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RESEARCH

The genetic structure of *Hancornia speciosa* (Apocynaceae) reveals two botanical varieties

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Abstract

Hancornia speciosa is an important native fruit tree species from Brazil, distributed in the Amazon, Cerrado, Caatinga and Atlantic Forest biomes. Six botanical varieties of *H. speciosa* have been described, although currently only two varieties (*pubescens* and *speciosa*) are recognized. However, there are no phylogenetic studies demonstrating the genetic relationships among the putative varieties. Here, we build a phylogenetic tree using *trn*H–*psb*A sequences from the chloroplast genome and analyze the genetic structure of six microsatellite loci from the nuclear genome. The results for the chloroplast sequence revealed 11 haplotypes, and the phylogenetic tree identified two main clades. The first clade included the botanical variety *H. speciosa* var. *speciosa*, while the other clade comprised the remaining putative varieties, which we recognized as *H. speciosa* var. *pubescens*. The microsatellite analysis also revealed two groups, one containing the botanical variety *H. speciosa* var. *speciosa*, and the other with the other putative botanical varieties. We concluded that the genetic diversity in *H. speciosa* is organized into two phylogenetic groups, supporting the classification of two botanical varieties.

Keywords Mangabeira · Microsatellite · Phylogeny · Taxonomy

Introduction

The mangabeira (*Hancornia speciosa*) is a species that occurs in Brazil, being endemic to the Amazon Forest, Cerrado, Caatinga and Atlantic Forest biomes (Maia et al. 2022, Collevatii et al. 2018, Silva et al. 2023). *Hancornia speciosa* belongs to the family Apocynaceae and is an economically important fruit species, with the fruit consumed *In natura* and as juice, sweet, ice cream, jams and jelly, liquor and medicinal purposes (Oliveira and Aloufa 2021).

Six botanical varieties were described by Monachino (1945)*:* (1) *H. speciosa* var. *speciosa* Gomes, (2) *H. speciosa* var. *maximiliani* A.DC., (3) *H. speciosa* var. *cuyabensis* Malme, (4) *H. speciosa* var. *lundii* A.DC., (5) *H. speciosa* var. *gardneri* (A.DC.) Muell.Arg. and (6) *H. speciosa* var. *pubescens* (Nees. & Martius) Muell.Arg. (Monachino 1945). These botanical varieties were distributed across the Atlantic Forest, Cerrado and Caatinga biomes, and the identification of these botanical varieties were based on morphological characteristics; however, the genetic relationships and possible hybrids among these varieties remain unclear (Collevatii et al. 2016; Flores et al. 2017). Recently, the classification in the Flora of Brazil (Koch et al. 2015) lists only two botanical varieties: *Hancornia speciosa* Gomes var. *speciosa* and *Hancornia speciosa* var. *pubescens* (Nees & Mart.) Müll. Arg.

Genetic studies in the species have been focused on genetic diversity, as in Collevatti et al. (2018), using SSR markers in accessions from the central region of Brazil,

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including the two varieties accepted by Koch et al. (2015). The results showed four different groups; however, they do not agree with the classification of only two botanical varieties, partially supporting Monachino's classification (1945). Maia et al. (2022) analyzed genetic diversity using SSR markers in accessions from the central and Northeast regions of Brazil, finding two groups, providing support for the two botanical varieties, and the genetic diversity using morphological data has supported the classification of the two botanical varieties (Morais et al. 2022; Morais et al., 2023).

However, there are no phylogenetic studies supporting this classification with sequence data, such as chloroplast markers, the existing studies have utilized SSR or morphological data. Here, we are exploring the hypothesis that the botanical varieties, as described by Monachino (1945), do not have phylogenetic support. To assess this hypothesis, we collected *Hancornia speciosa* accessions from the Cerrado, Caatinga and Atlantic Forest biomes and analyzed them using nuclear SSR markers and chloroplast genome sequences.

