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FULL PAPER

Genetic Diversity and Structure of Populations of *Annona crassiflora* Mart. of Brazilian Savanna and Its Association with Chemical Variability

by **Anary Priscila Monteiro Egydio-Brandão***, **Claudia Maria Furlan**, and **Déborah Yara Alves Cursino dos Santos**

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Annona crassiflora Mart. is a native tree from Brazilian savanna. Isoquinoline alkaloids are characteristic of species of Annonaceae. This work aimed to assess the magnitude of genetic diversity among different populations of *A. crassiflora* using AFLP markers, and verify the existence of any correlation between the AFLP data and previous reported alkaloid composition. *A. crassiflora* from eight populations in the states of São Paulo, Goiás, Minas Gerais, and Distrito Federal were analyzed. The data suggest a low, moderate, and high level of genetic diversity from different populations of *A. crassiflora*. Concentration of alkaloids was significantly correlated with AFLP data, suggesting interaction between chemical and molecular markers in *A. crassiflora*. The data of association between the chemical and genetic differentiation of *A. crassiflora* may be useful to establish cultivation areas allowing the definition of strategies to preserve their genetic diversity with an interest in specific chemotypes for genetic improvement programs focused on sustainable utilization of this specie.

Keywords: *Annona crassiflora*, Annonaceae, Amplified fragment length polymorphism (AFLP), Alkaloids, Population analysis.

Introduction

Brazilian Cerrado is included among the 25 biodiversity hotspots for conservation priorities [1]. However, during the past 40 years up to 60% of this biome has been changed due to extensive agriculture [2]. This biome presents distinct phytophysiognomies and edaphic conditions which have been correlated with phenotypic variations of some species [3]. The Cerrado consists of a vegetal formation complex, ranging from 'campo limpo' (grassland) to 'cerradão' (woodland). The intermediate physiognomies ('campo sujo' – a shrub savanna, 'campo cerrado' – a wooded savanna, and 'cerrado *sensu strict*' – a woodland) should be considered as ecotones [4]. Despite all this physiognomic variation, Cerrado can be characterized by no hydric limitation, low levels of soil nutrients, mainly N, and toxic levels of Al [5].

Annonaceae has been pointed out as one of the families with the highest values of floristic richness in Cerrado areas all over the Brazilian territory [6]. This family consists of about 108 genera and around 2400 species, which are widely distributed, especially in tropical and subtropical regions all over the world [7]. Some species of *Annona* are economically important, *e.g.*, *A. squamosa* (sugar apple), *A. muricata* (soursop), *A. reticulata* (custard apple), and *A. cherimola* (cherimoya). All abovementioned species are nonnative to Brazil. Moreover, *Annona* includes wild species that are appreciated by local communities living in the Brazilian Cerrado, despite having no significant economic importance.

Annona crassiflora Mart., also known as 'araticum', 'marolo' or 'pinha-do-cerrado' [8], is a native tree of Brazilian Cerrado, widely spread throughout the biome spanning across the states of São Paulo, Minas Gerais, Bahia, Mato Grosso do Sul, Mato Grosso, and Tocantins [9]. There are numerous important species threatened for the fragmentation in the Brazilian Cerrado, including *Dipteryx alata* [10], *Stryphnodendron adstringens* [11], *Caryocar brasiliense* [12], *Hymenaea stigonocarpa* [13], *Anemopaegma arvense* [14], and *Annona crassiflora* [15].

Conservation programs are essential for Cerrado vegetation. Evaluation of genetic structure and the stability of evolutionary lineages, may subsidize these decisions since they provide ideas about genetic diversity within and between populations.

Molecular markers provide an important tool for assessing the genetic variability and structure of natural populations [16]. Some techniques mostly used are isozyme profiles, restriction fragment length polymorphism (RFLP), microsatellites or simple sequence repeats (SSRs), random amplified polymorphic DNA (RAPD), and amplified fragment length polymorphism (AFLP).

