Intraspecific differentiation of *Hancornia speciosa* revealed by simple sequence repeat and random amplified polymorphic DNA markers

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ABSTRACT. *Hancornia speciosa*, popularly known as mangabeira, is a fruit tree native to the Brazilian Cerrado that shows great economic potential, due to its multiple uses. Intraspecific classification of this species is difficult because it shows high morphological diversity. An early study of the species reported that there are six botanic varieties that differ morphologically mainly in the shapes of their leaves and flowers. Except to note the wide morphological variation and economic potential of this species, few studies have been published about the genetic diversity of

mangabeira. Knowledge of the genetic variability of this species among populations would be useful for genetic conservation and breeding programs. Therefore, we tested the transferability of 12 simple sequence repeats from expressed sequence tags (EST-SSRs) from *Catharanthus roseus* to *H. speciosa* and used 10 random amplified polymorphic DNA markers to evaluate the genetic variability among botanical varieties of *H. speciosa.* We obtained a high transferability frequency of EST-SSR markers from *C. roseus* to *H. speciosa* (75%). However, EST-SSR markers showed low heterozygosity and locus variability (two or three alleles by locus), which suggest low genetic diversity in the mangabeira samples. The Jaccard dissimilarity index and an examination of geographic distances indicated a non-spatial structuring of the genetic variability. Our markers were unable to distinguish *H. speciosa* botanical varieties.

Key words: Cerrado; Genetic variability; *Hancornia speciosa*; Mangabeira; Molecular markers; Transferability frequency

INTRODUCTION

Hancornia speciosa Gomes, commonly known as “mangabeira”, is a plant species found in the Brazilian Cerrado or savanna-like vegetation. Interest in cultivating mangabeira for fruit production has been growing in recent years. Although the fruits are considered the main commercial product, other products from mangabeira have commercial value or at least potential. For example, the extract obtained from the leaves can have a hypotensive effect (Ferreira et al., 2007a,b; Silva et al., 2011). Different flavonoids, catechins, anthocyanins, and tannins extracted from the bark may be used to treat the gastritis caused by *Helicobacter pylori* (Moraes et al., 2008) or to stimulate liver functions and help treat diabetes, hypertension, and dermatitis (Ritter et al., 2002; Macedo and Ferreira, 2004). Recently, the latex from this species was shown to have anti-inflammatory (Marinho et al., 2011) and angiogenic properties (Almeida et al., 2014).

Despite the pharmacological and economic potential of this species, there are few commercial plantations in Brazil and most of the harvested fruit comes from extractive activity (Carvalho et al., 2002). This activity, in addition to expanding agricultural frontiers (WWF, 2011), has reduced the distribution of *H. speciosa* in the Brazilian Cerrado, which may reduce the genetic variability of the species due to founder or bottleneck effects (Young et al., 1996). Thus, genetic conservation programs are necessary to maintain the diversity of this species.

Mangabeira belongs to the class Dicotyledoneae, order Gentianales, family Apocynaceae, and genus *Hancornia*. The genus includes only one species, *Hancornia speciosa.* According to Monachino (1945), there are six botanical varieties of mangabeira that differ mainly in the shapes of their leaves and flowers: *Hancornia* variety *speciosa speciosa*, *maximilliani*, *lundii*, *cuyabensis*, *gardneri*, and *pubescens.* As the classification of these varieties is performed exclusively by morphological characteristics, it is very difficult to identify different strains and classify samples accurately (Moura, 2003).

The success of any breeding or genetic conservation program depends on understanding the amount and distribution of the genetic variation in the species genome. Traditionally, genetic diversity has been measured by examining a combination of morphological and agronomic traits.

However, *H. speciosa* is a heterogeneous plant with many overlapping morphological attributes. In addition, many vegetative characteristics are influenced by environmental factors and show continuous variation and high plasticity, which make it difficult to identify discrete taxonomic groups. Using molecular markers can overcome these problems and allow the monitoring of genetic variability (Gonzalés-Pérez et al., 2009; de Menezes et al., 2014; Song et al., 2014).

