Population structure of *Annona crassiflora*: an endemic plant species of the Brazilian Cerrado

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ABSTRACT. Habitat fragmentation has numerous consequences, particularly to endemic species, and has a negative impact on the genetic diversity of neglected species, leading to genetic drift. *Annona crassiflora* Mart. is a species that is endemic to Brazil, and its incidence in the Cerrado biome has decreased. The identification and characterization of its remaining diversity is necessary for its conservation. Our aim was to study the population structure of *A. crassiflora* populations from different Cerrado regions in Minas Gerais State, Brazil (Corinto, Curvelo, Carmo da Mata, Boa Esperança, and Paraguaçu) using inter-simple sequence repeat (ISSR) markers and DNA content. Nuclear DNA content was estimated by flow cytometry using 10 individuals from each population. ISSR markers were used for genotyping accessions in order to study their genetic diversity and population structures. We found considerable genetic variation among

populations, with the highest variability observed in the Curvelo population. There was a significant positive correlation between DNA content and latitude (r = 0.46, P = 0. 0003). A Bayesian-based cluster analysis grouped the populations into three clusters, which followed their geographical origins. There was some level of genetic diversity and differentiation among the populations, suggesting the need for a conservation plan for this species. The ISSR markers and DNA content analysis were effective in studying the genetic diversity and population structure of *A. crassiflora*.

Key words: Flow cytometry; Marolo; ISSR marker; *Annona crassiflora*

INTRODUCTION

Agricultural expansion and urbanization have resulted in the degradation of the Cerrado biome in Brazil (Brannstrom et al., 2008), which has had a direct impact on the population sizes of endemic species, and, therefore, constitutes a threat to Cerrado biodiversity. Despite the richness of its genetic resources (Mittermeier et al., 2005), the Cerrado has considerably decreased in area, and robust conservation measures are needed to avoid further biodiversity loss.

Of the several plant species that are endemic to the Cerrado, *Annona crassiflora* Mart. is particularly important for its potential as a food source and its pesticidal and allelopathic properties (de Omena et al., 2007; Inoue et al., 2010; Roesler, 2011; Ribeiro et al., 2013). *A. crassiflora* is commonly known as marolo in Brazil, and is distributed from Goiás State to Minas Gerais State. Minas Gerais has considerable importance in the genetic diversity of *A. crassiflora*, because the species has expanded its range in this state. Using random amplified polymorphic DNA (RAPD) markers, Cota et al. (2011) reported high genetic diversity among *A. crassiflora* populations in the north of Minas Gerais. Using simple sequence repeat (SSR) markers, Collevatti et al. (2014) found high genetic variability in populations of this species in Goiás State, highlighting the negative impact of habitat fragmentation, which can result in a high fixation index due to mating amongst closely related individuals. These results highlight the need for the conservation and characterization of the species' remaining diversity.

In order to design an appropriate conservation strategy, it is necessary to study the species' genetic diversity among different geographical regions. This can be conducted in several ways, including molecular marker techniques and quantification of the genomic DNA content. Inter-simple sequence repeats (ISSRs) are frequently used to study genetic diversity and population structures of different species, including endangered taxa such as *Ammopiptanthus* (Ge et al., 2005), montane plant species (Deshpande et al., 2001), and *Citrullus lanatus* landraces (Dje et al., 2010). This marker is particularly useful if there is insufficient information available on the species of interest, or if the study species has no specific markers, such as *A. crassiflora*. ISSR markers are multi-locus, species nonspecific, are amplified along the genome in a random manner, and are able to detect differences among accessions. We also analyzed *A. crassiflora* diversity using the DNA content, which is referred to as the C value (Bennett and Leitch, 1995) and indicates genome size. This value varies between species and between individuals of the same species (Schifino-Wittmann, 2001), is linked to species evolution, and can be correlated with various phenotypic and phenological traits (Bennett et al., 2000).