The AFLP technique has been considered as an ideal molecular tool in modern research, particularly in small-scale conservation projects [17]. This is a robust tool for DNA fingerprinting of genomes [18], which allows the study of genetic polymorphisms in populations, generally indicating *Mendelian* inheritance, and may be used to

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study relationship and genetic variability within and among populations.

Recently, some studies have been developed concerning the level of genetic diversity of *A. crassiflora* using isozymes [19], sequencing of cpDNA region [20], microsatellites [21], and RAPD [15]. However, these studies were restricted to populations of two Brazilian States, Goiás and Minas Gerais.

In addition to the molecular data which in general are neutral regarding the influence of abiotic and biotic factors [22], few phenotypic characters, such as secondary metabolites, can provide important data related to evolutionary survival strategy of individuals of a specific population.

There are several studies that report genetic data and others describing the chemical profile of plants, but works that discuss adaptative phenotypic characters in conjunction with molecular data are rare [22]. Certainly this information is very relevant to create conservation strategies and for genetic improvement programs [23]. One of the main problems in evaluating genetic resources is the lack of information allied to the establishment of strategies to ensure not only the conservation of natural populations, but also other potentially useful genetic resources.

Isoquinoline alkaloids are characteristic of species of Annonaceae [24]. Reticuline benzylisoquinoline [25], asimilobine and anonaine aporphines [26], and liriodenine oxoaporphine [27], have already been detected in *Annona* species. Such alkaloids were found in leaves and barks of *A. crassiflora* from the Guianas [28]. Previous data from our group show anonaine, annoretine, romucosine, and xylopine in leaves of *A. crassiflora* from eight populations in Brazilian Cerrado. Differences among populations were also demonstrated [29].

In this context, the main goal of this study was to assess the magnitude of genetic diversity among different populations of *A. crassiflora* using AFLP markers. Second, the existence of any correlation between the AFLP data and the previous published composition of alkaloids [29] was investigated.

Results and Discussion

Intrapopulational Genetic Diversity

The AFLP analysis using three primer combinations produced a total of 276 fragments when 106 individuals were analyzed. Parameters of intrapopulational genetic diversity, including the percentage of polymorphic *loci* (*P*), genetic diversity (*He*), and the *Shannon*'s index (*I*) are presented in *Fig. 1*. The data obtained in this study based on analysis of AFLP markers were compared to literature reports and used for analysis of genetic structure of populations from Federal District, and Goiás and Minas Gerais States, which presented different molecular markers (*Table 1*).

In this study, it was found that the percentage of polymorphic bands per population (*P*) ranged from 23.8% to

