



Genetic diversity analysis of *Croton antisyphiliticus* Mart. using AFLP molecular markers

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Genet. Mol. Res. 15 (1): gmr.15017461

Received August 17, 2015

Accepted November 6, 2015

Published February 19, 2016

DOI <http://dx.doi.org/10.4238/gmr.15017461>

ABSTRACT. *Croton antisyphiliticus* Mart. is a medicinal plant native to Cerrado vegetation in Brazil, and it is popularly used to treat urogenital tract infections. The objective of the present study was to assess the genetic variability of natural *C. antisyphiliticus* populations using AFLP molecular markers. Accessions were collected in the states of Minas Gerais, São Paulo, and Goiás. The genotyping of individuals was performed using a LI-COR® DNA Analyzer 4300. The variability within populations was found to be greater than the variability between them. The F_{ST} value was 0.3830, which indicated that the populations were highly structured. A higher percentage of polymorphic loci (92.16%) and greater genetic diversity were found in the population accessions from Pratinha-MG. Gene flow was considered restricted ($N_m = 1.18$), and there was no correlation between genetic and geographic distances. The populations of *C. antisyphiliticus*

exhibited an island-model structure, which demonstrates the vulnerability of the species.

Key words: Euphorbiaceae; Medicinal plant; Cerrado; Conservation; LI-COR

INTRODUCTION

Habitat destruction and fragmentation are among the major anthropic impacts that affect ecosystems (Young et al., 1996; Young and Clarke, 2000). Fragmentation not only reduces the population effective size and the wealth of the species, but it also increases the spatial isolation among them (Young et al., 1996). Plants can live in naturally fragmented habitats, and they are capable of adapting to new conditions associated with this process. However, the intensity by which anthropic fragmentation has occurred during the past decades has made it difficult for plants to adapt to changes due to partial habitat destruction (Lienert, 2004). Therefore, fragmentation threatens the survival of many species, and it can cause the extinction of local populations (Ehrlich and Wilson, 1991; Young and Clarke, 2000).

Several species of the Cerrado biome have been threatened by fragmentation, including *Croton antisiphiliticus* Mart., which is popularly known as “pé-de-perdiz”. The roots of this plant are widely used by the native people to treat urogenital tract infections (Munhoz and Felfili, 2007).

The practice of extractivism predominantly includes those plants whose active components are in their roots. These species become more vulnerable after being pulled up, and they subsequently disappear.

The use of tools for the measurement of genetic variability, such as molecular markers, is important for the determination of biodiversity conservation strategies (Avisé, 2010). Several markers are analyzed using PCR, including amplified fragment length polymorphism (AFLP) markers (Guimarães et al., 2009). The AFLP marker technique is robust, safe, and highly reproducible, and it allows the detection of high levels of polymorphism; therefore, it is widely used to assess the genetic variation in natural populations (Vos et al., 1995; Metin et al., 2014). Historically, AFLP has been used to analyze various species, including *Hipericum perforatum* L. (Percifield et al., 2007), *Tribulus terrestris* L. (Sarwat et al., 2008), *Croton draco* Schlttdl. and Cham. (Farias et al., 2009), *Anacardium humile* St. Hill. (Londe et al., 2010), *Maytenus ilicifolia* Mart. ex Reis (Ribeiro et al., 2010), *Stryphnodendron adstringens* (Mart.) Coville (Mendonça et al., 2012), and *Palicourea rigida* (Silva et al., 2013).

Therefore, the objective of the present study was to assess the genetic diversity of natural populations of *C. antisiphiliticus* using AFLP molecular markers to establish conservation strategies for the species.

MATERIAL AND METHODS

Young leaves were collected from 20 individuals from each natural population in the counties of Catalão and Jataí (GO), Sacramento, Pratinha and São Gonçalo (MG), and Pedregulho (SP). The choice of the collection areas was based on the natural occurrence of the species, and species localization was determined with GPS. The leaves were stored in paper bags and kept in a freezer at -20°C until extractions were performed.

The analyses were conducted at the Molecular Biology Laboratory of the Department of Medicinal Plant Biotechnology of Universidade de Ribeirão Preto.