Microsatellite markers (SSRs) have been widely used to answer questions related to population genetics (Gonzalés-Pérez et al., 2009; Madesis et al., 2014; Wang et al., 2014). However, microsatellites are expensive to use and it is time-consuming to develop specific primers for each locus of the native species (Zucchi et al., 2002). In many studies, primers designed for one species have been used in other species within the same genus or even belonging to different genera, demonstrating their transferability (Fan et al., 2013; Mathithumilan et al., 2013). SSRs from expressed sequence tags (EST-SSRs) are markers that may be used between species belonging to different genera. The EST provides a source of polymerase chain reaction (PCR)-based markers for SSR direction (Wang et al., 2014). In the present study, we evaluated the transferability of EST-SSR markers described for *Catharanthus roseus* to *H. speciosa*. *C. roseus* belongs to the same family as mangabeira and EST-SSR markers from *C. roseus* have been transferred to other medicinal plants (Mishra et al., 2011).

We also evaluated another type of molecular marker, random amplified polymorphic DNA (RAPD). This is a good option for species whose genetics have not been characterized, like mangabeira; only six DNA sequences of *H. speciosa* have been deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/). The use of RAPD markers also requires a lower level of skill and costs less per assay, and primers are readily available, allowing the entire genome to be scanned and the genotype to be characterized (Li et al., 2012).

The main objective of this research was to generate information for the future conservation, domestication, and breeding of mangabeira. Our specific goals were to 1) evaluate the transferability of microsatellite markers of *C. roseus* to *H. speciosa*; 2) estimate the genetic diversity of the species and determine whether genetic variation was related to geographical distribution; and 3) estimate the genetic relationships among different botanical varieties of *H. speciosa*.

MATERIAL AND METHODS

Plant material

A total of 34 leaf samples were collected in 27 locations in the Brazilian Cerrado, covering nine states (Figure 1). The leaves were transported to the laboratory for lineage identification and DNA extraction. DNA from young leaves was extracted using a modified cetyl-trimethylammonium bromide procedure (Pan et al., 2006). DNA concentration and quality was estimated electrophoretically and spectrophotometrically by Nanodrop (Thermo Scientific).

Oligonucleotide primers

A total of 12 SSRs from *C. roseus* (Table 1) were tested in our experiments with mangabeira DNA. The primer sequences were developed by Mishra et al. (2011). For RAPD experiments, we tested four oligonucleotide primers that were previously used by Silva et al. (2011) in mangabeira and six oligonucleotide primers used by Lal et al. (2011) (Table 1).

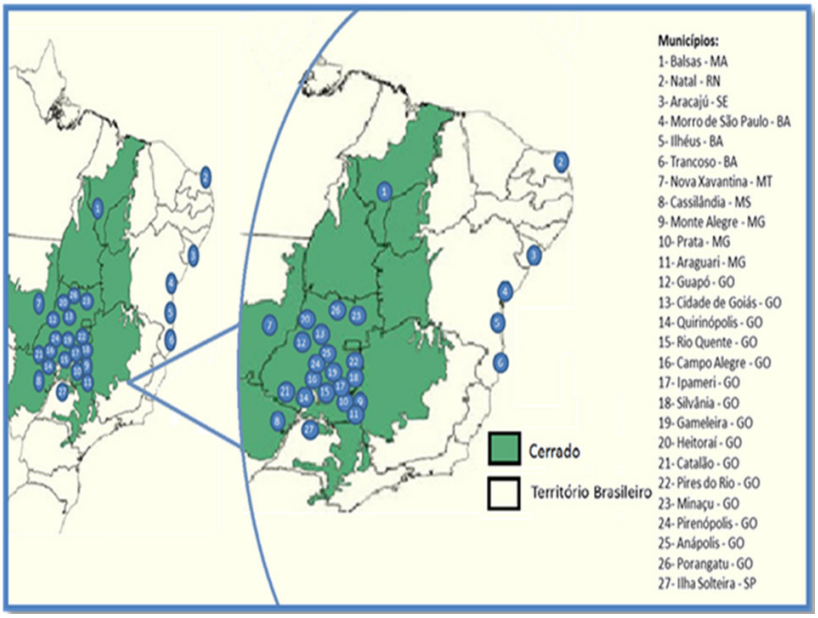

Figure 1. *Hancornia speciosa* geographic distribution in the Brazilian Cerrado biome (in green) and locations where the samples used in this study were collected.