Material and methods

Sampling and DNA extraction

Plants from natural populations of *H. speciosa* are present in three localities in the Cerrado, two in the Caatinga and three in the Atlantic Forest biome (Fig. 1). A total of 176 individuals were sampled, being sample by population ranging of 20–24 individuals. The samples correspond to a large area of the species distribution, allowing us to obtain robust inferences about the evolution of the species. These samples had not been previously identified as botanical varieties. To identify the samples as botanical varieties described by Monachino, nine individuals identified as the botanical varieties *gardneri*, *pubescens* and *cuyabensis* (three individuals for each botanical variety) were sampled from the Active Germplasm Bank of Mangaba of the Universidade Federal de Goiás, along with one sample of the *speciosa* from northeastern Brazil. These samples identified as botanical varieties were used as references in molecular identification, employing sequences from the chloroplast obtained in this study. DNA extraction was performed according to the protocol in Doyle and Doyle (1990), and its integrity verified in 1% (w/v) agarose gel electrophoresis stained with ethidium bromide.

cpDNA: amplification and sequencing

The amplification was performed for three regions of cpDNA (*trn*H–*psb*A intergenic spacer and *mat*K and *rbc*L genes) using primers described by Scarcelli et al. (2011). The reactions were performed in a total of 50 µL, containing 5 µL reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1.25 U *Taq* DNA polymerase, primers (0.5 µM each) and 200 ng DNA. Amplifications were performed with an initial denaturation at 94 °C for 3 min, followed by 40 cycles at 94 °C for 30 s and annealing at 48–60 °C for 30 s, extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. PCR reactions were performed in a thermal cycler (BioCycler), and PCR products were subjected to electrophoresis in 1% agarose gel to confirm amplification. PCR products were sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems®) and analyzed by electrophoresis in a 3500 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA, USA).

cpDNA: haplotype analysis

Sequences were edited using the Geneious R9 software and aligned with MAFFT (Katoh et al. 2002) implemented in Geneious. The alignment was performed using the default settings and optimized manually when necessary. Haplotype diversity (h), nucleotide diversity (π) (Rogers and Harpending 1992) and mismatch distribution (Rogers and Harpending 1992) were calculated using the DnaSP 5.10.01 software (Librado and Rozas 2009).

cpDNA: Molecular identification of botanical varieties

Nine samples from the Active Germplasm Bank of Mangaba at the Universidade Federal de Goiás, identified as the botanical varieties *gardneri*, *pubescens* and *cuyabensis*, along with a sample of the *speciosa* variety from northeastern Brazil, were used as references for identification. The sample identification was distributed as described in supplementary Online Resources 1. After analyzing all samples, sequences were aligned using MAFFT, revealing 11 haplotypes (H1–H11). These haplotypes were then utilized for phylogenetic and PCA analyses.

cpDNA: phylogenetic and PCA analyses

Sequences were aligned with MAFFT (Katoh et al. 2002) implemented in Geneious. The alignment was performed using the default settings and optimized manually when necessary. The best-fit substitution model was obtained using the MEGA7 (Kumar et al. 2016) to help guide the selection of a model for the molecular evolution of haplotypes. Also, the GTR + I + G model was used for subsequent phylogenetic inferences using Bayesian analysis. This analysis was performed using Beast v1.8.0 (Drummond and Rambaut 2007), and the subsequent distribution was approximated