Brazilian biomes are being continuously destabilized, so the existence of a large

number of endemic species makes species characterization a difficult task. Studies that focus on the recovery and conservation of genetic resources are necessary for sustainable economic development. The nuclear DNA content of *A. crassiflora* has not been described. Understanding the genetic diversity and population structure of *A. crassiflora* is the first step in making recommendations for its conservation and sustainable use. The information obtained from molecular markers is more stable and not as affected by environmental factors as phenotypic information, and can help in developing a conservation strategy for this species.

This study had the following objectives: 1) study the nuclear DNA content of *A. crassiflora* populations in different mesoregions of Minas Gerais State, and verify the feasibility of using flow cytometry as a tool for detecting genetic variability among isolated populations of *A. crassiflora*; and 2) study the population structure and genetic diversity of *A. crassiflora* populations in different mesoregions of Minas Gerais State using ISSR markers.

MATERIAL AND METHODS

Study area

Sample collection was conducted in the municipalities of Boa Esperança, Carmo da Mata, Paraguaçu, Corinto, and Curvelo (Figure 1). For each analysis, independent samples were collected.

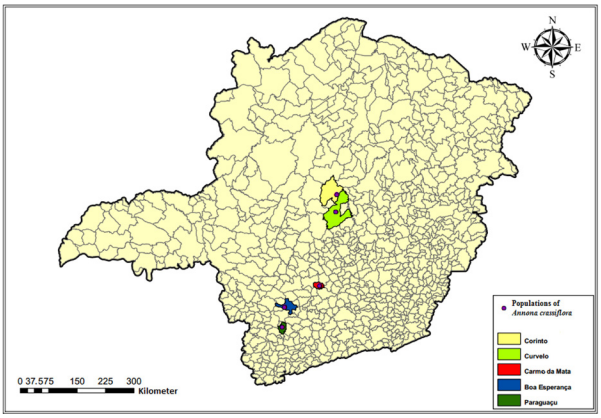

Figure 1. Populations of *Annona crassiflora* in Corinto (yellow), Curvelo (light green), Carmo da Mata (red), Boa Esperança (blue), and Paraguaçu (green) in Minas Gerais State, Brazil.

Genomic DNA content estimation

To estimate the C value of *A. crassiflora*, 10 adult plants from each *in situ* population were identified and georeferenced with a global positioning system device (eTrex Vista® HCx, Garmin, USA). Leaf samples that were in good phytosanitary condition were collected from adult plants and kept in a low-temperature container under high moisture. The collected samples were analyzed within 24 h at Laboratório de Cultura de Tecidos, Departamento de Agricultura, Universidade Federal de Lavras. The samples' DNA content was analyzed using flow cytometry according to the methodology described

by Magalhães et al. (2015), with some modifications. Tomato (*Solanum lycopersicum* L.) was used as an internal reference standard, the nuclear DNA content of which was 1.96 pg. The samples were kept under low temperature in the dark and analyzed using a flow cytometer (BD FACSCalibur™, BD Biosciences) to obtain histograms.

The 2C DNA content was estimated using the software WinMDI 2.8, and the estimated value was subjected to an analysis of variance (ANOVA) and a mean separation test. The relationship between DNA content and latitude was investigated using correlation analysis. All of the statistical analyses were performed using GENES (Cruz, 2013) and R (R Core Team, 2014).

ISSR analysis

Plant materials

Fifty-five *A. crassiflora* accessions from five different locations (11 from Corinto, 10 from Curvelo, 10 from Carmo da Mata, 12 from Boa Esperança, and 12 from Paraguaçu) were included. A list of the collection sites is presented in Table 1. The leaf samples were collected in the specified regions from randomly selected adult plants, and transported to the Laboratory of Plant Biotechnology, Empresa de Pesquisa Agropecuária de Minas Gerais, Caldas, Minas Gerais. The leaves were washed and stored in a freezer at -80°C for at least 2 h, then lyophilized until dry. The lyophilized leaves were ground and stored until DNA extraction.

Table 1. Characteristics of *Annona crassiflora* populations sampled in Minas Gerais State, Brazil.