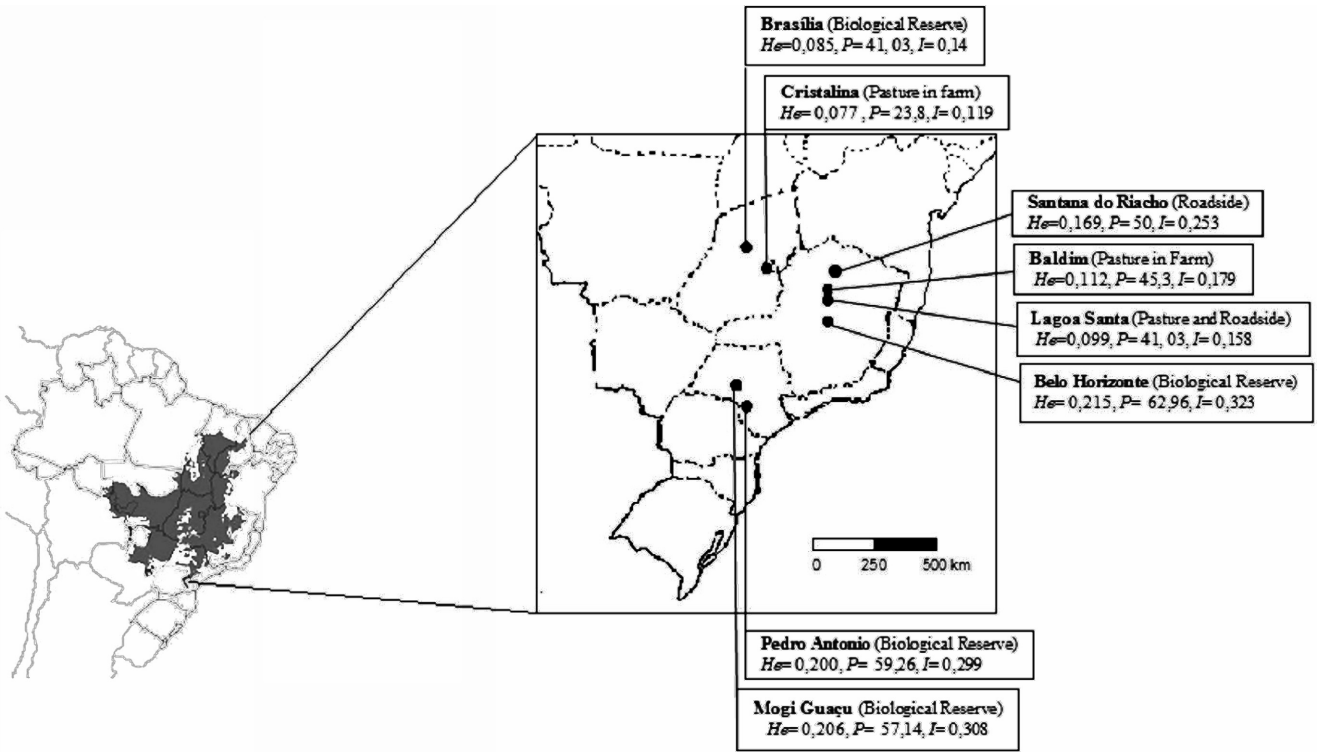


Fig. 1. Populations of *Annona crassiflora* used for genetic variation analyses from Brazilian Savanna. Parameters of intrapopulation variation and types of human intervention are presented into boxes. *P*: percentage of polymorphic loci, *He*: gene diversity of *Nei* [47], *I*: *Shannon* index.

Table 1. Parameters of intrapopulational genetic diversity of *Annona crassiflora*

Localization of populations	Marker	Na[a])	*He*[a])	*P*[a]) [%]	*I*[a])	π[a])	References
Federal capital							
Brasília	AFLP	10	0.085	41.03	0.140		Present study
Padre Bernardo	SSR	100	0.606				[30]
Reserva Ecológica de Aguas Emendadas	SSR	104	0.777				
State of Goiás							
Cristalina	AFLP	5	0.077	23.80	0.119		Present study
Cruzeiro	Isozyme	30	0.270				[19]
Dois Irmãos	Isozyme	30	0.250				
Goianésia	Isozyme	30	0.223				
Padre Bernardo	Isozyme	30	0.334				
Silvânia	Isozyme	30	0.332				
Vianópolis	Isozyme	30	0.303				
Rio Verde	SSR	32	0.807				[21]
Água Fria	*trn*L of *cp*DNA	7				0.0161	[20]
Alto Paraíso	*trn*L of *cp*DNA	8				0.0014	
Aragoiânia	*trn*L of *cp*DNA	7				0.0130	
Cabeceiras	*trn*L of *cp*DNA	6				0.0052	
Campos Belos	*trn*L of *cp*DNA	8				0.0129	
Itarumã	*trn*L of *cp*DNA	8				0.0127	
Mineiros	*trn*L of *cp*DNA	8				0.0335	
Orizona	*trn*L of *cp*DNA	8				0.0014	
Posse	*trn*L of *cp*DNA	6				0.0148	
Rio Verde	*trn*L of *cp*DNA	8				0.0245	
Serranópolis	*trn*L of *cp*DNA	8				0.0136	
State Minas Gerais							
Baldim	AFLP	20	0.112	45.30	0.179		Present study
Belo Horizonte	AFLP	17	0.215	62.96	0.323		
Lagoa Santa	AFLP	10	0.099	41.03	0.158		
Santana do Riacho	AFLP	12	0.169	50.00	0.253		
Campo Azul	RAPD	15	0.280	76.55	0.410		[15]
Campo Azul	RAPD	15	0.300	81.38	0.440		
Grão Mogol	RAPD	21	0.210	59.30	0.310		
Montes Claros	RAPD	21	0.250	71.05	0.370		
State of São Paulo							
Mogi-Guaçú	AFLP	10	0.206	57.14	0.308		Present study
Luis Antonio	AFLP	22	0.199	59.26	0.299		

[a]) Na: sample size, *P*: percentage of polymorphic loci, *He*: gene diversity of *Nei* (1978), *I*: *Shannon* index, π: nucleotide diversity.