Due to the difficulty in obtaining intact genetic material, two DNA extraction protocols were evaluated for *C. antisiphiliticus*. The first was proposed by Doyle and Doyle (1987), and the second was designed by Deshmukh et al. (2007). Both methods were performed with modifications.

AFLP protocols

AFLP analyses were performed using the method described by Vos et al. (1995), which utilizes the commercial IRDye® fluorescent AFLP® Kit designed for large plant genome analysis. This method also included a protocol (LI-COR Biosciences, Lincoln, NE, USA) The combination of eight primers included E-ACA/M-CGA, E-AAC/M-CTA, E-AAG/M-CAT, E-ACC/M-CTG, E-ACG/M-CAG, E-AGC/M-CTA, E-AGG/M-CAT, and E-ACT/M-CTG, and the AFLP amplification products were loaded on a polyacrylamide gel (6.5%). We visualized the results using an automated AFLP analysis program (LI-COR SAGA^{MX} v.3.3). Only clear and unambiguous bands were scored and numbered for each fragment based on the molecular weight.

Statistical analysis

The resulting binary data were used to estimate the allele frequencies, which were then subjected to variance decomposition analysis of total genetic variance both within and between the populations (AMOVA). This analysis allowed the assessment of the variability structure, as proposed by Excoffier et al. (2005). The descriptive analysis of total variability was performed by calculating the percentage of polymorphic loci and Nei's gene diversity index (*h*). The unweighted pair group method with arithmetic mean (UPGMA) was used for both the analysis of grouping and the assessment of genetic divergence between the study populations, based on the *h* values (Nei, 1978). The geographic distance was calculated using the TrackMaker v.13.8 software, and Mantel's test was performed with 1000 simulations. The programs Popgene 32 and Genes v 2009.7.0 were used for these analyses.

The genotypic structure was assessed by performing principal coordinate analysis (PCoA), using the GenAlEx v6.5 (Peakall and Smouse, 2006) and STRUCTURE v.2.2.4 (Pritchard et al., 2000) software. Genotypic structure was also determined using grouping analyses based on Bayesian and admixture models with a burn-in of 200,000 followed by a run-length of 500,000, and the analysis included five independent interactions and K-values ranging from 1 to 7. With this, we sought to determine the number of genetic groups based on the criterion proposed by Evanno et al. (2005).

RESULTS

DNA extraction

The protocol proposed by Deshmukh et al. (2007) resulted in genetic material with the highest integrity as well as a mean DNA concentration of 393.43 ng/μL.

Analyses using the SAGA^{MX} program enabled the visualization of 255 bands, and the use of eight primer combinations yielded a number of bands (all polymorphic) per combination that

ranged between 10 to 51 (Table 1). Regarding the percentage of polymorphism, the populations from Pratinha and Jataí showed the highest values (92.16 and 91.76%, respectively), whereas the population from Sacramento had the lowest value (78.43%) (Table 2).

Values of h ranged from 0.2733 to 0.3540, and the Shannon's index values ranged from 0.4136 to 0.5195. The correlation coefficient between both indices was 0.998, which demonstrated a strong positive correlation. The population from Pedregulho exhibited the lowest diversity for both indices, whereas those from Catalão and Pratinha had the highest values (Table 2). The Pratinha population also showed the best results for the following parameters: percentage of polymorphic loci, number of observed alleles, and number of effective alleles.

The variability within the population was greater (61.97%) than that between populations (38.03%). The gene flow between populations was low ($N_m = 1.18$).

The allele fixation index (F_{ST}) was 0.3803 (Table 3), which indicated a very high value for genetic population structuring based on the limit of >0.25 proposed by Wright (1978). The spatial pattern determined by Mantel's test based on 1000 permutations was $r = 0.17$, thus indicating the absence of a significant correlation between geographic and genetic distances.

Analysis of the dendrogram and PCoA indicated the formation of three groups: 1) populations from Catalão and Jataí; 2) populations from São Gonçalo and Pedregulho; and 3) populations from Sacramento and Pratinha (Figures 1 and 2).

The results generated by the STRUCTURE software distributed the 120 individuals into six genetic groups (Figure 3). Regarding the STRUCTURE results, the same color for different genotypes indicates that they belong to the same group, and different colors for the same individual indicate the percentage of genome shared by each group.