Location (County)	Latitude (south)	Longitude (west)	Altitude (m)	Number of samples	Minas Gerais (Mesoregion)
Corinto	18°24'02"	44°27'44"	674	11	Central
Curvelo	18°49'10"	44°29'19"	724	10	Central
Carmo da Mata	20°33'59"	44°52'09"	869	10	West
Boa Esperança	21°04'59"	45°41'53"	814	12	South/Southeast
Paraguaçu	21°33'03"	45°45'29"	846	12	South/Southeast

DNA extraction

DNA was extracted according to the method described by Nunes et al. (2011) that is based on the cetyltrimethylammonium bromide method, using 70 mg of fully developed lyophilized leaf. The DNA obtained was quantified using an ultraviolet spectrophotometer (UV mini 1240, Shimadzu, Japan) at 260 nm and 280 nm in two replicates. DNA quality was estimated by dividing the reading at 260 nm by that at 280 nm. The quantified DNA was diluted using TE [Tris-HCl and ethylenediaminetetraacetic acid (EDTA)] to 50 ng/µL for analysis. DNA quality was verified by 0.7% agarose gel electrophoresis (Figure 2).

ISSR genotyping

ISSR genotyping was performed according to Nunes et al. (2013), with some modifications. Twenty-four ISSR primers that had been previously characterized were used to detect genetic variation among the *A. crassiflora* plants. The primers, their sequences, and their annealing temperatures are presented in Table 2.

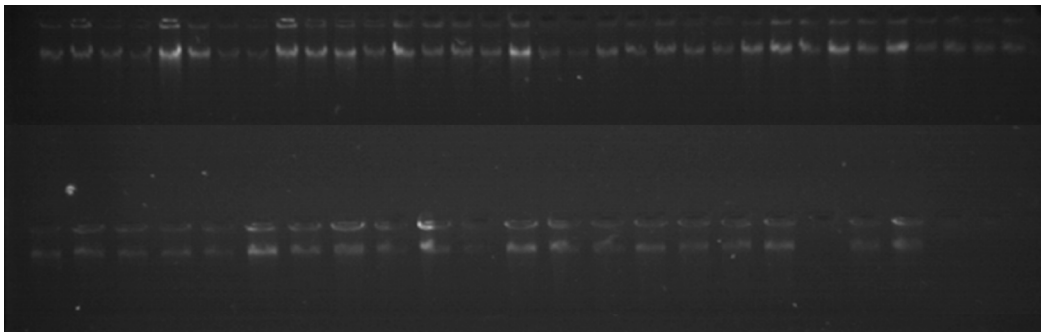

Figure 2. Agarose gel electrophoresis showing DNA samples.

Table 2. Primer characteristics of the inter-simple sequence repeat markers used in the study.

Name	Primer sequence (5'-3')	Motif	Annealing temperature (°C)
UBC807	AGAGAGAGAGAGAGAGT	$(AG)_8T$	50
UBC809	GAGGAGAGAGAGAGAGG	$GAGG(AG)_6G$	59
UBC810	GAGAGAGAGAGAGAGAT	$(GA)_8T$	52; 44.6
UBC812	GAGAGAGAGAGAGAGAA	$(GA)_8A$	51.6
UBC817	CACACACACACACACAA	$(CA)_8A$	54
UBC818	CACACACACACACACAG	$(CA)_8G$	54
UBC823	TCTCTCTCTCTCTCTCC	$(TC)_8C$	54
UBC827	ACACACACACACACACG	$(AC)_8G$	50
UBC834	AGAGAGAGAGAGAGAGYT	$(AG)_8YT$	59
UBC846	CACACACACACACACAAGT	$(CA)_8AGT$	38
UBC849	GTGTGTGTGTGTGTGTCTA	$(GT)_8CTA$	54
UBC850	GTGTGTGTGTGTGTGTYC	$(GT)_8YC$	52
UBC855	ACACACACACACACACYT	$(AC)_8YT$	52
UBC856	ACACACACACACACACYA	$(AC)_8YA$	48
UBC857	ACACACACACACACACYG	$(AC)_8YG$	52; 54.3
UBC859	TGTGTGTGTGTGTGTGRC	$(TG)_8RC$	55
UBC889	AGTCGTAGTACACACACACACAC	$AGTCGTAGT(AC)_7$	62
UBC890	VHV GTGTGTGTGTGTGTT	$VHV(GT)_7T$	52
UBC891	HVH TGTGTGTGTGTGTG	$HVH(TG)_7$	52; 51.6
VBV	VBVACACACACACACAC	$VBV(AC)_7$	51
BDV	BDVAGAGAGAGAGAGAG	$BDV(AG)_7$	47
HBH	HBHCTCTCTCTCTCTCT	$HBH(CT)_7$	47
BDB	BDBCACACACACACACA	$BDB(CA)_7$	51
GCV	GCVTCTCTCTCTCTCTC	$GCV(TC)_7$	49

