

## Chloroplast diversity of *Casearia grandiflora* in the Cerrado of Piauí State

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**ABSTRACT.** *Casearia grandiflora* (Salicaceae) is a typical Cerrado species adapted to disturbed environments, making it useful for restoration projects. Knowledge of genetic diversity is important for establishing conservation strategies for this species. This study aimed to compare chloroplast haplotype diversity and structure of *C. grandiflora*, under the assumption that protected areas hold greater genetic diversity than disturbed areas. The populations studied are from Parque Nacional de Sete Cidades Conservation Unit and from the surroundings of the city of Cocal de Telha, both located in the State of Piauí. Molecular analyses were performed with seven chloroplast

microsatellite loci. The number of private haplotypes and haplotype diversity were higher in the conservation unit, which reinforces the importance of these areas in maintaining biodiversity. Analysis of molecular variance showed that most of the genetic variation is found within populations, with a moderate divergence between them ( $F_{ST} = 0.14$ ). The Bayesian analysis and discriminant analysis of principal components suggested that populations are not structured, revealing that a set of individuals from Parque Nacional de Sete Cidades were more divergent within populations than between them. Since literature has little information on *C. grandiflora*, the results of this study provide important contribution to a better understanding of the specie's genetic diversity.

**Key words:** Chloroplast microsatellites; Genetic structure; Haplotype networks; Parque Nacional de Sete Cidades

## INTRODUCTION

The Brazilian Cerrado holds forests, savannas, and grasslands, comprising 11 major phytophysiognomic types, showing a gradient from open fields to dense forest formations. These formations are controlled by climate interactions, soil type, and disturbance factors such as fires and herbivory (Tarazi et al., 2013).

Due to the abundance of species and their high degree of endemism, the Cerrado is considered a hotspot for global biodiversity conservation (Myers et al., 2000); however, it is the Brazilian most threatened biome.

The conservation is a necessary condition for maintaining biodiversity levels and is an important component for the sustainability of natural populations (Moura et al., 2012). The fragmentation of natural habitats and the spatial isolation of populations may cause changes in basic evolutionary processes, such as genetic drift, selection, and migration, resulting in changes in their genetic diversity and structure (Sebbenn et al., 2011).

Several studies evaluated the genetic structure of native plant populations in the Cerrado (Collevatti et al., 2010; Telles et al., 2010; Tarazi et al., 2010; Martins et al., 2011; Moura et al., 2011). These studies aimed at determining the distribution of diversity in populations of conservation units and inferring on historical events related to the effects of human activities on ecosystems fragmentation.

In view of this, there is need to establish methodologies for the preservation and maintenance of genetic diversity of the Cerrado populations. In this context, molecular markers are important tools used in genetic research to assess diversity in natural populations. Chloroplast microsatellites are among the molecular markers that have contributed to this issue. Chloroplast DNA (cpDNA) is a maternally inherited marker that undergoes little or no recombination, and exhibits a high level of genetic diversity and significant population structure (Petit et al., 1993; Liu et al., 2016).

Piauí is the state with the highest representation of the Cerrado in the Northeast Brazil, where the biome occupies about 12 million ha of which 70.4% are in your domain area and 29.6% in the transition area (Matos and Felfili, 2010). This state has three Cerrado conservation units: the Ecological Station Uruçuí-A, the Nascentes do Rio Parnaíba, and Parque Nacional de Sete Cidades.

The Parque Nacional de Sete Cidades, located in the northeastern portion of Piauí state, contains a mosaic of physiognomies that occur along its 6,221 ha. It is a focal for studies, particularly the long-term ones, because it is a federal conservation unit of integral protection (Matos and Felfili, 2010). The vegetation of the park is characterized by great diversity of communities, occurring in mosaics included in areas of Cerrado and Cerrado/Caatinga ecotones (Mendes et al., 2012).

*Casearia grandiflora* is a relatively important Cerrado species, appearing in several different surveys (Machado and Oliveira, 2000). Its flowers are greenish white, with about 7 mm. Its leaves contain diterpene and clerodanicosides, which can be used to treat wounds and snake bites (Xia et al., 2015). It easily grows in disturbed areas, such as roadsides. Despite having a wide distribution making it useful for restoration projects, there are no reports on the biological and genetic characteristics of this species.

This study aims to evaluate the diversity and structure of two populations of *C. grandiflora* of Piauí Cerrado with chloroplast microsatellite markers. One of the populations is located within the Parque Nacional de Sete Cidades Conservation Unit and the other in the municipality of Cocal de Telha. We tested if the individuals of the conservation unit are more genetically diverse than those in the disturbed area. Since there are no studies related to the distribution of diversity and haplotype structure of natural populations of Piauí Cerrado, this presents novel results.