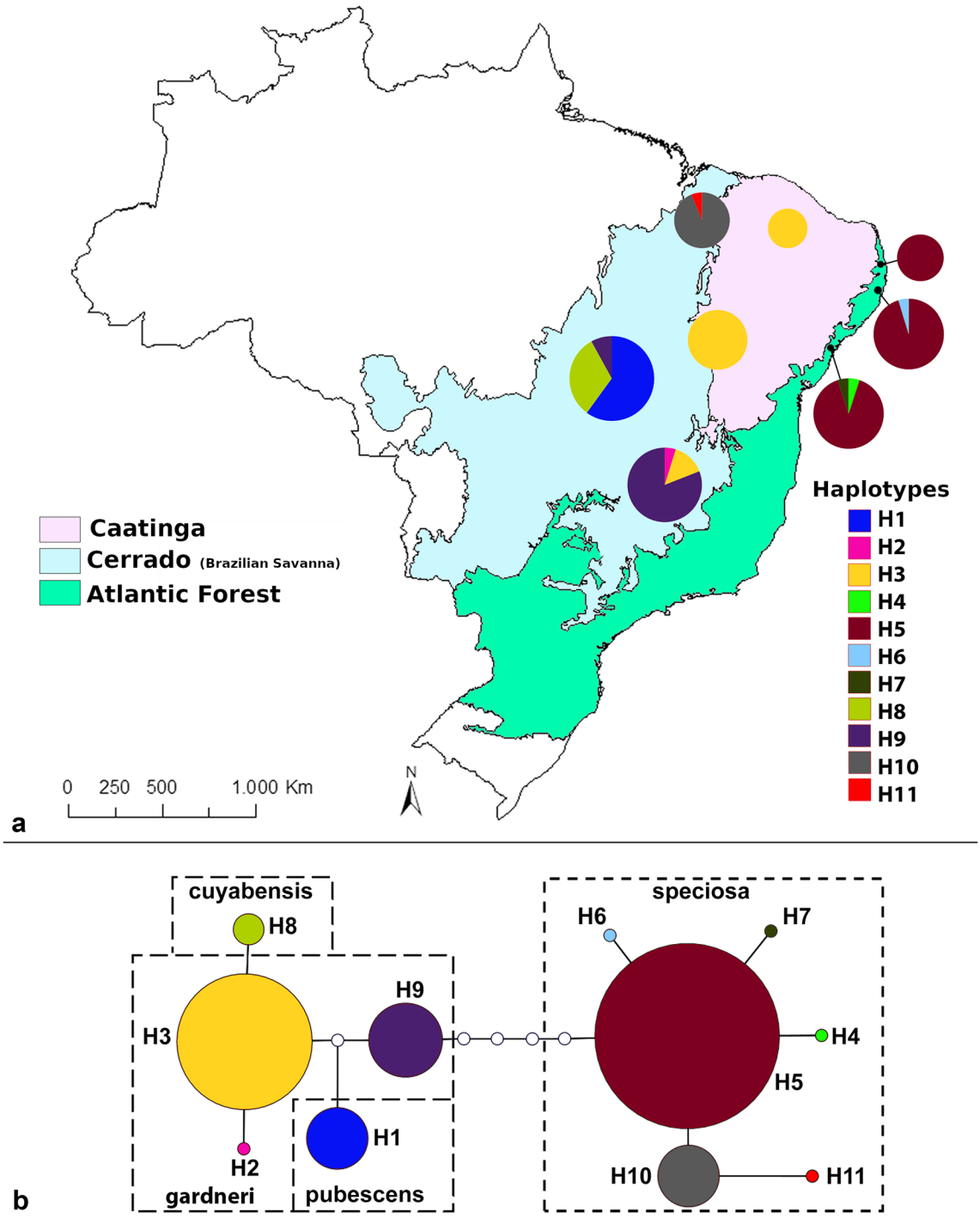

Fig. 1 Patterns of distribution of *Hancornia speciosa* haplotypes. (**a**) Frequency of haplotypes for the *trn*H-*psb*A intergenic spacer per region site. The size of the pie graphic corresponds to the size of the sample; the colored portions represent the Caatinga biome (pink), Cerrado biome (sky blue) and Atlantic Forest biomes (green). The colors on the pie graph correspond to haplotypes following the names in the legend. (**b**) Haplotypes network obtained by maximum parsimony statistics using the haplotype software. The sizes of the circles correspond to the proportion of each haplotype, and the names correspond to the botanical varieties

using the Markov Chain Monte Carlo method for 10 million generations. The convergence of parameters was assessed using the Tracer 1.5 software (Rambaut and Drummond 2018). Genealogical relationships between haplotypes were estimated using the maximum parsimony statistical method (Templeton et al. 1992), implemented in the R package

haplotypes. The genetic relationships among the identified haplotypes for each species were also investigated using principal component analysis (PCA). This was performed with the function 'glPca' in from the R package adegenet (Jombart and Ahmed 2011), and graphical obtained using the 'plot3D and 'rgl' functions from the R package plot3D (Soetaert 2021).

cpDNA: genetic structure

The analysis of genetic structure was performed using the software structure (Pritchard et al. 2000), implemented in the R package strataG (Archer et al. 2016). The genetic structure analysis was conducted with 100 replications and k groups ranging from 1 to 4. After phylogenetic, PCA and structure analyses, two genetics groups were identified, which we classified as two botanical varieties: *pubescens* and *speciosa*.