Table 1. Nucleotide combination and number of amplified fragments.

EcoRI-MseI combination	Total number of fragments
ACA/CGA	51
AAC/CTA	39
AAG/CAT	41
ACC/CTG	42
ACG/CAG	28
AGC/CTA	24
AGG/CAT	20
ACT/CTG	10
TOTAL	255
MEAN	31.87

Table 2. Basic descriptive statistics for *Croton antisiphiliticus* populations assessed using AFLP markers.

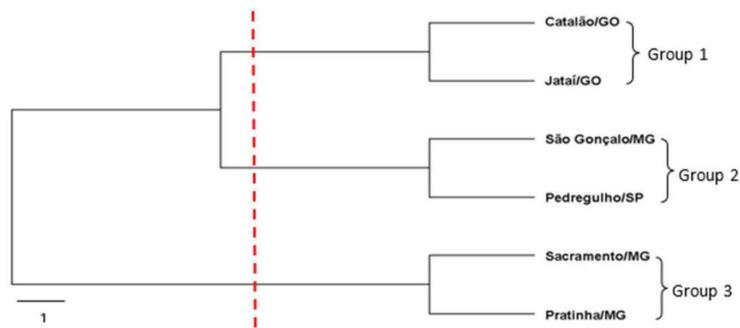
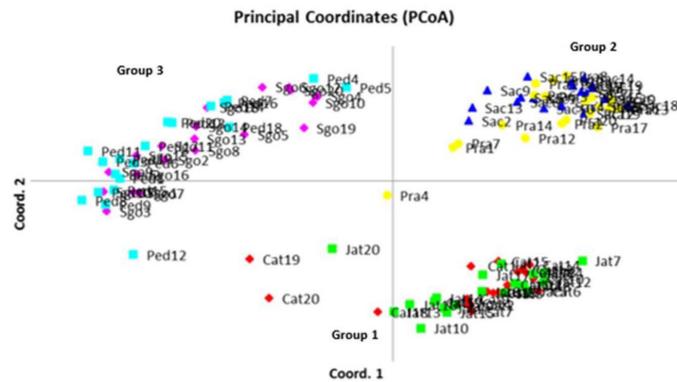
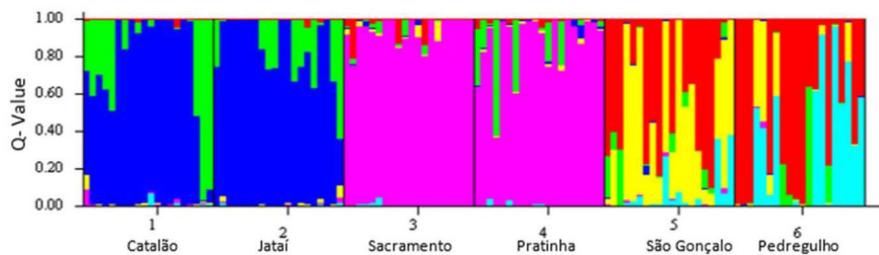
Populations	N	N_A	P	N_E	h	I
Catalão	20	1.9176	91.76	1.5583	0.3209	0.4770
Jataí	20	1.8627	86.27	1.5407	0.3104	0.4602
Sacramento	20	1.7843	78.43	1.4931	0.2841	0.4212
Pratinha	20	1.9216	92.16	1.6262	0.3540	0.5195
São Gonçalo	20	1.8627	84.71	1.4916	0.2896	0.4358
Pedregulho	20	1.8471	86.27	1.4614	0.2733	0.4136
Mean	20	1.8660	86.60	1.5286	0.3054	0.4546

Mean size of sample (N); number of observed alleles (N_A); percentage of polymorphic alleles (P); number of effective alleles (N_E); Nei's genetic diversity (1978) (h); Shannon's index (I).

Table 3. Analysis of molecular variance (AMOVA) between and within populations of *Croton antispyhiliticus* assessed using AFLP markers.