## MATERIAL AND METHODS

### Study sites

The study was carried out in two areas of Cerrado in the State of Piauí: Cocal de Telha city (04°33'32"S and 41°58'20"W) and Parque Nacional de Sete Cidades (04°02'08"S and 41°40'45"W), with a distance of 67 km between them, both occurring within the vegetation profile of Cerrado *sensu stricto*. Parque Nacional de Sete Cidades is a conservation unit established in 1961, located between Brasileira and Piracuruca cities in northern Piauí State (Mendes et al., 2012). Eighty individuals were collected, including two populations.

The vegetation of Cocal de Telha region is characterized by a great variety of communities occurring in mosaics included in the Cerrado. The rainfall is defined as Equatorial Maritime regime, with annual isohyets between 800-1600 mm, with about 5-6 months as the rainy season and the remainder of the year as the driest season. The months between February and April correspond to the wettest trimester of the region (Aguar and Camargo, 2004).

The choice of these sites aimed at comparing the diversity in the population of the Parque Nacional de Sete Cidades Conservation Unit to the population of Cocal de Telha located around the BR-343 highway, which is under greater human disturbance.

### Extraction and quantification of DNA

Samples of young leaves were collected and transported in liquid Nitrogen to the Molecular Biology Laboratory of Piauí Federal University, where they were conserved in freezers. DNA extraction was performed with the DNeasy Plant Mini kit (Qiagen Inc., Valencia, CA, USA) according to supplier's recommendations.

The quantification of the extracted DNA was performed by electrophoresis on 0.8%

agarose gels (w/v), prepared with 0.5X TBE buffer (Tris-borate-EDTA) and stained once with SYBR<sup>®</sup> Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, USA), by comparison with  $\lambda$ -DNA bands diluted at a concentration of 100 ng/ $\mu$ L. DNA samples were then diluted to a concentration of 5 ng/ $\mu$ L and stored at - 20°C.

### **Amplification and genotyping of chloroplast microsatellite loci**

For chloroplast microsatellite analyses, we employed universal primer pairs described to dicotyledonous angiosperms by Weising and Gardner (1999) and Chung and Staub (2003).

The microsatellite markers were amplified with the following program: a denaturation step at 94°C for 4 min; 30 cycles composed of a denaturation step at 94°C for 1 min, an annealing step at 58°C for each pair of primers for 1 min, an extension step at 72°C for 40 s, and a final extension step at 72°C for 10 min.

After the amplification of the chloroplast microsatellite loci, the size of the alleles was determined in the LI-COR DNA Analyzer 4300s (LI-COR Corporate) automated sequencer. The genotypes of individuals were automatically determined with the aid of 50-350-bp IRDye-700 and IRDye 800 (LI-COR Corporate) molecular size standards, with the program Saga<sup>™</sup> (LI-COR Corporate), and then visually inspected.

### **Statistical analyses**

We estimated the number of haplotypes, the number of private haplotypes for each population (NPH), the proportion of private haplotypes, the haplotype diversity (*h*), and the effective number of haplotypes, using GenAIndex 6.5 (Peakall and Smouse, 2012). Analysis of molecular variance (AMOVA) was performed using Arlequin 3.5 (Excoffier and Lischer, 2010) in order to estimate the proportion of genetic variation between and within populations.

The admixture of individuals between populations was evaluated using the Bayesian analysis of genetic structure described by Pritchard et al. (2000) with STRUCTURE 2.3.1. Ten independent simulations were carried out for each number of *K* groups, with *K* ranging from 1 to 10, using the correlated allele frequencies and admixture models of ancestry, with 500,000 MCMC replicates after the burn-in period of 100,000. The most likely number of clusters was found using Structure Harvester (Earl and vonHoldt, 2012) following the  $\Delta K$  criteria (Evanno et al., 2005).

The genetic structure of populations was also evaluated with discriminant analysis of principal component (DAPC; Jombart et al., 2010), using the adegenet package for the R version 2.11 platform (R Core Team 2010 Development). The DAPC is based on data transformation by principal component analysis. The adegenet package proposes the  $\alpha$ -score calculation to assess the optimal number of principal components to be retained in the analysis. Using this procedure, only the first principal component, corresponding to 53.43% of the total variation, was retained for the analysis.

The haplotype network was built with the Median Joining method, using Network v.4.610 (Bandelt et al., 1999). This method of clustering is based on coalescence theory and has been used as an alternative to Wagner's parsimony, for providing better connections between haplotypes (Excoffier and Smouse, 1994). In this analysis, DNA haplotypes are related by mutation and arranged in a network. Network nodes represent each haplotype, while the distance between them is proportional to the number of mutations. The number of mutations

throughout the network was used as an evolutionary divergence measure between any two haplotypes (Excoffier et al., 1992).