The analysis of molecular variance (AMOVA) was performed for explore the distribution of genetic variation whiting and between the *pubescens* and *speciosa* groups. The AMOVA was performed using de function 'gl.amova' from R package dartR (Gruber et al. 2018). The estimative of Fst for differentiation between botanical varieties was obtained using 'pairwiseTest' function from R package strataG (Archer et al. 2016).

nSSR: amplification and genotyping

A set of six SSR markers developed by Rodrigues et al. (2015) was used for polymorphism analysis (Online Resources 2). The reactions were performed in a total volume of 50 µL containing 5 µL reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1.25 U *Taq* DNA polymerase, primers (0.5 µM each) labeled with 5-FAN and 200 ng DNA. Amplifications were performed with an initial denaturation at 95 °C for 10 min, followed by 15 cycles at 94 °C for 30 s and annealing at 55–60 °C for 1 min; 20 additional at 89 °C for 1 min and annealing at 55–60 °C for 1 min, extension at 72 °C for 1 min and a final extension at 72 °C for 1 h. PCR reactions were performed in a thermal cycler (BioCycler), and PCR products were subjected to capillary electrophoresis in a 3500 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA, USA), using the GeneScan™ 600 Liz® (Applied Biosystems) as a size standard.

nSSR: statistical analysis

The allele number (*Na*), expected heterozygosity (H_E), observed heterozygosity (H_O) and deviation from Hardy–Weinberg equilibrium (HWE) were obtained using the R package Pegas (Paradis et al. 2016) and the R package PopGenReport 3.0 (Adamack and Gruber 2014). The minimum-spanning networks were produced in the R package poppr 2.6.1 (Kamvar et al. 2014), using the genetic distance measure of Bruvo et al. (2004) to examine the genetic relationships among multilocus microsatellite genotypes, and PCA was produced in the R package adegenet (Jombart and Ahmed 2011). The genetic structure, AMOVA and Fst for differentiation between botanical varieties were performed using the methods described for cpDNA. For the SSR analyses, the *pubescens* and *speciosa* varieties were used as genetic groups.

Results

Haplotype networks and phylogenetic analyses

Of the three chloroplast regions sequenced, only the *trn*H–*psb*A region exhibited polymorphism in the 151 samples of the 176 individuals that were sampled, resulting in 427 bp length. The 25 samples were not analyzed due to the low quality of the sequences. The analysis of the sequences revealed 11 haplotypes, designated with the letter "H" followed by numbers from 1 to 11 (Online Resources 3). The haplotype diversity (h) was 0.683 ± 0.022 and the mean nucleotide diversity (π) was 0.00458 ± 0.004. Following the botanical varieties as described by Monachino, the haplotypes H1 being specific to *H. speciosa* var. *pubescens*, H2, H3 and H9 to *H. speciosa* var. *gardneri;* H8 to *H. speciosa* var. *cuyabensis* and H4, H5, H6, H7, H10 and H11 being specific to *H. speciosa var. speciosa* (Figs. 1 and 2).

Markedly, the only haplotype dispersed in the Caatinga biome was H3 (belonging to *H. speciosa* var. *gardneri*), which was also observed in the Cerrado, along with H2 and mainly H9, of the same variety (Fig. 1). Additionally, haplotypes H1 (*H. speciosa* var. *pubescens)*, H8 (*H. speciosa* var. *cuyabensis)* and H9 (*H. speciosa* var. *gardneri*) were dispersed in the Cerrado biome (Fig. 1).

The relationship among haplotypes using phylogenetic tree and PCA strong evidence for the delineation of botanical variety within *H. speciosa* (Fig. 2). *Hancornia speciosa* var. *speciosa* formed a distinct group, while the putative varieties *H. speciosa* var. *gardneri*, *H. speciosa* var. *cuyabensis* and *H. speciosa* var. *pubescens* clustered together as another group. Within the pubescens group, the putative *H. speciosa* var. *gardneri* exhibited shared haplotypes with both *pubescens* and *cuyabensis* (Fig. 2b). When analyzed using the PCA approach, the results displayed two clearly delineated groups, with three components explaining 80.98% of variation (Fig. 2a). According to the network results, haplotypes from *H. speciosa* var. *speciosa* exhibited four nucleotide substitutions when compared with *pubescens* group, while within of *pubescens* and *speciosa* group showed one or two variation (Fig. 1b).

