

## Population genetic structure of *Attalea vitrivir* Zona (Arecaceae) in fragmented areas of southeast Brazil

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**ABSTRACT.** *Attalea vitrivir* Zona (synonym *Orbignya oleifera*) is one of the six species of Arecaceae known as “babassu”. This species is used to make cosmetics, food, and detergents due to the high concentration of oil in the seeds. It is found only in fragmented areas of southern Bahia State and northern Minas Gerais State, southeast Brazil, and this fragmentation has affected both its ecological and genetic characteristics. We evaluated the genetic diversity and population genetic structure of *A. vitrivir* in six areas of two different regions at the extremes of its geographical range, in order to gain a better understanding of the factors that affect the distribution and partitioning of its diversity. Nine inter simple sequence

repeat (ISSR) markers amplified 74 polymorphic bands, resulting in large diversity values (Shannon diversity index, 0.37-0.47; intrapopulation genetic diversity, 0.25-0.34). Analysis of molecular variance (AMOVA) revealed considerable differentiation between sampling sites (30.03%) and regions (12.08%), although most of the diversity was observed within sampling sites (69%). Further differentiation between sampling sites was noted more in the northern region than in the southern region, highlighting the genetic connectivity between the sampling sites within Rio Pandeiros Environmental Protection Area (southern region). The identification of two distinct genetic clusters ( $K = 2$ ) corresponded to the northern and southern regions, and corroborated the AMOVA results. We suggest that the northern area, outside Rio Pandeiros Environmental Protection Area, must be included in future management plans for this species.

**Key words:** Babassu; Habitat fragmentation; Cerrado.

## INTRODUCTION

Naturally occurring fragmentation processes have recently been intensified by anthropogenic activity (Rimbaldi and Oliveira, 2003), particularly by intensive agriculture and land conversion to pasture, which have generated mosaics of original vegetation fragments embedded within highly transformed matrices (Arroyo-Rodríguez et al., 2007). Fragmentation affects ecological and genetic processes (Couvét, 2002; Lowe et al., 2005) by altering rates of genetic drift, endogamy, and selection in isolated, remnant populations (Rimbaldi and Oliveira, 2003).

One of the principal conceptual models used to examine what occurs after the subdivision of large and randomly organized populations was developed by Wright (1946). Loveless and Hamrick (1984) highlighted the importance of this model, particularly the ecological attributes of each species for the dynamic organization of genotypes, including factors such as gene flow, mating system, and dispersal.

Within this context, genetic diversity and population structure measured by molecular markers can be used to elucidate the biological factors inherent to a particular species, including divergence between populations, gene flow, and reductions in diversity levels (Frankham et al., 2002). This information, together with the biological characteristics of a given species, can aid germplasm conservation programs and the definition of evolutionarily significant units (ESUs) and/or management units for conservation purposes (Eizirik, 1996).

Molecular markers have been widely used as auxiliary tools for accessing genetic information (Frankham et al., 2002; Nybom, 2004). Inter simple sequence repeat (ISSR) markers were developed by Zietkiewicz et al. (1994), and, although dominant, they do not require a detailed knowledge of the genome of the target species, and produce large quantities of fragments with a high reproducibility (Brandão et al., 2011). Because of these advantages, ISSR markers have been used in numerous studies for genetic characterization, estimates of diversity, analyses of intra and interspecific similarities, evaluations of the effects of environmental change on genetic fragmentation and variability, and species conservation (Brandão et al., 2011; Gaiero et al., 2011; Kozyrenko et al., 2011).

*Attalea vitrivir* Zona (synonym *Orbignya oleifera*) is one of six species of palm (family Arecaceae) that are popularly known as “babassu” (babaçu in Portuguese) (Lorenzi et al.,

2010). These species are socioeconomically important to traditional agricultural communities, mainly because harvesting occurs during the inter-harvest periods of the principal cultivated crops of the region (Lucena, 2008). The seeds of *A. vitrivir* have a high oil content, and are used in the cosmetic and food industries to make detergents and lubricants (Lima et al., 2007). This species is restricted to northwestern Minas Gerais State and southern Bahia State, Brazil, where it is found on pasture lands formerly occupied by gallery forests and Cerrado vegetation (neotropical savanna) (Lorenzi et al., 2010).