## RESULTS AND DISCUSSION

Of the universal chloroplast microsatellite primers tested, 12 could be amplified (ccpm1, ccpm2, ccpm7, ccSSR-3, ccSSR-4, ccSSR-5, ccSSR-6, ccSSR-9, ccSSR-10, ccSSR-11, ccSSR-20, and ccSSR-22), but only 7 were polymorphic: ccSSR-3, ccSSR-4, ccSSR-6, ccSSR-9, ccSSR-10, ccSSR-11, and ccSSR-22. This set of chloroplast microsatellites obtained 10 haplotypes (Table 1).

**Table 1.** Constitution of haplotypes.

Haplotypes	ccSSR-3	ccSSR-4	ccSSR-6	ccSSR-9	ccSSR-10	ccSSR-11	ccSSR-22
1	191	178	191	200	130	122	114
2	191	178	197	200	130	122	108
3	191	178	197	200	130	124	114
4	191	183	197	196	135	122	114
5	191	183	197	200	130	122	108
6	191	183	197	200	135	122	114
7	191	183	191	200	130	122	114
8	191	183	197	200	130	122	114
9	191	183	197	200	130	124	114
10	193	183	197	200	130	124	114

The locus ccSSR-10 had the highest number of alleles for the population of Parque Nacional de Sete Cidades, suggesting that this locus underwent a larger number of mutations. On the other hand, most of the loci were monomorphic for the population of Cocal de Telha, where only the ccSSR-3 was polymorphic.

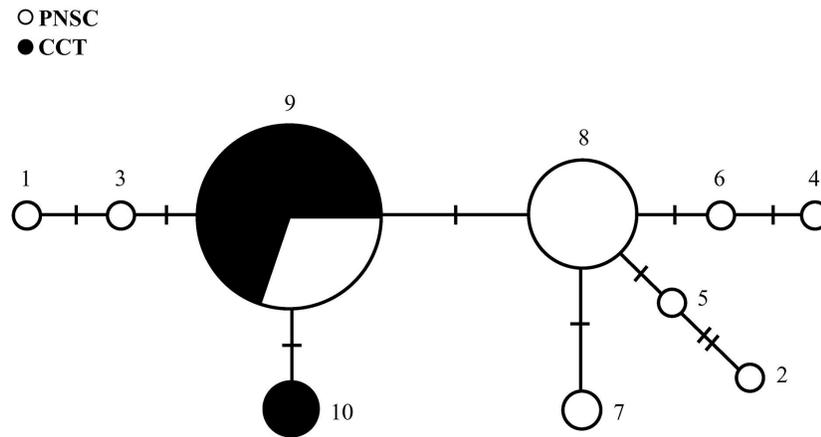
The population of Parque Nacional de Sete Cidades had higher number of haplotypes ( $H = 9$ ) and higher haplotype diversity ( $h = 0.674$ ) than the population of Cocal de Telha ( $H = 2$ ;  $h = 0.139$ ). The number of private haplotypes was also higher for Parque Nacional de Sete Cidades ( $NPH = 8$ ) than in individuals from Cocal de Telha ( $NPH = 1$ ). The estimates of haplotype diversity reveal that the population in the conservation unit had greater genetic diversity and confirms the effectiveness of protected areas for the preservation and maintenance of genetic diversity of natural species.

Based on the data of haplotype frequencies (Table 2), haplotype 9 is the most common in both populations with frequency of 40% for the population of Parque Nacional de Sete Cidades, and 92.5% for Cocal de Telha.

**Table 2.** Haplotype frequency in populations of *Casearia grandiflora*.

cpDNA haplotypes	Parque Nacional de Sete Cidades	Cocal de Telha
1	00.025	0.00
2	00.025	0.00
3	00.025	0.00
4	00.025	0.00
5	00.025	0.00
6	00.025	0.00
7	0.050	0.00
8	0.400	0.00
9	0.400	0.925
10	0.00	0.075

The haplotype network (Figure 1) reveals that haplotype 9 is shared between the two populations and it is possibly the ancestral haplotype according to the theory of coalescence (Posada and Crandall, 2001), where the most frequent haplotypes are probably the oldest ones. The haplotype 9 differs from haplotypes 10, 3, and 8 by a single mutation, as well as haplotype 8 differs from haplotypes 5, 6, and 7 also for one mutation, while haplotype 5 differs from haplotype 2 by two mutational steps.