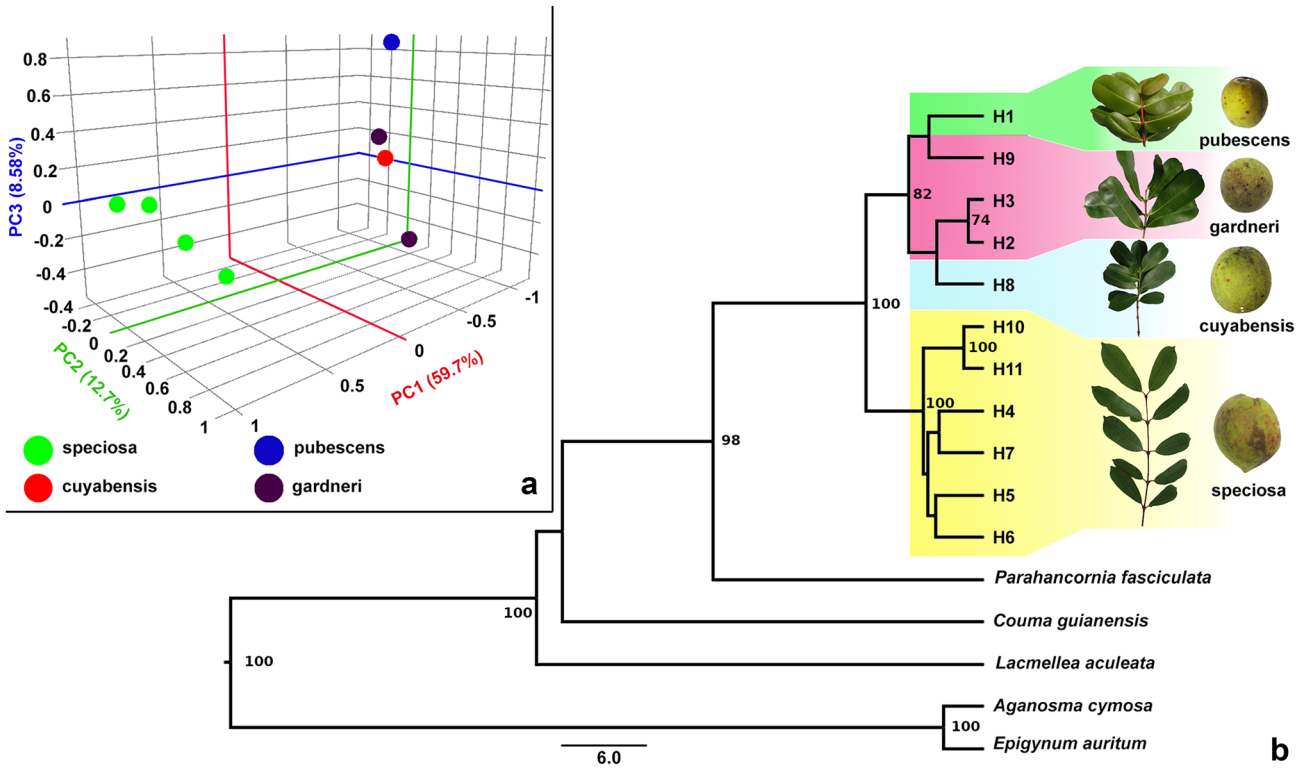

Fig. 2 Genetic relationships among haplotypes of the *trn*H-*psb*A sequence. (**a**) Principal component analysis (PCA) reveals a three-dimensional distribution of *Hancornia speciosa* genotypes according to the botanical varieties. (**b**) Phylogenetic analysis using Bayesian analysis, with support inferred by posterior probability (here, represented in percentage). Support values < 50% were suppressed

Genetic structure for cpDNA

The sequences were analyzed for genetic structure, considering k groups ranging from 1 to 4. The results indicated that genetic diversity is organized into two groups (Fig. 3), which aligns with the findings from phylogenetic and PCA analyses. These two groups correspond to the *pubescens* and *speciosa* botanical varieties. Further analysis using AMOVA revealed that the variation between the *pubescens* and *speciosa* varieties accounted for 96.33% of the total variation. Additionally, genetic differentiation, as measured by Fst according to Weir and Cockerham (1984), detected an Fst value of 0.84 between the two varieties (Table 1).

SSR diversity

The results displayed 138 alleles with 23 alleles on average by *locus*, ranging from 13 to 41 alleles/locus (Online Resources 2). The observed heterozygosity (H_O) ranged from 0.34 to 0.79, and the expected heterozygosity (H_E) ranged from 0.66 to 0.91. The genetic relationships among genotypes using a minimum-spanning network, revealed that majority of genotypes from *H. speciosa* var. *speciosa* formed a large cluster (called *speciosa*), while the other cluster was constituted by individuals from the *pubescens* (including putative botanical varieties described by Monachino: *H. speciosa* var. *gardneri*, *H. speciosa* var. *cuyabensis* and *H. speciosa* var. *pubescens* (Fig. 4a).