Using ISSR markers, the present study aimed to characterize the population genetic structure of *A. vitrivir* in order to answer the following questions: i) How is the genetic variability of this species distributed within and between the study areas selected? ii) Are these areas and/or regions genetically isolated? iii) Do the areas included within Rio Pandeiros Environmental Protection Area effectively conserve the species' genetic diversity? The answers to these questions can be used to assist future conservation programs, and protect the genetic resources of this economically important species.

## MATERIAL AND METHODS

### Sampling

Preliminary field inspections were undertaken within the geographical range of this species in Brazil (as described by Lorenzi et al., 2010). We identified two principal regions of occurrence, separated by approximately 90 km along a north-south axis, and sampling was undertaken in these two isolated regions: one in the north (in southern Bahia State and in the extreme north of Minas Gerais State) and one in the south (in central-northern Minas Gerais State), subsequently referred to in the text as north (N) and south (S). Individuals were sampled in three sampling sites in the north (N1, N2, and N3) and the south (S1, S2, and S3) (Figure 1). The study areas in the north were located in the municipalities of Montalvânia, Minas Gerais (N2 and N3), and Cocos, Bahia (N1). The study areas in the south were within Rio Pandeiros Environmental Protection Area, which includes the municipalities of Januária, Cônego Marinho, and Bonito de Minas, Minas Gerais (Nunes et al., 2009).

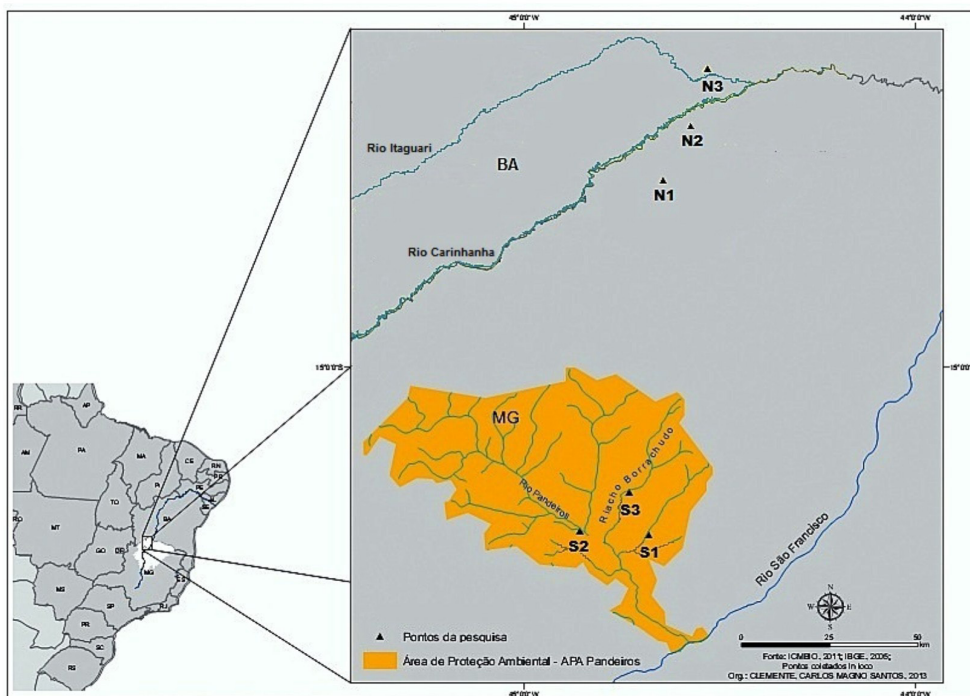
The three sampling sites in the north were separated by an average of 22.4 km, with a minimum distance of 16.5 km between N2 and N3 and a maximum of 33.6 km between N1 and N3. In the south, the average distance between sampling sites was 16.3 km, with a minimum of 12.9 km between S1 and S3 and a maximum of 18.6 km between S1 and S2. An average of 21 individuals from each population were sampled [varying from 12 (S3) to 30 (N3)] (Table 1), with a minimum distance of 50 m between individuals.