The amplification reactions were performed in a final volume of 25 µL, which contained 50 ng DNA, 5 µL 5X reaction buffer, 1.5 µL $MgCl_2$ (1.5 mM), 0.5 µL dNTPs (200 µM each), 1.5 µL primer (0.6 µM, Sigma, USA), and 0.75 U *Taq* DNA polymerase (GoTaq® Flexi, Promega, USA). The reactions were programmed for an initial denaturation step of 2 min at 95°C followed by 40 cycles of denaturation at 95°C for 45 s, annealing temperature of primers (50°C) for 1 min and extension of primers at 72°C for 2 min, followed by a final extension step at 72°C for 5 min. Samples were subjected to electrophoresis on 1.5% agarose gel immersed in TBE buffer (90 mM Tris-borate, pH 8.0, 10 mM EDTA) at 110 V. Subsequently, they were stained with ethidium bromide (0.2 mg/mL) and photographed. The data were plotted onto a binary matrix, where 0 denoted "absence of band" and 1 denoted "presence of band".

Data analysis

The molecular weight of each fragment was estimated based on a DNA marker [50 mg (1.0 mg/μL) 50-bp DNA ladder, Invitrogen, USA]. In order to investigate genetic diversity, Nei's genetic diversity index (Nei, 1973), the Shannon diversity index, and the percentage of polymorphic bands were estimated using the Popgene software version 1.3 (Yeh et al., 1997). A principal coordinates analysis (PCoA) and analysis of molecular variance (AMOVA) were performed using the software GenAlex 6.1 (Peakall and Smouse, 2006), which was also used to prepare the data for Structure 2.3.4 (Pritchard et al., 2000). To analyze the genetic structure of the accessions and determine the probable number of groups (K), the data were analyzed using a Bayesian-based method of clustering using Structure 2.3.1. The burn-in period was 10,000, and the number of Markov chain Monte Carlo iterations was 100,000 using an admixture model. Runs were performed from K = 2 to K = 12, with 20 iterations for each K. The result obtained from Structure was used to determine the appropriate number of clusters using Structure Harvester (Earl, 2012).

RESULTS

DNA content analysis using flow cytometry

The DNA content was estimated using histograms generated by flow cytometry. All of the peaks analyzed had a coefficient of variation (CV) that was below 1%. According to Dolezel and Bartos (2005), CV values below 3% are acceptable. ANOVA revealed significant differences in DNA content among the populations (Table 3). The DNA content of samples from Curvelo ranged from 1.44 to 1.85 pg, which was the largest range observed and corresponded to a 22% difference between the minimum and the maximum (Table 3).

Table 3. Nuclear DNA content of *Annona crassiflora* populations in different regions of Minas Gerais State, Brazil.