The relationship among genotypes, as illustrated by PCA, revealed clear groups comprised of individuals from *speciosa*, and another group formed by individuals from *pubescens* (Fig. 4b), thus reinforcing the findings sequence data.

Population genetic structure for nSSR

The microsatellites results were analyzed in the genetic structure, considering k groups ranging 1–4. The results showed the genetic diversity is organized in two groups (Fig. 3), consistent with the findings obtained for cpDNA, representing the *pubescens* and *speciosa* botanical varieties. The distribution of genetic variation was further analyzed through AMOVA, which demonstrated that the variation between the *pubescens* and *speciosa* varieties accounted for 29.95% of the total variation. Moreover, genetic differentiation, calculated using Fst according to Weir and Cockerham (1984), revealed Fst value of 0.22 between the two varieties (Table 1).

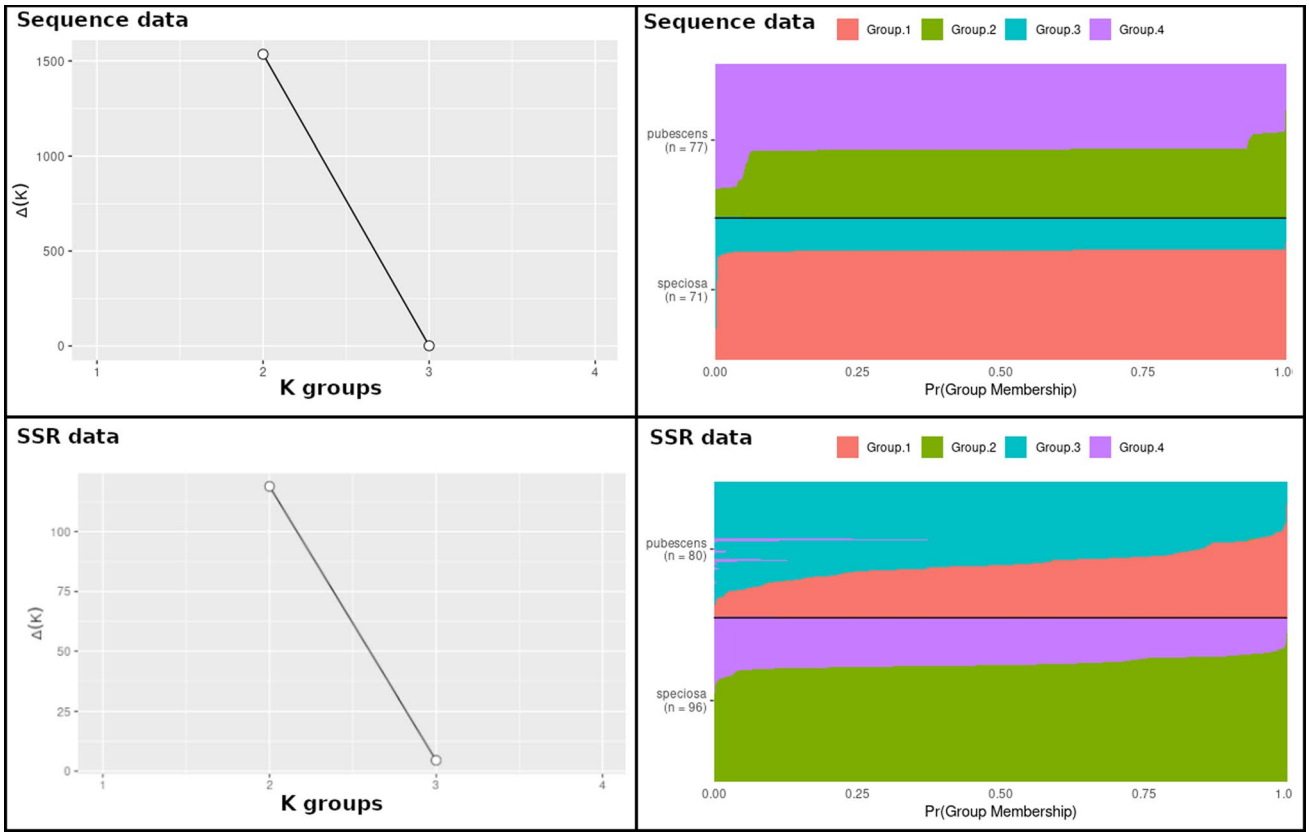

Fig. 3 Genetic structure for cpDNA and microsatellites obtained by STRUCTURE software