### DNA extraction and amplification

DNA extraction was performed according to Machado et al. (2002). Polymerase chain reactions (PCRs) were performed using a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems), with the following combination of reagents: 10X PCR buffer (500 mM Tris-HCl, 200 mM KCl, pH 8.0), 0.17 mM dNTPs, 1.25 mM MgCl<sub>2</sub>, 1.7 mM primer, 1 U Taq DNA polymerase, 10 ng DNA, and ultra-pure water. Nine ISSR primers were used (Table 2).

After an initial denaturation at 94°C for 5 min, 35 amplification cycles (identical for each of the primers) were conducted. Each cycle consisted of 94°C for 1 min, 30 s at 47°C, and

1 min at 72°C. At the end of the last cycle, there was a final extension of 7 min at 72°C. The fragments were separated on 5% polyacrylamide gel using vertical electrophoresis, and subsequently stained with silver nitrate as described by Benbouza et al. (2006). Fragment sizes were estimated by comparing them to an internal 50-bp DNA ladder standard.



**Figure 1.** Sampling sites (S1, S2, S3, N1, N2, and N3). The orange-shaded area corresponds to Rio Pandeiros Environmental Protection Area (Pandeiros-EPA); BA, Bahia State; MG, Minas Gerais State.

**Table 1.** Sampling site locations, with descriptions in terms of their relative extensions, land use, and the number of individuals sampled in each area (N).

Sampling site	Location	N	Description
N1	Montalvânia, MG (14°31'34.0"S; 44°38'38.8"W)	20	Area with few, widely spaced babassu palms inter-dispersed with tree species.
N2	Montalvânia, MG (14°23'12.7"S; 44°34'25.5"W)	22	Area with a larger population of babassu palms than N1 with a highly altered surrounding matrix. There were small agglomerations of babassu palms surrounding this site that could facilitate connections with N3.
N3	Cocos, BA (14°14'45.6"S; 44°31'27.8"W)	30	Located within a highly altered pasture land matrix, this was the smallest sampling site. It was located near a river and surrounded by other agglomerations of babassu palms.
S1	Januária, MG (15°25'26.7"S; 44°40'55.0"W)	22	Area showing the effects of extractive activities, cattle raising, and burning. The babassu palms were widely spaced, as this area was occupied by farms.
S2	Januária, MG (15°24'54.5"S; 44°51'17.8"W)	20	A larger and better-conserved area than S1, with high densities of babassu palms mixed in with other species.
S3	Bonito de Minas, MG (15°19'01.3"S; 44°43'45.5"W)	12	Area with a small cluster of densely grouped babassu palms within an altered matrix used as cattle pasture.

**Table 2.** Primers used in the study, including their nucleotide sequences, the number of fragments generated, and their polymorphic information content (PIC) values.

Primer	Sequence	No. of fragments	PIC
UBC 807	5' (AG) <sub>8</sub> T 3'	9	0.42
UBC 813	5' (CT) <sub>8</sub> T 3'	8	0.45
UBC 814	5' (CT) <sub>8</sub> A 3'	7	0.50
UBC 829	5' (TG) <sub>8</sub> C 3'	4	0.49
UBC 834	5' (AG) <sub>8</sub> YT 3'	8	0.47
UBC 840	5' (GA) <sub>8</sub> YT 3'	8	0.46
UBC 845	5' (CT) <sub>8</sub> RG 3'	11	0.41
UBC 857	5' (AC) <sub>8</sub> YG 3'	8	0.45
TERRY	5' (GTG) <sub>8</sub> RC 3'	11	0.41
		74 (Total)	0.45 (Mean)

## Statistical analyses

Polymorphic information content (PIC) values of the samples were estimated using the methodology described by Bhat (2002). The GENES software (Cruz, 2001) was used to calculate the optimal number of bands, which indicated how many bands would be necessary for the results of these analyses to be safely generalized to the study areas.

Shannon's diversity index was used to determine the genetic variability within populations, as calculated using the POPGENE 1.32 software (Yeh et al., 1997).

An analysis of molecular variance (AMOVA) was conducted in order to determine how the genetic variability was partitioned between and within the sampling sites and regions, using the ARLEQUIN 3.11 software (Excoffier et al., 2005). Four AMOVAs were performed: the first considered all six sampling areas as a single group (AMOVA 1), the second divided them according to the north and south geographical regions (AMOVA 2), and the third and fourth analyses considered each region separately.