62.96% (*Fig. 1*). Population from Belo Horizonte (Minas Gerais) presented the highest value of percentage of polymorphic bands, while the population of Cristalina (Goiás) presented the lowest value. *Blanco et al.* [20] found 28.3% of polymorphism analyzing the *trn*L region of cpDNA of 11 populations of *A. crassiflora* from state of Goiás. *Cota et al.* [15] found 59.3 – 81.38% of polymorphic *loci* among four populations from northern of state of Minas Gerais (*Table 1*). Intrapopulational genetic diversity (*He*) ranged from 0.077 to 0.215 based on AFLP analysis (*Fig. 1*). Cristalina (Goiás), Lagoa Santa (Minas Gerais), and Brasília (Distrito Federal) populations presented lower values than other populations of Belo Horizonte (Minas Gerais), Mogi-Guaçu (SP), and Pedro Antonio (São Paulo). In the State of Goiás, *Telles et al.* [19] observed values which ranged from 0.223 to 0.334 and *Cota et al.* [15] verified values ranging from 0.210 to 0.300 at state of Minas Gerais (*Table 2*). The *Shannon*'s

Table 2. Analysis of molecular variance (AMOVA) within and among samples of eight populations of *Annona crassiflora*

Source variation	DF[a])	SSQ[a])	VC[a])	% Total variation	*P*[a])	F_{ST}
AMOVA						
Between regions	2	103.251	0.809	8	0.001	0.401
Interpopulational	5	169.145	2.214	22		
Intrapopulational	89	632.615	7.108	70		

[a]) DF: degrees of freedom; SSQ: sum of squares; VC: variance component; *P*: level of significance for estimation of genetic variation based on 10 000 permutations. Regions: Southeast and Central-West of Brazil.

index ranged from 0.119 (Cristalina-Goiás) to 0.323 (Belo Horizonte – Minas Gerais) (*Fig. 1*). *Cota et al.* [15] verified values that ranged from 0.310 to 0.440 (*Table 1*).

Based on the results obtained in this study and in literature, distinct levels of genetic diversity were found for populations of *A. crassiflora*. In this study, the highest values of intrapopulational genetic diversity were observed in the populations of protected areas (Mogi-Guaçu, Luis Antonio, and Belo Horizonte – Biological Reserve), except for Brasília population (*Fig. 1*, *Table 1*). Lower values of intrapopulational genetic diversity found for populations from areas of high anthropogenic activity (Cristalina, Baldim, Santana do Riacho, and Lagoa Santa) agree with [31] data, which demonstrated significant differences in these parameters between two populations from Distrito Federal, one obtained from conservation area and another from impacted region.

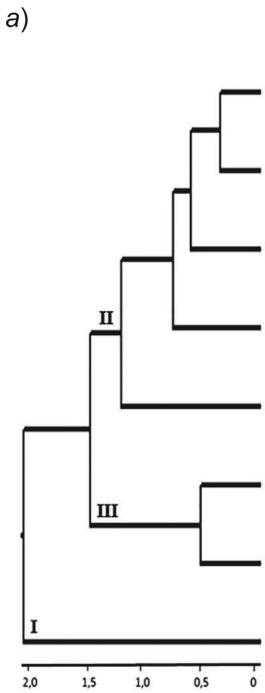
Estimated values of populational genetic diversity can be achieved using several molecular markers including the isozymes [19], sequences of the *trn*L [20], microsatellites [21][31], and RAPD [15] and AFLP (*Table 1*). Depending on the chosen marker, the observed values can vary. However, many studies using AFLP markers have confirmed the effectiveness of this technique to access the diversity and the population genetic structure for several plant species, for example, *S. adstringens* [11], *Daphne laureola* [32], *Phaseolus coccineus* [33], *Silene chlorantha* [34], *Capsicum baccatum* [35].