Table 1 Analysis of molecular variance (AMOVA) between pubescens and speciosa varieties

Source of variation	cpDNA		nSSR	
	Variance Components	Percentage of variation	Variance Components	Percentage of variation
Among varieties	0.132	96.33	1.12	29.95
Within varieties	0.005	3.67	2.62	70.05
Total	0.167	100	3.74	100
	F_{ST}	0.84 ($p < 0.001$)*	F_{ST}	0.22 ($p < 0.001$)*

*Fst fromWeir and Cockerham (1984)

Discussion

Hancornia speciosa was initially categorized into six botanical varieties by Monachino in 1945. However, more recently, Koch et al. (2015) reclassified it into two botanical varieties. To date, no phylogenetic studies have been conducted on this species. In this study, we present the first phylogenetic analysis using chloroplast DNA sequences and examine the genetic structure using SSR data. Our findings show clear genetic differentiation among varieties of *H. speciosa*, of which *H. speciosa* var. *speciosa* shows high differentiation and distinct clade when compared with the putative botanical varieties *H. speciosa* var. *cuyabensis*, *H. speciosa* var. *gardneri* and *H. speciosa* var. *pubescens*. This clear differentiation was observed using both chloroplast DNA sequence and SSR data.

Among the varieties described by Monachino, there was a lower distinction, in which the *H. speciosa* var. *cuyabensis* was displayed in one clade and *H. speciosa* var. *pubescens* in another clade; however, *H. speciosa* var. *gardneri* showed haplotypes close to *H. speciosa* var. *cuyabensis* or *H. speciosa* var. *pubescens,* suggesting that *H. speciosa* var. *gardneri* may have been originated by cross-pollination. The hybridization among *H. speciosa* varieties was demonstrated by Collevatti et al. (2016), showing that there is gene flow