The intrapopulation genetic diversity ( $H_s$ ), the total diversity [ $H_t$ , which is analogous to Nei's (1973) genetic diversity index], and the Bayesian genetic divergence ( $\theta^b$ ) between pairs of populations were estimated using HICKORY 1.0 (Holsinger and Lewis, 2003). Values were extracted from the optimal statistical model according to the deviance information criterion (DIC; Holsinger and Lewis, 2003). Correlations between genetic distances and geographical distances were investigated by conducting a Mantel test, using the NTSYS software (Rohlf, 2001).

The Bayesian approach of Pritchard et al. (2000) was used to infer the most probable number of genetically distinct groups ( $K$ ) in the whole data set, using the STRUCTURE 2.2 software. STRUCTURE was run using the admixture model ( $Q$ , with non-correlated allele frequencies) for a  $K$  of 1-10. Ten independent runs for each  $K$  were conducted. All of the runs were performed with a burn-in period of 100,000 steps followed by 100,000 Markov Chain Model iterations. The most probable number of clusters was determined using the ad-hoc measure,  $\Delta K$  (Evanno et al., 2005).

## RESULTS

### Genetic diversity

The number of fragments amplified per primer varied from four (UBC 829) to 11 (UBC 845 and Terry), totaling 74 fragments. The percentage of polymorphic loci varied from

84% (S3) to 93% (S2). All of the markers were polymorphic when the full set was considered. The PIC values for each primer varied from 0.41 (Terry) to 0.50 (UBC 814), with a mean of 0.45 (Table 2), demonstrating that all of them were satisfactory in revealing genetic differences between individuals. Regarding the optimal number of bands, at least 68 fragments are needed for reliable results, according to Silveira et al. (2003).

Mean Shannon diversity index values varied from 0.38 ( $\pm$  0.26) for N3 to 0.52 ( $\pm$  0.19) for S1 (Table 3). The overall mean for all of the six areas was 0.57 ( $\pm$  0.13). The Hs and Ht values exhibited the same pattern (Table 3).

**Table 3.** Shannon diversity index (SI), intrapopulation diversity index (Hs), and total heterozygosity (Ht) based on mean allele frequencies. The values in parentheses represent standard errors.

Area	SI	Hs
N1	0.3965 (0.24)	0.2878 (0.010)
N2	0.4221 (0.25)	0.3007 (0.008)
N3	0.3774 (0.26)	0.2590 (0.008)
S1	0.5164 (0.19)	0.3406 (0.013)
S2	0.4753 (0.25)	0.3309 (0.011)
S3	0.3953 (0.27)	0.3004 (0.011)
SI Total	0.5650 (0.13)	
Ht		0.41(0.009)

## Population genetic structure

AMOVA 1 demonstrated that approximately 30% of the total observed genetic variability was due to differences between the sampling sites (Table 4). When the sampling regions (north and south) were also considered, approximately 12% of the total genetic variability was between regions, while differences between sampling sites within each of the two regions were responsible for approximately 21% of the variability (Table 5). Additionally, AMOVAs within each region indicated that there was greater differentiation within the north ( $\Phi_{ST} = 0.31$ ) than within the south ( $\Phi_{ST} = 0.22$ ). In all cases, the largest proportion of genetic variability was observed between individuals within each sampling site.

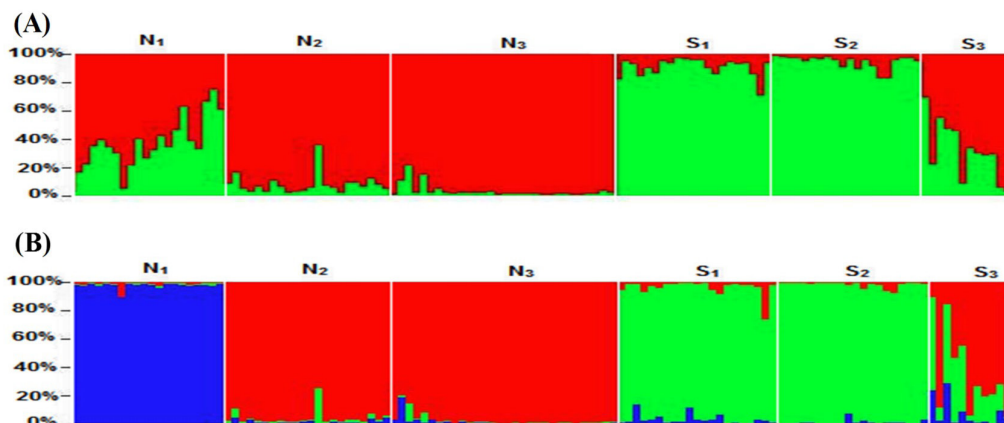
**Table 4.** Results of molecular variance analysis of *Attalea vitrivir*, including sampling sites within regions and individuals within each sampling site (AMOVA 1).