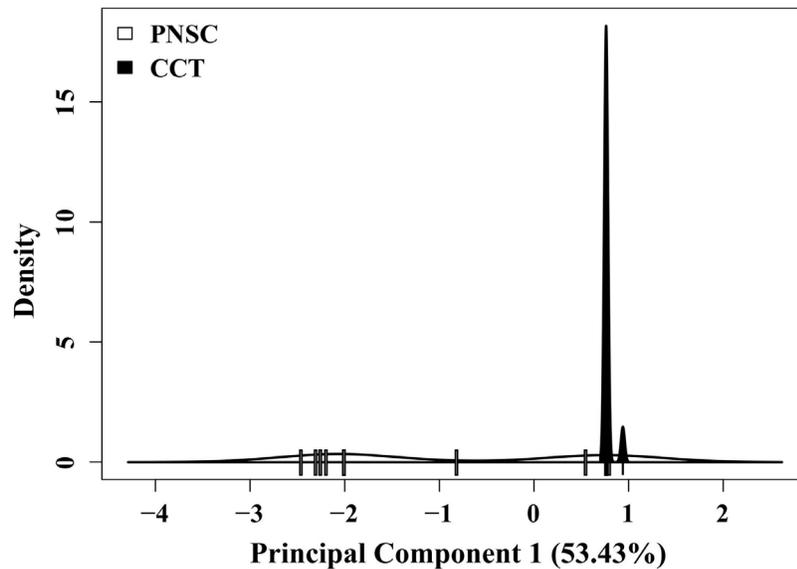
**Figure 1.** Haplotype network, the size of each circle is proportional to the overall frequency of each haplotype. The colors of the circles indicate the occurrence of these haplotypes in the two population groups. Each line represents one mutational step. PNSC = Parque Nacional de Sete Cidades and CCT = Cocal de Telha.

Bayesian clustering analysis did not detect the formation of clusters, and any of the individuals had similar probabilities of belonging to either one of the populations. This suggests that the populations are not genetically differentiated.

Discriminant analysis of principal components is an excellent tool for exploring the genetic structure of populations without strong genetic assumptions of Bayesian clustering, such as adherence to Hardy-Weinberg equilibrium and linkage equilibrium between loci (Jombart et al., 2009). Thus, this analysis provides an alternative to Bayesian clustering algorithms, such as the implemented in STRUCTURE (Pritchard et al., 2000). The analysis evidenced low haplotype differentiation between the populations of Parque Nacional de Sete Cidades and Cocal de Telha, confirming the absence of genetic structure.

The reproductive biology of *C. grandiflora* may be an important factor to explain the observed genetic uniformity. According to Moura et al. (2012) in natural populations, one of the main factors that influence the genetic structure of populations is the mating system, which determines the extent of gene flow between populations. Outcrossing plants, unlike autogamous ones, tend to have high levels of genetic variability within populations and low genetic differentiation between populations (Zanella et al., 2012).

Although the populations are not genetically structured, it can be seen that some individuals of Parque Nacional de Sete Cidades are more divergent from the population of Cocal de Telhas, which shows a more restricted distribution (Figure 2). This result may be due to greater anthropic disturbance in the population of Cocal de Telha, which may compromise the maintenance of genetic variability due to habitat fragmentation, for example.



**Figure 2.** Discriminant principal components analysis. PNSC = Parque Nacional de Sete Cidades and CCT = Cocal de Telha.

The destruction of natural habitats for the human occupation causes ecosystem fragmentation, leading to a loss of biodiversity, which can impose evolutionary constraints on the species. These, in turn often have negative effects on the biota and can lead to increased frequency of deleterious alleles compromising the persistence and adaptation of species to the environment (Habel and Zachas, 2012).

AMOVA derived from a matrix of squared distances between pairs of haplotypes produces estimates of variance components, reflecting the haplotype diversity in different levels of hierarchical subdivision. The results of AMOVA showed that most of the genetic variation was found within populations (85%).

The observed value of  $F_{ST}$  (0.14) suggests moderate genetic structure among populations (Wright, 1978). The analysis of haplotype frequency revealed that, although both populations share the haplotype 9, the population of Parque Nacional de Sete Cidades had eight haplotypes that were not found in Cocal de Telha, which may explain the moderate level of population structure.

Based on the results of Bayesian and multivariate analyses, it can be initially rejected the hypothesis of strong population structure. It is possible that pollen flow between populations still exists, despite the fact that the populations are 67 km apart. It is also possible that there is restriction to pollen flow, but it may not be older enough leading to genetic structure between populations. However, forest fragmentation and anthropic disturbances may lead to increasing  $F_{ST}$  estimates over time.

According to Haddad et al. (2015), fragmentation of habitats has a long term effect on biodiversity, and for decades there was a reduction of about 50% of some species diversity distributed across the five continents. In addition, about 70% of forests in the world are threatened by human activities.

From the molecular analysis it was possible to estimate the haplotype diversity, which suggested that there are differences in the genetic diversity of *C. grandiflora* population of the Parque Nacional de Sete Cidades Conservation Unit and under human intervention area of the population of the municipality of Cocal de Telha. These results show that populations in less urbanized areas have greater genetic diversity and confirm the effectiveness of protected areas for the conservation of genetic diversity of the species. The results obtained for *C. grandiflora* will certainly bring benefits to the conservation of other native species that occur in this area the Cerrado of Piauí.

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