Location	DNA content					CV (%)
	Minimum (pg)	Maximum (pg)	Fold variation (%)	Average[a] (pg)	Genome size (Mb)[b]	
Corinto	1.49	1.67	10.77	1.57c	1535	0.66
Curvelo	1.44	1.85	22.16	1.60c	1564	0.56
Carmo da Mata	1.52	1.66	8.43	1.60c	1564	0.55
Boa Esperança	1.64	1.80	8.88	1.72a	1682	0.52
Paraguaçu	1.56	1.74	10.34	1.65b	1613	0.84

[a]Values followed by the same letter did not differ significantly by the Scott-Knott test ($P < 0.05$). [b]Calculated as 1 pg = 978 Mbp (Dolezel and Bartos, 2005). CV, coefficient of variation.

Individuals from Carmo da Mata had the smallest range of nuclear DNA content, with values ranging from 1.52 to 1.66 pg, which corresponded to an 8.4% difference (Table 3). The mean DNA content values suggest that a high DNA content was most commonly found among individuals located at higher latitudes, such as south/southeast Minas Gerais (Table 3). The nuclear DNA content estimates did not statistically differ among the populations in west and central Minas Gerais (Table 3). Our data suggest a positive relationship between nuclear DNA content and geographical location, and populations with similar genome sizes were geographically close. We found a positive, significant correlation between latitude (south) and DNA content ($r = 0.46$, $P = 0.0003$) (Figure 3).

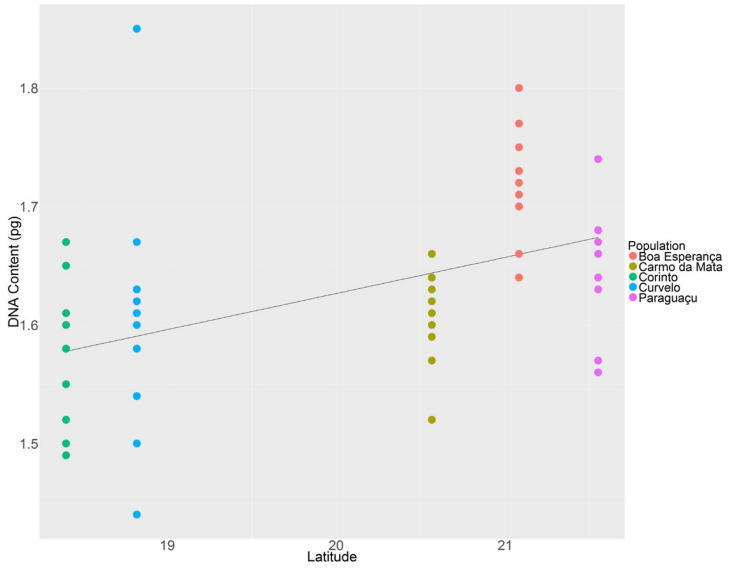

Figure 3. Relationship between DNA content and latitude.

The geographical locations, altitudes, and annual mean climatological characteristics (2000-2013) of the regions where the samples were collected are presented in Table 4.

Table 4. Climatic conditions of the regions where the *Annona crassiflora* populations were collected in Minas Gerais, Brazil.

Location	Altitude (m)	Rainfall (mm/year)	Annual mean		Humidity (%)
			Maximum temperature (°C)	Minimum temperature (°C)	
Corinto	674	1008	30.70	17.04	64.98
Curvelo	724	1008	30.70	17.04	64.98
Carmo da Mata	869	1300	29.02	15.61	66.14
Boa Esperança	814	1294	27.54	15.54	70.62
Paraguaçu	846	1294	27.54	15.54	70.62

Source: Instituto Nacional De Metereologia (www.inmet.gov.br).

Population structure and genetic diversity

The 24 ISSR primers produced 137 alleles, with a mean of six alleles per primer. Primer GCV did not produce polymorphic alleles. The number of alleles observed per primer varied from two to eleven, and the minimum number of alleles was observed in UBC890 and the maximum in HBH and UBC810 (Figure 4).

The multi-population analysis revealed that the primers HBH and UBC810 produced the most information within the populations studied. Most of the alleles produced by these primers were informative regarding the genetic diversity of the *A. crassiflora* accessions. The individual population diversity indices (Nei's genetic diversity and the Shannon information index) confirmed that there was high genetic diversity among accessions from Paraguaçu, Corinto, and Curvelo (Table 5).