Source of variance	d.f.	Sum of squares	% of variance	P	Statistical $\Phi$
Between populations	5	394.65	30.03	(<0.0001)**	$\Phi_{ST} = 0.3003$
Between individuals within populations	119	955.574	69.97	(<0.0001)**	$1 - \Phi_{ST} = 0.6997$
TOTAL	124	1350.224	100.00		

\*\*Highly significant.

The Mantel test indicated that there was no correlation between the genetic ( $\theta^B$ ) and geographical distance matrices ( $r = 0.47$ ;  $P = 0.07$ ). The STRUCTURE analysis revealed that the most probable number of real populations was two ( $K = 2$ ), followed by  $K = 3$  (which in spite of not demonstrating a large  $\Delta K$  was still high compared to subsequent values). If  $K = 2$  was assumed, the two clusters coincided with the north and south regions, with areas S3 and N1 demonstrating the greatest percentages of admixture ( $Q$ ) compared to other areas in their regions (Figure 2A). If  $K = 3$  was assumed, there was a separation of N1 from the other study areas (Figure 2B).





**Figure 2.** Clustering of individuals made by STRUCTURE at  $K = 2$  (A) and  $K = 3$  (B). Individuals are represented by vertical colored lines. Same-colored individuals indicate that they belong to the same cluster. Different colors in the same individual indicate the percentage of the genome that was inherited from each cluster, and is numerically represented on the left-hand side of the figure on a scale from 0 to 100% (see Prichard et al., 2000).

**Table 5.** Results of an analysis of molecular variance for *Attalea vitrivir*, including regions (north and south), sampling sites within regions, and individuals within sampling sites (AMOVA 2).

Source of variance	d.f.	Sum of squares	% of variance	P	Statistical $\Phi$
Between regions	1	151.795	12.08	(<0.0001)**	$\Phi_{CT} = 0.1208$
Between populations within regions	4	242.855	21.39	(<0.0001)**	$\Phi_{SC} = 0.2139$
Between individuals within populations	119	955.574	66.53	(=0.1) <sup>ns</sup>	$1 - \Phi_{ST} = 0.6653$
TOTAL	124	135.022	100.00		

\*\*Highly significant; ns, not significant.

## DISCUSSION

The results of the PIC analysis, as well as the analysis of the optimal numbers of bands, indicated that the primers were informative (Botstein et al., 1980), with satisfactory numbers of fragments observed. Shannon's diversity index values for *A. vitrivir* were higher than those obtained for other species, demonstrating a predominance of cross-fertilization (e.g., *Rhodiola rosea*; Kozyrenko et al., 2011) by insect and/or wind pollination (e.g., *Nelumbo nucifera*; Han et al., 2009) and barochoric dispersal (e.g., *Orbignya phalerata*; Dos Santos et al., 2011). The intrapopulation and total values of diversity reported here are relatively high when compared to similar studies, but the two indices were congruent, in spite of differences in their absolute values.