PCR analysis

PCR was performed in an MJ Research PTC-200 thermocycler with the thermal gradient software to select the best annealing temperature for each marker. For EST-SSR experiments, PCR mixtures included 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM dNTPs, 0.2 mM of each primer, 0.5 U AmpliTaq Gold Polymerase (Life Technologies™), and 50 ng DNA in a 10-µL reaction volume. The PCR conditions were as follows: initial denaturation at 94°C for 10 min, followed by 35 cycles at 94°C for 30 s (denaturation), 50° to 65°C for 30 s (annealing; the temperature depended on the individual microsatellite marker; Table 1), extension at 72°C for 30 s, and a final extension at 72°C for 7 min. RAPD experiments used the same PCR mixture, except 0.5 U Taq Polymerase (Thermo Scientific) was used. The PCR conditions were as follows: initial denaturation at 94°C for 1 min, followed by 35 cycles at 94°C for 30 s (denaturation), 40° to 42°C for 30 s (annealing; the temperature depended on the individual microsatellite marker, Table 1), extension at 72°C for 30 s, and a final extension at 72°C for 7 min.

The PCR products were electrophoresed through 3% agarose gels in 1X TBE buffer containing ethidium bromide and photographed under UV light. PCR products were scored 1 for present and 0 for absent amplification and the data were entered into a binary matrix as discrete variables.

Data analysis

The SSR and RAPD products were scored 1 for present and 0 for absent to determine the number of amplified fragments, polymorphic fragments, and percentage of polymorphism. Genetic diversity across 34 samples was calculated using the GENALEX 6.5 program (Peakall and Smouse,

Table 1. Sequence of primers used for amplification of *Hancornia speciosa* DNA, using simple sequence repeats from expressed sequence tags (EST-SSRs) and random amplified polymorphic DNA (RAPD) markers.

Marker	ID	Sequence	Annealing temperature (°C)	Reference
SSR-RG01	gi|164561865	F: GGAACCAAGGATGTTAGAGTGG R: CTGCAACGGTTACTAGAGAGTAGAGC	50	Mishra et al., 2011
SSR-RG04	gi|164556897	F: CGTCAAGACCTACCCAGGAG R: GTCTCCTCCGTCACCAGAAA	50	Mishra et al., 2011
SSR-RG05	gi|164556756	F: CTGCTAGGCATGGTGGTGTAG R: GATCCCAGCGGTGACTCTTA	56	Mishra et al., 2011
SSR-RG07	gi|164556449	F: GAGGAGGTGTCTCTCATGCTG R: CGACCTCAACAGAAGGTTCG	50	Mishra et al., 2011
SSR-RG08	gi|164555839	F: AGAAGGAAGTGGTGGTGCTG R: GTTTACAGGGGGAGGAGGAG	56	Mishra et al., 2011
SSR-RG11	gi|164561349	F: GGCACGAGGCATCCTTACTC R: CCACAGCTCTGGTAGCTCCT	56	Mishra et al., 2011
SSR-RG12	gi|164561153	F: GGACAAGCTGGAGCAGCA R: CTGCAACCAGAGGCTTCC	50	Mishra et al., 2011
SSR-RG13	gi|164560959	F: CCGGAGGTGATGAGGTTCTG R: GAGGCTGCTTTGGAGGAG	56	Mishra et al., 2011
SSR-RG15	gi|164556819	F: GAGAGAGAGAGAGCGGCAAG R: GTGGGTCTCCCACAATAGCG	60	Mishra et al., 2011
SSR-RG18	gi|164561715	F: CATTCTCTTCTCGAGGCTTCTG R: ACCCCCATGACAGTCAAGATAG	53	Mishra et al., 2011
SSR-RG21	gi|164559832	F: CCCTTCCTGAGAGACTCAAATG R: CCAAGCACTCTTCATCTCAGG	53	Mishra et al., 2011
SSR-RG30	gi|164554493	F: GCCTCCAGTTACCCTTCCTC R: ACAGCAGGATCACCAAGACC	53	Mishra et al., 2011
RAPD-ITD04	-	TGATCCCTGG	37	Silva et al., 2011
RAPD-ITD11	-	ACGGATCCTG	37	Silva et al., 2011
RAPD-ITD13	-	CTACGGAGGA	37	Silva et al., 2011
RAPD-ITD14	-	GGCACTGAGG	37	Silva et al., 2011
RAPD-OPA03	-	AGTCAGCCAC	42	Lal et al., 2011
RAPD-OPC12	-	TGTCATCCCC	42	Lal et al., 2011
RAPD-OPD20	-	AACCCGGTCA	42	
RAPD-OPN15	-	CAGCGACTGT	42	Lal et al., 2011
RAPD-OPAF5	-	CCCGATCAGA	42	Lal et al., 2011
RAPD-OPAF15	-	CACGAACCTC	42	Lal et al., 2011
RAPD-ITD13/ITD11	-	CTACGGAGGA ACGGATCCTG	42	Silva et al., 2011
RAPD-ITD13/ITD14	-	CTACGGAGGA GGCACTGAGG	42	Silva et al., 2011
RAPD-ITD4/ITD11	-	TGATCCCTGG ACGGATCCTG	42	Silva et al., 2011
RAPD- OPA03/ OPAF5	-	AGTCAGCCAC CCCGATCAGA	42	Lal et al., 2011