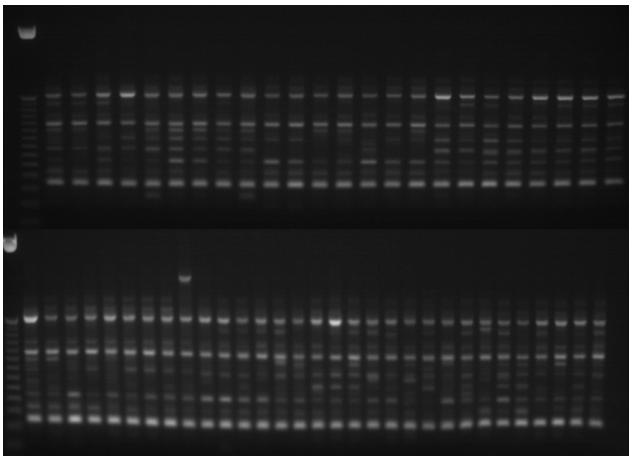

Figure 4. Agarose gel showing the amplified product of *Annona crassiflora* DNA using primer UBC810.

Table 5. Genetic diversity of *Annona crassiflora* populations.

Population	Number of samples	H		I		n poly	Pol
		Mean	SD	Mean	SD		
Corinto	11	0.1532	0.1767	0.2379	0.2591	69	50.36
Curvelo	10	0.15	0.1868	0.2277	0.2721	61	44.53
Carmo da Mata	10	0.1187	0.1739	0.1817	0.2557	50	36.5
Boa Esperança	12	0.1438	0.1753	0.2237	0.2568	66	48.18
Paraguaçu	12	0.1556	0.1834	0.2387	0.2674	67	48.91
Overall	55	0.1796	0.1802	0.2833	0.2518	101	73.72

H, Nei's genetic diversity; I, Shannon information index; n poly, number of polymorphic loci; Pol, percentage of polymorphic loci.

The lowest genetic diversity was observed among accessions from Carmo da Mata. Populations with high genetic diversity also had a high percentage of polymorphic loci (Table 5). The Bayesian model classified the accessions into three groups (K = 3), based on the method proposed by Evanno et al. (2005) (Figure 5).

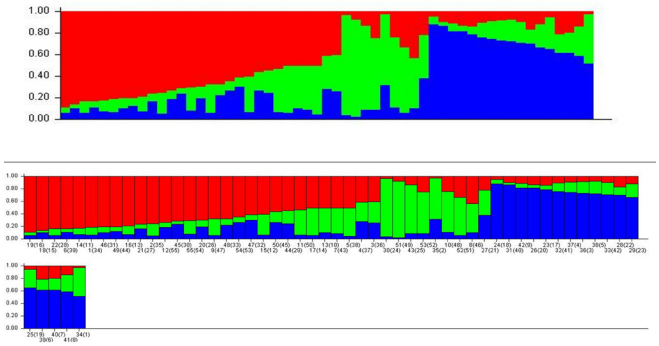

Figure 5. *Annona crassiflora* accessions subjected to cluster analysis and individual lines represented by numbers in the horizontal axis.

The grouping pattern followed that of the collection regions. Cluster I (predominantly red) comprised accessions from Boa Esperança, Carmo da Mata, and some accessions from Paraguaçu; Cluster II (predominantly green) comprised some accessions from Paraguaçu; and Cluster III (predominantly blue) comprised accessions from Corinto and Curvelo (Figure 5). The accessions that were collected from southern Minas Gerais were included in Clusters I and II, whereas accessions from central Minas Gerais were grouped in Cluster III. There was a high level of admixture among the populations, particularly concerning the accessions collected from southern Minas Gerais that had a high level of admixture among them (Clusters I and II). The PCoA supported the results obtained by the Bayesian model; the accessions were classified into two major groups, in which the accessions collected from Corinto and Curvelo were on the left-hand side of the y-axis and the others were on the right-hand side (Figure 6).