A number of authors have observed that most palms display a predominance of cross-pollination, long lifecycles, and long-distance pollen dispersal, partitioning most of their genetic variability within populations (Nybom, 2004; Oliveira et al., 2008). AMOVA, using data from dominant markers in cross-pollinating arboreal species, have indicated that up to 28% of the genetic diversity resides between populations (Nybom, 2004; Oliveira et al., 2008). Our results suggest that there is a degree of isolation between the areas of occurrence of *A. vitrivir*, as differences between populations were responsible for approximately 30% of the observed variation. The differentiation between the north and south regions was less pro-

nounced (21.39%), although the  $\Phi_{CT}$  value (12.08%) suggests genetic divergence has occurred between them. According to Wright (1978),  $\Phi_{ST}$  values between 0.15 and 0.25 are indicative of a high degree of intrapopulation differentiation. Our results with *A. vitrivir* similarly reflect its low-dispersal-capacity barochoric system, although differential consumption by mammals or insects could also have increased differentiation between the areas (as observed by Quiroga-Castro and Roldán, 2001). Furthermore, *A. vitrivir* has a narrow geographical range that is fragmented (Lorenzi et al., 2010).

According to Gitzendanner and Soltis (2000), fragmentation and the consequent alteration of the matrix between fragments can contribute to increased divergence between areas. Molecular variance analyses performed separately for the two regions demonstrated this effect- as the areas in the north (where the matrix is more altered and palm populations are more fragmented) showed the greatest  $\Phi_{ST}$  values (0.31), while those in the south had the smallest differentiation values (0.23). The southern region included areas with greater connectivity, as it lies within the Rio Pandeiros Environmental Protection Area. According to Loveless and Hamrick (1984), ecological characteristics such as breeding system, floral morphology, life cycle, and successional stage can promote gene flow and lead to decreased population differentiation.

The results of the AMOVA corroborated the results obtained by STRUCTURE, which grouped individuals into two clusters ( $K = 2$ ). These clusters corresponded to the north/south regional delimitation, and it appears that the limitations of pollen and fruit dispersal may contribute to differentiation. The primary dispersal mode of babassu fruits involves barochory, and although there is no information available about the specific pollinator(s) of *A. vitrivir*, pollination in palm trees is generally mediated by beetles (Silberbauer-Gottsberger, 1990) that have only limited flight capacities (Han et al., 2009), which is incompatible with the approximately 90-km distance that separated the northern and southern regions in this study. Abreu et al. (2012) suggested that the aggregated distribution of the palm *Acrocomia aculeata* could influence pollinator behavior, as animals would tend to pollinate more efficiently within closely spaced groups, increasing genetic divergence between populations. This same reasoning could be applied to *A. vitrivir*, as the study areas were separated by landscape fragmentation- a common and very pronounced situation in the Cerrado biome where our study areas were located (Klink and Machado, 2005).

In addition, if  $K = 3$  (Figure 2B) and the sub-structuring suggested by the variance analyses performed separately in each region was accurate, reduced gene flow is the principal factor favoring differentiation in *A. vitrivir*, whether by limitations on fruit or pollen dispersal and/or by geographical distances between populations, and their limited connectivity. This isolation by distance is quite evident at a regional scale, as the importance of fragment quality and conductivity is obvious when the results of the percentage of admixture are compared between the northern and southern study regions. At  $K = 3$ , N1 was separated from the other populations of the same region. This population contains few individuals, which may have favored endogamy and genetic drift.

According to Moritz (1994), it is possible to draw up short- and long-term conservation strategies by examining information concerning nuclear and/or mitochondrial allele frequencies and delimiting management units and/or ESUs. In spite of the conceptual differences that characterize them, these two types of conservation units have the same goal: preserving areas with significant allele frequency divergences. Our results suggest that the six sampling sites studied form two distinct management units, and that Rio Pandeiros Environmental Protection Area (southern study region) does not retain the totality of the extant genetic diversity of *A. vitrivir*, making it necessary to incorporate the northern study region into management



plans for this species.

## CONCLUSIONS

The levels of genetic diversity found in *A. vitrivir* were considered moderate to high, as compared to other species with similar systems of crossing and dispersal. Genetic differentiation between the sampling sites and regions examined in the present study suggest the existence of two distinct potential management units that correspond to the northern and southern regions of the species' geographical range. There was significant intraregional differentiation between the sampling sites in the north, but less so in the south, indicating the importance of maintaining connectivity between populations. In spite of the greater connectivity between the areas included within Rio Pandeiros Environmental Protection Area in the south, these sites did not contain all of the genetic diversity found for this species, making it necessary to incorporate the northern region into future plans for the management and conservation of *A. vitrivir*.

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