Interpopulational Genetic Structure

Three main clusters are observed in UPGMA analysis based on AFLP (*Fig. 2*). Cluster I is formed only by population of Cristalina (GO); Cluster II includes four populations: Baldim (MG), Lagoa Santa (MG), Luís Antonio (SP), Belo Horizonte (MG), and Brasília (DF); and Cluster III comprises populations of Mogi-Guaçú (SP) and Santana do Riacho (MG). No significant correlation between geographic and genetic distances was detected through *Mantel* test ($r = 0.107$; $P = 0.154$), suggesting that geographic distance alone does not explain the spatial pattern of genetic diversity among populations.

AMOVA results confirmed a distinct population structure between the analyzed populations of *A. crassiflora*. The molecular variance within populations was much higher (70%, $P = 0.001$) than the variation among populations (22%, $P = 0.001$). Comparing regions, the variation was even smaller between southeast and Central-West regions (8%, $P = 0.001$) (*Table 2*). *Blanco et al.* [20] and *Cota et al.* [15] have already reported that interpopulational genetic diversity of *A. crassiflora* is less expressive than intrapopulation variations. *Blanco et al.* [20] emphasized that some populations of *A. crassiflora* appear to be on an imminent process of differentiation.

Genetic differentiation as proposed by *Wright* [36], which means the change in allele frequency among populations (F_{ST}), reflects the probability that two random genes of two populations are identical by descent [37].



b)

	Annoretine	Anonaine	Romucosine	Xylopine
Baldim (MG)	63.9	**743.6**	240.2	20.9
Lagoa Santa (MG)	18.9	**779.7**	4.1	33.7
Luís Antonio (SP)	58.5	**445.7**	0	0
Belo Horizonte (MG)	130.3	**442.8**	210.8	0
Brasília (DF)	186.8	**2254.3**	300.7	66.2
Mogi-Guaçú (SP)	106.4	**114.6**	0	0
Santana do Riacho (MG)	**361.2**	247.2	13.6	94.2
Cristalina (GO)	**559.9**	365.7	2.5	126.5

Fig. 2. *a*) Affinity relationships among populations of *Annona crassiflora* obtained with *Nei*'s unbiased genetic distance and UPGMA method of clustering based on the AFLP fragments (see *Experimental Part*). *b*). Average amounts of alkaloids (μg/g of dry mass) for each population [29]. Numbers in bold: main alkaloid for each population.

According to *Solferini and Selivon* [38], a F_{ST} value > 0.25 indicates a high degree of genetic structuring. *Frankhan et al.* [16] reported that F_{ST} values > 0.15 are often an indication of fragmentation of the population. In the present study, the value of genetic differentiation F_{ST} is 0.401 (P = 0.001) (*Table 2*), suggesting high level of fragmentation of sampled populations.

Other populations of native species from Brazilian savanna have been characterized with high level of genetic differentiation among them, *e.g.*, *C. brasiliense* (F_{ST} = 0.260) [12], *Eugenia dysenterica* (F_{ST} = 0.270) [39], *D. alata* [10], *H. stigonocarpa* (F_{ST} = 0.692), *Jacaranda decurrens* (F_{ST} = 0.263) [13], *S. adstringens* (F_{ST} = 0.290) [11], and *A. arvense* (F_{ST} = 0.283) [14]. This genetic divergence found between populations of *A. crassiflora* and other species of Cerrado may possibly reflects the isolation between populations due to the processes of fragmentation and the reduction in size of the population. In the past 40 years, extensive agricultural practices have caused changes in up to 60% of this ecosystem [2]. For several authors, this fragmentation represents a huge barrier that hinders the gene exchange between populations, which might explain the high level of genetic differentiation among them [14]. *Loveless and Hamrick* [40] suggested that species characterized by entomophily are more sensitive to fragmented ecosystems due limited pollen movement, which can accentuate the differentiation among populations. Since *A. crassiflora* is mainly pollinated by beetles, this feature could also contribute to low interpopulational genetic variation.