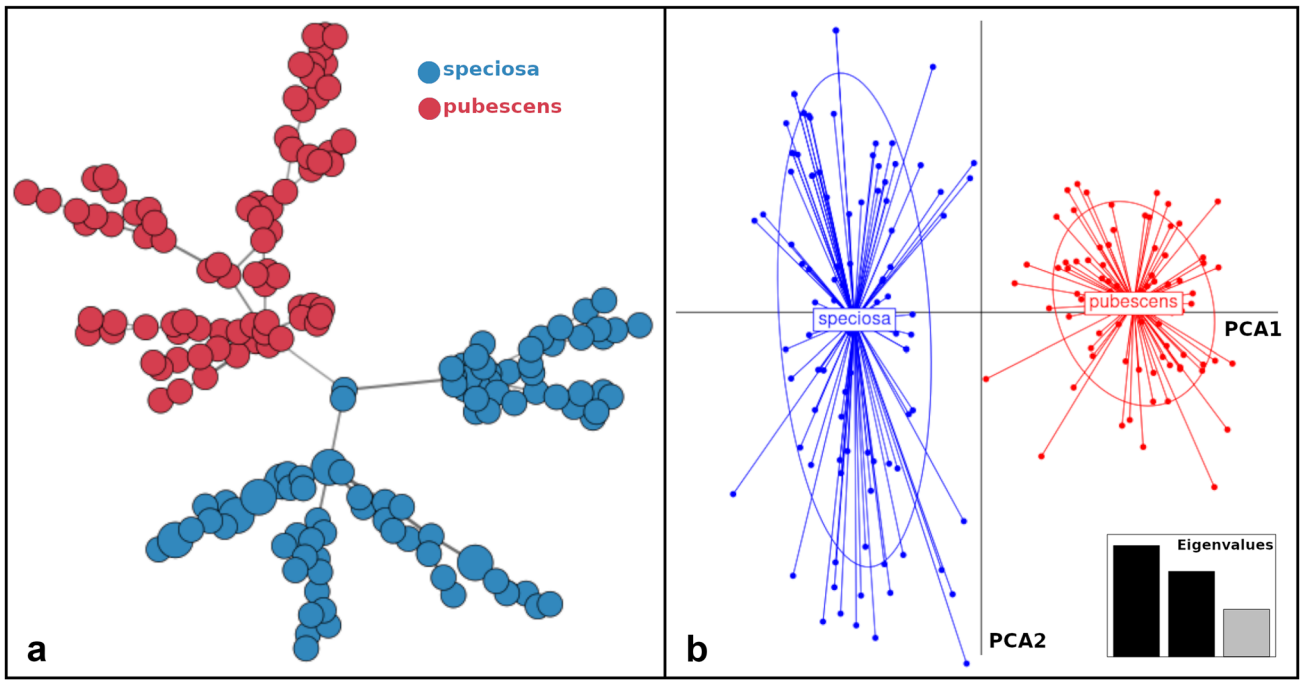

Fig. 4 Genetic relationships among genotypes for SSR loci. (**a**) Minimum-spanning network depicting the distribution of *Hancornia speciosa* with multilocus microsatellites. (**b**) Principal component analysis (PCA) reveals a two-dimensional distribution of *H. speciosa* genotypes

among putative botanical varieties. These results are very important for breeding, suggesting that crosses between different genetic groups occur spontaneously. We recommend further studies using a genomic approach to clarify whether spontaneous hybridization occurs or if there is variation within the *pubescens* variety.

The high similarity among individuals from the Cerrado biome was observed by Collevatti et al. (2018) using SSR data, in which the varieties *H. speciosa* var. *cuyabensis*, *H. speciosa* var. *gardneri* and *H. speciosa* var. *pubescens* generally clustered together. Also, considering the similarities between the three varieties (*pubescens*, *gardneri* and *cuyabensis*) in this study, all three belong to the same clusters in both cpDNA and nSSR analysis. Two botanical varieties have also been recognized by Koch et al. (2015), which belong to the *H. speciosa* var. *speciosa and H. speciosa* var. *pubescens*. Studies using SSR data (Collevatti et al. 2018) suggested partial support for Monachino's classification at least for the four sampled varieties; however, our study used samples from Caatinga and Atlantic Forest biomes and two different genetic data (sequence data from chloroplast and SSR data from the nuclear genome).

Recently, study using SSR molecular markers analyzed the genetic diversity in accessions from the central and Northeast regions of Brazil, finding two groups, providing support for the two botanical varieties (Maia et al. 2022). Additionally, genetic diversity studies using morphological data have also endorsed the two-variety classification (Morais et al. 2022; Morais et al., 2023). Chemometric analyses further demonstrated that *H. speciosa* var. *speciosa* differs from other varieties (Flores et al. 2017), aligning with our findings that support the identification of two botanical varieties: *H. speciosa* var. *speciosa* from the Atlantic Forest and *H. speciosa* var. *pubescens* from central and northern Brazil.

In this study, we employed three chloroplast regions, but only one (*trn*H–*psb*A) exhibited polymorphisms. This suggests that *H. speciosa* has limited variation in chloroplast sequences. However, the *trn*H-*psb*A region provided evidence that the botanical varieties described by Monachino lack phylogenetic support. To strengthen our conclusions, we also utilized SSR data from the nuclear genome, and the results were consistent with the sequence data. We concluded that *H. speciosa* exhibits two main genetic groups, and Monachino's classification lacks phylogenetic support, suggesting the existence of only two botanical varieties.

Information on Electronic Supplementary Material

Online Resource 1. The distribution of *Hancornia speciosa* populations is shown. Blue crosses represent the species' distribution, while red crosses denote the sampled individuals. The colors on the map correspond to the Brazilian biomes.

Online Resource 2. *Loci* described by Rodrigues et al. (2015) utilized in the study. Alleles size (bp), numbers allele, observed heterozygosity (*Ho*) and expected heterozygosity (*He*).

Online Resource 3. Position sites for SNPs and indels for the *trn*H-*ps*bA intergenic spacer. Ft represent frequency of the haplotype.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00606-024-01919-w.

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Authors' contributions J.T. and A.K.M. conducted the experiment. L.J.C, J.F.S.J and E.F.S conceived the project. C.A. analyzed the results and drafted the manuscript. All authors contributed to the final manuscript draft.

Data availability Haplotype sequence data for intergenic regions have been submitted to GenBank (MN249239 to MN249393).

Declarations

Conflict of interest The authors declare no competing interests.

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