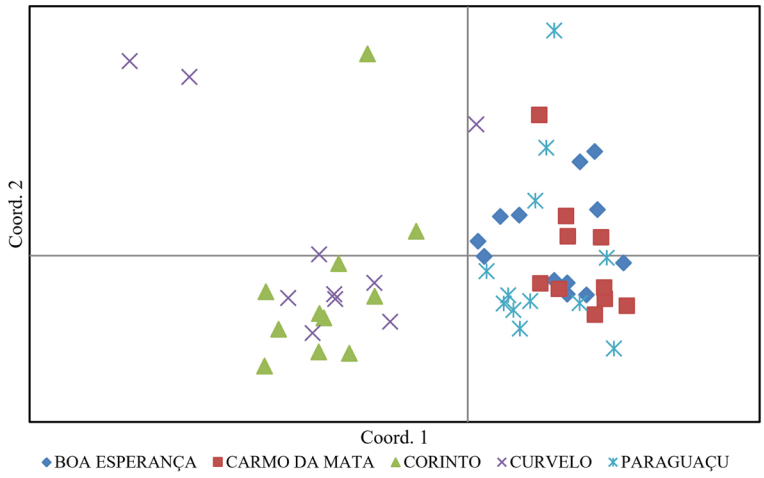

Figure 6. Principal coordinates analysis of 55 accessions collected from five regions of Minas Gerais State, Brazil.

This grouping pattern was also related to the collection site, where the cities of Corinto and Curvelo are in the central part of the state, whereas the other three sites are in southern Minas Gerais. The second group was divided into two subgroups (Figure 6). The first two principal components explained 18.41% of the total variation. Although the amount of variation explained was low, the analysis adequately categorized the accessions based on their respective locations. AMOVA showed that 86% of the total variation occurred within the populations and 14% occurred among them (Table 6).

There was a high level of variability among individuals within the populations, and there was some differentiation between the populations.

Table 6. Analysis of molecular variance of *Annona crassiflora* populations from different regions of Minas Gerais State, Brazil.

Source	d.f.	SS	MS	Estimated variance	%
Among populations	4	133.621	33.405	1.948	14
Within populations	50	600.688	12.014	12.014	86
Total	54	734.309		13.962	100

SS = sum of squares; MS = mean square.

DISCUSSION

Some environmental factors, such as temperature and precipitation, vary with latitude and altitude, and can act as agents of selection pressure, promoting the development of adaptive advantages and favoring changes in nuclear DNA content. Individuals with high DNA content are rare in environments with low water availability and extreme temperatures (Knight and Ackerly, 2002). The accessions that were collected from northern Minas Gerais, which receives little rainfall, had lower DNA content than those from southern Minas Gerais, which has high precipitation. In general, individuals with small genomes are found under a wide variety of conditions, whereas those with larger genomes are only found under moderate conditions, outside extreme environmental zones (Knight et al., 2005). In our study, the accessions collected from moderate environments had a higher DNA content than those collected from drought-affected areas.

Tavares et al. (2013) reported differences in genome size between *Eryngium duriaei* populations at different altitudes. Isolation at high altitudes favors the development of morphological differences between populations, particularly in the composition of essential oils and genome size. According to Murray (2005), variation is common among species from different habitats, and so it is possible that this variation may also occur in individuals of the same species that are under different conditions, particularly in populations that are in regions with a plurality of environmental conditions, such as *A. crassiflora*.

The variety of genome sizes in a single species is related to the adaptive capacity of that species. Li et al. (2013) did not find any intraspecific differences in nuclear DNA content among 36 populations of three *Miscanthus* species at different temperatures and altitudes. In this case, environmental change did not result in adaptive change. Although the environments included in the present study did not exhibit extreme variations in temperature, precipitation, or humidity, some degree of variability in nuclear DNA content was detected, which suggests that efforts directed to the collection, conservation, and exploitation of these genetic resources should be encouraged.