Correlation between Molecular and Chemical Data

Previous alkaloid data for populations of *A. crassiflora* [29] were compared with AFLP clusters. Annoretine and anonaine were the main alkaloids among populations. The distribution of romucosine and xylopine was more variable. Cluster II includes all populations with anonaine at least threefold higher than annoretine. Populations from clusters I and III are characterized by higher or similar amounts of annoretine against anonaine (*Fig. 2*).

Considering AFLP data combined with alkaloid amounts for PCA analysis, three distinct groups could be observed (*Fig. 3*), almost completely coincident with UPGMA (*Fig. 2*). The distinction of Brasília population from the others from cluster II is clearer with combined data. The first principal component (PC1) explained 9.89% of the total variance and the second 8.33% of the variance. Although the axis explained a lower percentage of variability, it allowed separating some individuals. The first axis was positively correlated with annoretine and xilopine. The second axis was positively correlated with anonaine and romucosine.

Mantel test confirmed that the concentration of alkaloids was significantly correlated with AFLP data (*Table 3*). In this study, the total concentration of

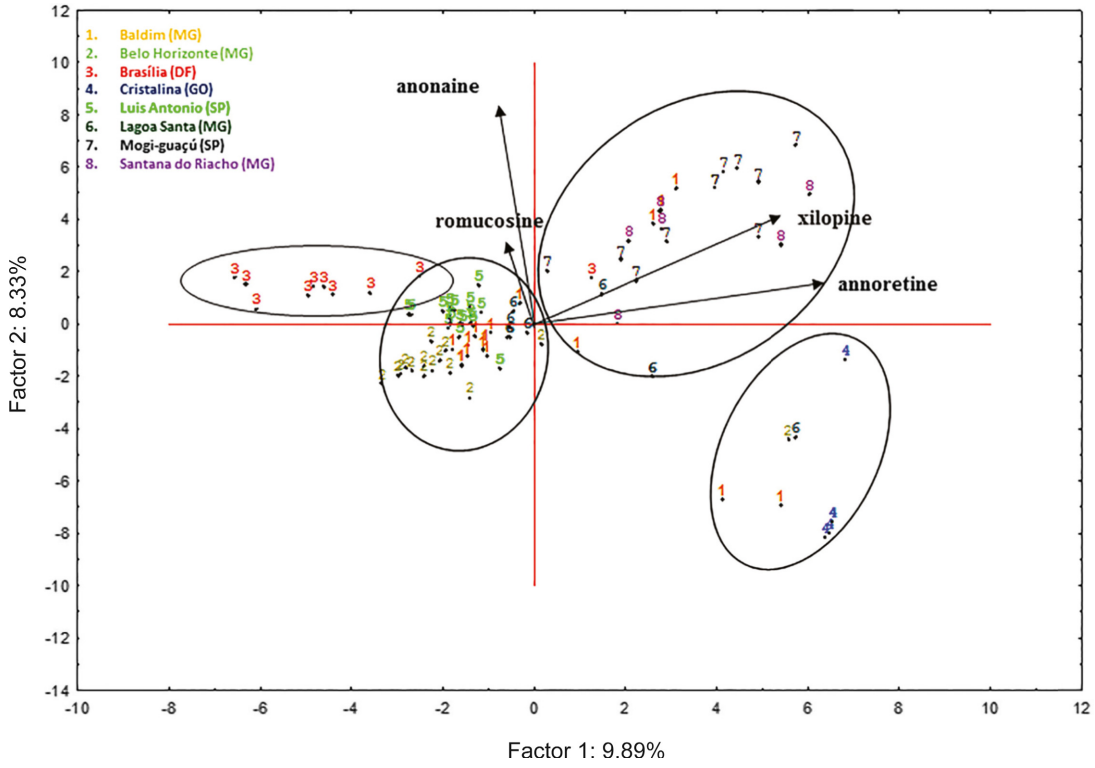


Fig. 3. PCA using AFLP polymorphism and alkaloids data from *Egydio et al.* [29]. The numbers and different colors represent the individuals of each population.

