzed groups. The results were similar when compared to the observed indices for other asexual propagating species. The authors suggest that they are potentially duplicate cultivars, or at least a minimal genetic difference between them. In a study of genetic diversity of 16 accessions of the genus Ananas by means of 148 RAPD markers, Ruas et al. (2001) verified a great genetic similarity (80%) among five varieties of A. comosus and attributed this result to its asexual reproduction.

The low genetic variability observed within the

assessed curauá collections may be due to two main factors: 1 – collection formation from few individuals; 2 – low genetic variability in the collection regions. This second hypothesis seems to be less likely since Costa et al. (2002) found great genetic divergence among curauá accessions; they assessed 16 accessions using 104 RAPD markers (Active Germplasm Bank of Embrapa Amazônia Oriental) and found 79 (75.96%) polymorphic ones. Therefore, there is a slight relationship between geographical origin and pattern of genetic variability distribution.

Due to the cultivation method of curauá, there is a possibility of low genetic variability in the original regions of its cultivation, which can be due to two main factors: 1 – a reduction of variability resulting from the domestication process; and 2 – the genetic erosion caused by crop abandonment by indigenous people. Ferreira and Bustamante (2004) report that curauá is a relative of pineapple which have been domesticated by indigenous people a long time ago and probably can no longer survive in nature without human interference.

RAPD markers allowed the detection of variation within groups, but this variation was also, in general, quite low. The major differences observed among the nine groups of analyzed accessions, which were collected at six different locations, suggesting that a greater variability will be sampled if a larger number of sampling sites are sampled. In this case, there is no need to collect a large number of samples per site since the intra-site variability is generally low.

The data presented here regarding the genetic variability among accessions of the same region are divergent from those found by Costa et al. (2002), who used 104 RAPD markers and observed variability among accessions of some of the assessed locations. Among these accessions are white curauá samples from Bragança (B_{WC}) and Ponta de Pedra (PP_{WC}). In this study, no polymorphism was detected among the analyzed accessions from both sources with data from ten pairs of microsatellite primers. RAPD data demonstrate polymorphism between both groups; however, the genetic similarity between B_{WC} and PP_{WC} was quite high (76.6%), actually indicating 2 molecularly similar groups. The divergence between the data obtained in this study and that of Costa et al. (2002) is probably due to the difference of the assessed samples. There is no correlation between the degree of genetic similarity observed in genetic markers and the origin of accessions (Salla et al., 2002). On the other hand, our findings are in agreement with those of Costa et al. (2002), who demonstrated a high variability between accessions from different locations.

CONCLUSIONS

The greater genetic diversity will be sampled if a larger number of sampling sites are experimented, and there is no need to collect a large number of accessions per site since intra-site variability is low. Primers resulting from this study may decrease the time and expenses involved in the isolation of microsatellite markers, such as the construction of a genomic DNA library and DNA sequencing.

These primers are an extra and new set for assessing *Ananas comosus* var. *erectifolius* and will be useful for other species from the genus Ananas.

CONFLICT OF INTERESTS

The authors have not declared any conflicts of interest.

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