2012). The data matrix for the combined markers was used to calculate the dissimilarity matrix using the Jaccard coefficient, according to the method of Sneath and Sokal (1973) using the DARwin V5.0 program (Perrier and Jacquemoud-Collet, 2006). We determined the genetic relationships of 34 samples using a dendrogram based on the unweighted pair-group method of averages (UPGMA) with the MEGA5 program (Tamura et al., 2011). In addition, the cophenetic correlation was measured to evaluate the degree of fit of the clusters in the dendrogram to the data in the dissimilarity coefficient matrix. Principal component analysis (PCA) was applied to all genotypes for the clustering analysis.

RESULTS

Morphological classification

The samples included five of the six morphological lineages of *H. speciosa*, identified according to Monachino (1945). *H. speciosa speciosa* samples had glabrous leaves, petioles 0.9

to 0.15 cm long, and leaf lamina up to 6 cm long and 2 cm wide. This botanical variety was collected in Natal (Rio Grande do Norte, RN), Aracaju (Sergipe, SE), Morro de São Paulo (Bahia, BA), Trancoso (BA), and Ilhéus (BA). *H. speciosa gardineri* samples had glabrous leaves, petioles from 0.3 to 0.5 cm long, and leaf blades 6 to 12 cm long and 3 to 6 cm wide. This botanical variety was collected in Guapó (Goiás, GO), Cidade de Goiás (GO), Quirinópolis (GO), Rio Quente (GO), Campo Alegre (GO), Ipameri (GO), Silvânia (GO), Heitoraí (GO), Catalão (GO), Pires do Rio (GO), Minaçu (GO), Pirinópolis (GO), Anápolis (GO), Porangatu (GO), and Ilha Solteira (São Paulo, SP). *H. speciosa pubescens* samples had short petioles, pubescent blades at the bottom, and leaves that were 6 to 12 cm long and 3 to 6 cm wide. This botanical variety was collected in Balsas (Maranhão, MA), Gameleira (GO), and Ipameri (GO). *H. speciosa maximiliani* samples had glabrous leaves, petioles about 0.8 cm long, and limbus 5 to 6 cm long and 2 to 2.5 cm wide. This botanical variety was collected in Prata (Minas Gerais, MG) and Araguari (MG). *H. speciosa cuyabensis* samples had petioles between 0.3 to 0.5 cm long, limbus 4 to 10 cm long and 1.5 to 3 cm wide, and calyx and corolla glabrous externally. This botanical variety was collected in Monte Alegre (MG). *H. speciosa lundii* samples had petioles 0.3 to 0.5 cm long, limbo 5 to 7 cm long and 3 cm wide, and pedicels pubescent. This botanical variety was collected in Nova Xavantina (Mato Grosso, MT) and Cassilândia (Mato Grosso do Sul, MS). Some samples were deposited at the State University of Goiás Herbarium (Universidade Estadual de Goiás, Anápolis, GO, Brazil).

Transferability of microsatellite markers of *C. roseus* to *H. speciosa*

The 12 primers tested were classified according to the PCR results: 75% (nine pairs of primers) amplified clear EST-SSR products, 16.7% presented nonspecific fragment amplification, and 8.3% did not amplify any fragments. Of the nine primer pairs that showed amplification, four were monomorphic and five were polymorphic. In all, 14 alleles were identified, ranging from two to three bands per locus. Table 2 showed the genetic characterization of five microsatellite loci in 34 mangabeira samples. While some of the heterozygous loci studied displayed a relative excess (F_{IS} < 0) or deficiency (F_{IS} > 0), the average genetic diversity (H_E = 0.226 ± 0.017) did not differ significantly from the observed heterozygosity (H_O = 0.194, ± 0039) when the standard error of the mean was considered. Thus, there was no standard or intra/interlocus distribution, as indicated by an average rate of inbreeding that did not differ from zero (F_{IS} = 0.119 ± 0.157).

Table 2. Genetic characterization of five microsatellite loci in 34 *Hancornia speciosa* samples.