Table 3. Pairwise correlation test between AFLP data and alkaloids amounts (μg/g of dry mass)

Variable	Correlation (*r*)	*P*-value
Annonaine	−0.432	0.009
Annoretine	−0.556	0.012
Romucosine	−0.697	0.008
Xilopine	−0.559	0.071
Alkaloid not identified	−0.476	0.010
Total Concentration	−0.413	0.013

alkaloids showed correlation of −0.413 ($P = 0.013$) to AFLP data, annonaine −0.432 ($P = 0.009$), annoretine −0.556 ($P = 0.012$), romucosine −0.697 ($P = 0.008$), xylopine −0.559 ($P = 0.071$), and the not identified alkaloid −0.476 ($P = 0.01$).

As far as we know, this is the first study which tentatively explored the correlation between genetic and chemical differentiation among populations of *A. crassiflora*. Other authors have already reach success using genetic markers (AFLP and/or SAMPL) and essential oil compounds [41][42].

Conclusions

The data obtained in this study may contribute to the establishment of conservation strategies for Brazilian savanna, looking forward avoiding genetic erosion of this species. The higher intrapopulation genetic variation compared with the interpopulation unveils the danger of loss of genetic diversity due to the decreased gene flow between populations. Furthermore, the expressive intrapopulation genetic variation in populations which occur in preserved areas strengthens the importance of conservation. Genetic data taken together with chemical data, based on alkaloid profiles, may be useful for selecting germplasm resources of high quality considering medicinal purposes, since these compounds have already been demonstrated with several biological activities.

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Experimental Part

Plant Material

Leaf samples of 106 individuals of *A. crassiflora* were collected from eight distinct populations from Brazilian Cerrado areas in Luís Antonio and Mogi-Guaçu (state of São Paulo); Baldim, Belo Horizonte, Lagoa Santa, and Santana do Riacho (state of Minas Gerais); Cristalina (state of Goiás); and Brasília (Brazilian capital). For each population were collected 10 – 20 individuals. The shortest distance between two individuals in the same population was 1 m, while the shortest distance among populations was 30 km. Distances among populations were determined using the computer program GPSTRACKMAKER 11.7. Voucher specimens were deposited in the Herbarium of Department of Botany, University of São Paulo (SPF) (*Table 4*).

AFLP Analysis

DNA was extracted from silica gel dried leaf material using the CTAB method described by [43] with minor modifications.

Table 4. Localization and sample size of eight populations of *Annona crassiflora*

Localization of populations	Voucher	PHYs[a])	N[a])
Distrito federal (DF) (15°57.045′S, 47°52.353′W)	A. P. M. Egydio 1	Woodland 'cerradão'	10
Cristalina (GO) (16°59.599′S, 47°45.843′W)	A. P. M. Egydio 2	Wooded savanna 'campo cerrado'	05
Santana do Riacho (MG) (19°20.234′S, 43°37.594′W)	A. P. M. Egydio 5	Woodland 'cerradão'	12
Baldim (MG) (19°15.520′S, 43°57.028′W)	A. P. M. Egydio 6	Wooded savanna 'campo cerrado'	20
Lagoa Santa (MG) (19°33.831′S, 43°57.565′W)	A. P. M. Egydio 4	Wooded savanna 'campo cerrado'	10
Belo Horizonte (MG) (20°01.718′S, 44°00.596′W)	A. P. M. Egydio 3	Woodland 'cerradão'	17
Luís Antonio (SP) (21°58.654′S, 47°77.819′W)	A. P. M. Egydio 8	Woodland 'cerradão'	22
Mogi-Guaçu (SP) (22°15.231′S, 47°09.386′W)	A. P. M. Egydio 7	Woodland 'cerradão'	10

[a]) N: sample size, PHYs: phytophysionomies of Cerrado Biome.

Several primer combinations were tested for *Annona crassiflora* samples. The final AFLP analysis was carried out with three primer combinations *Eco*-AC/*Mse*I–CTG, *Eco*–AT/*Mse*I-CTT, and *Eco*-TG/*Mse*I-CAA since they allowed higher number of fragments.