We found considerable variability in genetic diversity, and the maximum genetic diversity was observed in the populations from Corinto and Curvelo. Similarly, Cota et al. (2011) reported high genetic diversity among *A. crassiflora* accessions collected from northern Minas Gerais using RAPD molecular markers. In addition, Collevatti et al. (2014) reported high levels of genetic diversity among accessions collected from Goiás State using SSR markers.

A possible reason for the low genetic diversity found in our study may be the number of accessions collected in each region (10 on average). In both previous studies (Cota et al., 2011; Collevatti et al., 2014), a greater number of accessions per region was collected. We selected the most diverse regions in order to include a variety of sites and environmental conditions. Even with only a small sample size at each site, the accessions exhibited considerable genetic diversity, suggesting that we were able to sufficiently characterize genetic variation in this species to support its conservation.

The largest proportion of the total variation (86%) was within the populations, with a high level of variability among accessions within the same population. This is observed in allogamous species with a high level of cross-fertilization. This result explains the high level of heterozygosity found within the populations studied, which is in agreement with the individual population genetic diversity analysis. A high level of intrapopulation variability in *A. crassiflora* populations has been described previously (Cota et al., 2011; Egydio-Brandão et al., 2016).

Variation among the populations was 14%, suggesting some differentiation among them.

Collevatti et al. (2014) argued that habitat fragmentation could cause low genetic diversity, because it favors crosses among closely related individuals leading to a high fixation index. The high level of variation found within the populations highlights the allogamous nature of this species. Cavalcante et al. (2009) reported that *A. crassiflora* is preferentially cross-pollinated. Cota et al. (2011) also reported high diversity within populations of this species.

The PCoA and Bayesian-based cluster analysis showed that the grouping pattern followed that of the collection sites, in which cities in central Minas Gerais (Corinto and Curvelo) were clustered together. These two locations have similar climatic conditions and are adjacent to each other, which explain the high level of gene exchange between them. In addition, accessions from southern Minas Gerais were grouped in the same cluster, and are subject to similar climatic conditions. Therefore, the clustering revealed that the accessions have adapted to their environments through genetic change. We found a high level of admixture among the populations, indicating that some level of genetic exchange occurs among them. The admixture could also have been caused by *A. crassiflora* reproductive system, which is allogamous and favors pollen exchange among individuals within the same population (Cavalcante et al., 2009). The high similarity observed among the accessions from southern and western Minas Gerais could have been caused by their proximity, which favors genetic exchange. Collevatti et al. (2014) reported that habitat fragmentation results in mating occurring between closely related *A. crassiflora* individuals. The results of this and previous studies highlight the importance of conserving the variability of related species and their sustainable use. When planning collections, representative samples should be collected from each region in order to obtain as high a diversity as possible and a wide genetic pool.

The DNA content and ISSR molecular marker analyses were effective in studying the population structures and genetic diversity of *A. crassiflora* accessions. The low genetic diversity found suggests that habitat fragmentation has had an impact on genetic exchange among different populations; therefore, it is important to prevent further environmental degradation caused by urbanization and agricultural expansion. The remaining genetic diversity of an *in situ* germplasm allows species to respond to adverse conditions in the long term, resulting in adaptation. This process leads to the development of adaptive advantages (Lowe et al., 2005), and the loss of this capacity is an indication of a critical reduction in natural genetic variability (Young et al., 1996).

Few studies have investigated the genetic diversity, conservation, and breeding of *A. crassiflora*, which is what is required if we want to continue economically exploiting this valuable genetic resource. Further studies are required that include more samples and different molecular markers, in order to devise better strategies for germplasm conservation and breeding. Our study has revealed variability in genome size among *A. crassiflora* individuals in the same geographical locations, and among populations. It has also revealed the existence of considerable genetic diversity among accessions at each site. In addition, the study highlighted the effect of habitat fragmentation on the genetic diversity of *A. crassiflora*, and the importance of an appropriate conservation strategy for this species.

Conflicts of interest

The authors declare that they have no conflict of interest.

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