Source of variation	d.f.	S.S.	S.M.S.	Components of variance	% total of variance	P	Statistics - PHist
Between populations	5	2571.325	514.265	23.776	38.0293	<0.001	0.3803
Within population	114	4416.85	38.7242	38.7743	61.9707	-	-
Total	119	6988.175	58.7242	62.5203	100.00	-	-

Degrees of freedom (d.f.); sum of squares (S.S.); sum of mean squares (S.M.S.); significance level for genetic variation estimation using 1000 permutations (P); genetic variation estimated for the variation source analogue to Wright's F_{ST} (PHI_{ST}). Results were obtained using the GENES software.

**Figure 1.** Dendrogram of *Croton antispyhiliticus* populations.**Figure 2.** Results of the principal coordinate analysis (PCoA) of *Croton antispyhiliticus* populations.**Figure 3.** Composition of genetic groups of *Croton antispyhiliticus* populations identified by the STRUCTURE analysis.

DISCUSSION

It has been reported that it is difficult to obtain intact DNA from tropical species because of the presence of secondary metabolites, particularly those in the phenolics class (Rath et al., 1998; Deshmukh et al., 2007). During the *C. antisiphiliticus* DNA extraction process, both viscosity and oxidation were observed in the samples, and this problem was solved using the protocol developed by Deshmukh et al. (2007). The effectiveness of this protocol is related to the higher number of washings to which the samples were subjected.

The results indicated a high percentage of polymorphism in *C. antisiphiliticus* individuals, which is likely related to the pollination type and seed dispersal. Although no studies of the phenology of this species have been conducted, *Croton* research has shown the predominance of cross-pollination and seed dispersal by insects (Dominguez and Bullock, 1989; Almeida et al., 2003), and this may be a pattern of the genus that explains the high level of polymorphism in *C. antisiphiliticus*.

As gene flow is directly related to the types of reproduction and dispersal that occur in a species, and this case of *C. antisiphiliticus* dispersal is mainly performed by ants. This is because its seed has a structure called a caruncula, which is capable of attracting these insects (Leal et al., 2007). Although *Croton* studies have demonstrated that pollen is primarily dispersed by wind and secondarily by insects (especially bees) (Dominguez and Bullock, 1989; Juhász et al., 2009), the present study provides evidence that *C. antisiphiliticus* has low N_m . This result is probably due to the difficulty associated with pollen and seed dispersal, since the localization of the collected individuals was restricted to small bands of vegetation along roads and permanent protection areas.

Smouse et al. (2001) stated that the greater the structuring, the lower the dispersal and N_m . This was evidenced with the high allele fixation value ($F_{ST} = 0.3803$), indicating that *C. antisiphiliticus* populations are highly structured. According to Frankham et al. (2007), when F_{ST} values are high, the dispersal capacity is low. Moreover, studies have also suggested that the greater the probability of the populations becoming endogamic, the lower the probability that the species will adapt to environmental changes, which leads to a higher risk of extinction (Ellstrand and Elam, 1993; Silva et al., 2013). Based on these assumptions, the values of both F_{ST} and N_m indicated that the *C. antisiphiliticus* populations at the collection sites were highly anthropic and fragmented regions, which resulted in island-model structured populations.

The data obtained from the STRUCTURE, dendrogram, and PCoA analyses reflected how the populations were structured, providing evidence for the fragmentation of *C. antisiphiliticus* populations into smaller ones (Figures 1, 2, and 3). Moreover, Figure 3 shows a trend in which the São Gonçalo and Pedregulho populations are divided into sub-populations, because virtually 50% of their genomes were assigned to each gene group. This finding deserves special attention, because a consequence of fragmentation is the restriction of individuals to small areas, which will isolate them partially or completely from other populations. Research has shown that fragmentation, from the genetic point of view, can alter the N_m among populations in such a way that they become more susceptible to endogamic processes and genetic drift (Young et al., 1996).

In addition, the effective population size is reduced with habitat fragmentation, because even individuals carrying favorable allelic compositions may be lost. Therefore, over the generations, the remaining individuals begin to accumulate identical alleles as a result of high endogamy levels, and this reduces the adaptive value of these individuals (Young et al., 1996). Based on the composition

data of the groups identified by the STRUCTURE analysis, it was possible to establish a collection strategy by prioritizing populations from Catalão, Sacramento, and Pedregulho, because these showed greater genetic diversity.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by UNAERP and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPQ).

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