The AFLP technique was performed according to an *AFLP™ Plant Mapping* protocol of *Applied Biosystems* with modifications [44]. Total DNA was submitted to the cleavage reaction, including 2 μl of buffer T4 DNA ligase, 0.25 μl of *Mse*I (50 U/ml), 0.50 μl of *Eco*RI (10 U/ml) for 2 h at 37 °C, and 15 min at 70 °C. For the binding reaction, 5.5 μl of cleavage reaction product were added to 2 μl of buffer T4 DNA ligase 5×, 1 μl of 0.5M NaCl, 0.5 μl of BSA, 1 μl of each of the adapters for *Mse*I and *Eco*RI (*Applied Biosystems*, Foster City, CA, USA), and 1 μl of the enzyme T4 DNA ligase. The mixture was incubated overnight at 15 – 20 °C. The preselective amplification was made using 2 μl of a diluted aliquot (1:10) of binding reaction product, 7.5 μl of core soln. mix (*Applied Biosystems*), and 0.5 μl of primers. The preamplification cycles started with 2 min at 72 °C, followed by 20 cycles of 20 s at 94 °C, 30 s at 56 °C, 2 min at 72 °C, and ended at 60 °C for 30 min. For selective amplification, 1.5 μl of a diluted aliquot (1:10) of preselective reaction product were added to 7.5 μl of core soln. mix (*Applied Biosystems*) and 0.5 μl of each *Mse*I and *Eco*RI primers combined. The selective amplification cycles started with 2 min at 94 °C, followed 10 cycles of 20 s at 94 °C, 30 s at 66 °C with reduction of 1°/s, and 2 min at 72 °C, followed 20 cycles of 20 s at 94 °C, 30 s at 56 °C, 2 min at 72 °C, and ended at 60 °C 30 min, 4 °C indefinitely.

Fragment analyses were performed with a DNA *ABI Prism 310* automatic sequencer using the protocol of *AFLP Plant Mapping Protocol* [44]. For the analysis of the fragments, 1.5 μl amplified product, 11 μl of formamide, and 0.5 μl of standard fragment sizes (ROX) were mixed and heated 95 °C for 5 min. The fragments detected were analyzed by size of base pairs (bp) using *Genescan 500 ROX* Standard.

Analysis of Genetic Data

Raw data were analyzed using the ABI Prism Genescan analysis software [45]. Fragments of 50 – 500 bp were scored as present (1) or absent (0) using ABI Prism Genotyper 2.5 Software [46].

Percentage of polymorphic bands (*P*), total genotypic diversity (*He*) (*Nei*'s measures of gene diversity – 1972, 1978) [47][48], *Shannon*'s index (*I*) and the coefficient of gene differentiation (F_{ST}) were calculated using GenAlEx 6.3 [49]. Furthermore, the partitioning of variation at different levels was calculated by analysis of molecular variance (AMOVA) using 10 000 permutations. All parameters were estimated assuming *Hardy–Weinberg* equilibrium.

The unweighted pair group method with averages (UPGMA) clustering analysis, based on the *Nei*'s unbiased genetic distance, was calculated using the TFPGA ver. 1.3, and the bootstrap values were derived from 10 000 permutations [50].

Association between the matrix of direct line geographic distance (km) and genetic distances were analyzed using *Mantel* test with 9999 permutations [51] in GenAlEx 6.3 program [50]. Geographical distance matrices among populations were computed from GPS coordinates, while genetic distance matrices were calculated in the form of F_{ST}.

Leaf Alkaloids as Related to AFLP

To investigate the congruencies between chemotypic and genomic distances, the matrix of genetic distance (F_{ST}) based on the combined data set of AFLP and matrix of an *Euclidean* phytochemical distance were compared using *Mantel* tests using GenAlEx 6.3 program [50].

Principal component analysis (PCA) was performed to show the overall pattern of variation and relationships among the populations. To eliminate the effects of different scales of measurement, the AFLP and chemical data were standardized using the Standardization Module in Statistica ver. 12 software [52].

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