Locus	Alleles (bp)	N_A	H_O	H_E	F_{IS}
RG07	159, 177, 193	3	0.095	0.260	0.625
RG08	63, 75, 99	3	0.273	0.255	-0.119
RG12	113, 177, 195	3	0.107	0.168	0.351
RG21	193, 313	2	0.273	0.241	-0.158
RG30	326, 447, 603	3	0.222	0.205	-0.106
Total		14	0.194	0.226	0.119
Standard deviation		-	0.039	0.017	0.157

N_A = number of alleles; H_O = observed heterozygosity; H_E = expected heterozygosity; F_{IS} = inbreeding coefficient.

Estimation of the genetic diversity of the species and association with geographical distribution

Analysis of the Jaccard dissimilarity coefficients, calculated on the basis of the presence

and absence of RAPD and EST-SSR markers, showed that the similarity coefficients were highly similar, indicating a high degree of association between the dendrogram cluster and dissimilarity matrices. Due to this result, both markers were used to estimate the genetic diversity. The average dissimilarity obtained was 0.80, with an observed maximum dissimilarity of 1.0 and a minimum of 0.34. The analysis between the genetic dissimilarity and geographic distance of *H. speciosa* from different regions was positively correlated but not significant (correlation coefficient r = 0.17). Genetic cluster analysis was conducted using the UPGMA. Cluster analysis divided the 34 genotypes into six main groups (Figure 2). There was no correlation between genetic diversity and geographical distribution, as lineages collected from distant regions were grouped together. For example, samples from Bahia (Morro SP 1) and São Paulo (Ilha Solteira) were grouped with different samples from Goiás; samples from Maranhão (Balsas), Rio Grande do Norte (Natal), and Goiás (Guapó) were grouped with Minas Gerais (Monte Alegre); samples from Mato Grosso (Nova Xavantina) were grouped with Bahia (Morro SP2); and samples from Sergipe (Aracaju), Goiás (Pires do Rio), and Bahia (Trancoso) were grouped with Minas Gerais (Prata). This result suggested a non-spatial structuring of the variability for the different lineages of *H. speciosa.*

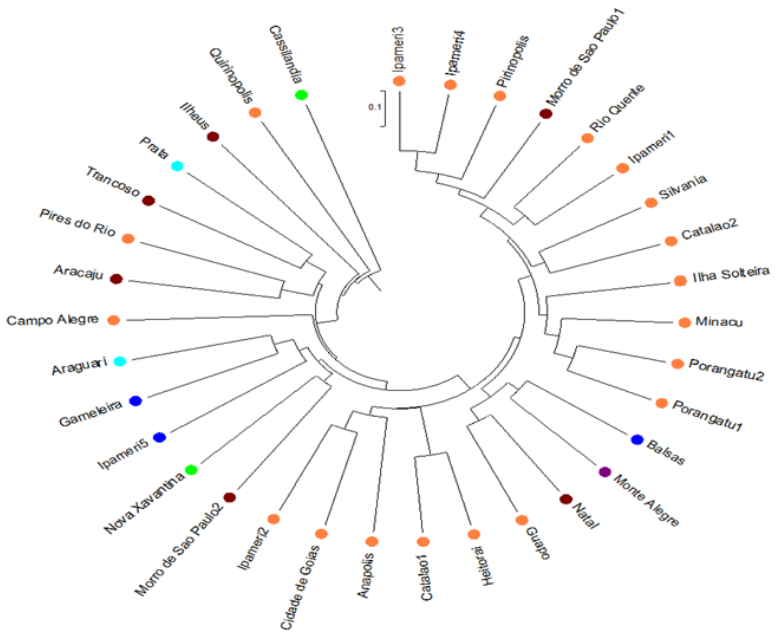

Figure 2. Genetic distance dendrogram for *Hancornia speciosa* samples, created using simple sequence repeat and random amplified polymorphic DNA markers.

Assessment of the genetic relationships among different *H. speciosa* varieties

Analysis of molecular variance and PCA were performed to evaluate the genetic variability among the botanical varieties. The molecular variance between the botanical varieties was not significant (0.03) and the PCA did not detect any differentiation among the botanical varieties (Figure 3). Therefore, the markers employed in this study were not able to distinguish variation as well the morphological traits could distinguish differences.

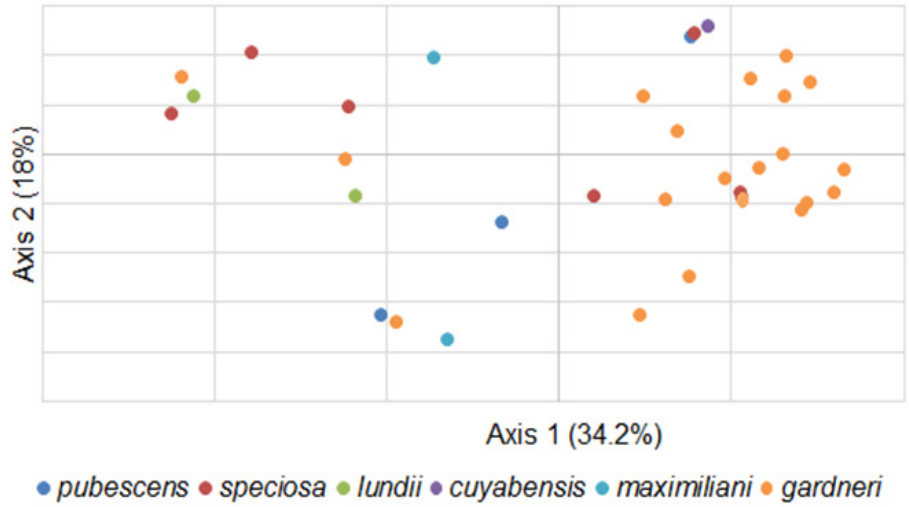

Figure 3. Genetic relationships among botanical varieties of *Hancornia speciosa* using principal component analysis.

DISCUSSION

The main objective of this study was to generate specific markers for use in future conservation and genetic improvement studies involving *H. speciosa*. To date, there have been very few studies of genetic diversity in mangabeira populations, and all of them use RAPD markers as the molecular tool (Moura et al., 2005, 2011; Costa et al., 2011; Silva et al., 2011) due to the absence of the species genome information. As noted, only six sequences of *H. speciosa* appear in the GenBank database. In addition, all the studies of *H. speciosa* that have been cited here based their analysis on regional or local samples and evaluated only small dispersion areas of this species. The present analysis, in contrast, used samples collected from an extent dispersion area that included nine Brazilian states.

One of our secondary objectives was to transfer SSR markers from *C. roseus* to *H. speciosa.* We used the EST-SSR markers described by Misha et al. (2011), which were selected due to their easy availability, hypervariability, and suitability for high throughput analysis, high polymorphism, and transferability compared with other available markers. Our EST-SSR results showed a high transferability frequency (75%) of *C. roseus* to *H. speciosa*, which indicates that other SSR markers described by Misha et al. (2011) may be used to detect the genetic variability of *H. speciosa.* However, as the EST is a highly conserved DNA region, there is a high probability of finding low genetic variability among closely related species. Indeed, our results showed this low locus variability, with four monomorphic markers and five markers with two or three alleles per locus. The low H_E value (0.2) obtained in our analysis is similar to that reported in a previous study (Costa et al., 2011) and suggested low genetic diversity in mangabeira populations. The polymorphic loci are important indicators of the level of genetic variation in a given area. A specimen with a high rate of polymorphism in the locus has a stronger capability for adaptation to environment, whereas one with a weaker capability will be eliminated by natural selection.

The Jaccard dissimilarity index and analysis of geographic distances indicated that the genetic variability was not spatially structured. This is probably due to the low number of markers

used and the low genetic variability detected by the markers that were employed.

Classification of *H. speciosa* varieties is often hindered by the great morphological diversity of the species. Most taxonomists still use the Monachino (1945) classification, which identified six lineages for *H. speciosa* (*speciosa, gardineri*, *pubescens*, *maximiliani*, *cuyabensis*, and *lundii*). However, a recent reclassification (Koch et al., 2014) suggested that there were many synonymies and that mangabeira may be classified into only two lineages (*speciosa* and *pubescens*). We compared the genetic variability among the *H. speciosa* lineages and verified whether there was a significant difference in the botanical varieties evaluated. We observed a low divergence between the botanical varieties and an absence of specific fragments for different genotypes. In conclusion, the markers used in this study are not able to distinguish botanical varieties for *H. speciosa*.

The mangabeira is one of the fruiting trees that is most threatened with extinction in the Brazilian Cerrado and the Northeast, due to the large reduction in its distribution. The reduction has occurred as a result of deforestation, land speculation, and expansion of the agricultural frontier for grain crops and pastures (Lederman et al., 2000). It is necessary to characterize the distribution and the genetic composition of the different populations in order to outline conservation and breeding projects. Our contribution to this study was to identify reliable molecular markers to be used in future research and to show the low genetic variability among botanical varieties of *H. speciosa*.

Conflicts of interest

The authors declare no conflict of interest.

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