Universidade de Brasília
Instituto de Ciências Biológicas
Programa de Pós-Graduação em Ecologia

EFEITO DOS IMPACTOS ANTRÓPICOS NA DIVERSIDADE GENÉTICA DE POPULAÇÕES DE ARATICUM (*Annona crassiflora* Mart.) E CAGAITA (*Eugenia dysenterica* DC.)

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Doutorado em Ecologia
pela Universidade de
Brasília

Dezembro – 2016

UNIVERSIDADE DE BRASÍLIA

INSTITUTO DE CIÊNCIAS BIOLÓGICAS

PROGRAMA DE PÓS-GRADUAÇÃO EM ECOLOGIA

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Tese apresentada ao Programa de Pós-Graduação em Ecologia, Instituto de Ciências Biológicas da Universidade de Brasília, como parte dos requisitos necessários para a obtenção do título de Doutor em Ecologia

Brasília – DF, 2016

AGRADECIMENTOS

Agradeço aos amigos do Laboratório de Genética Vegetal da Embrapa: Lorena Ferraz, Lucileide, Ana Flávia, Neide, Peter, Natasha por todo o apoio para conseguir finalizar essa tese. Agradeço também a Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) pela estrutura concedida para realizar toda a análise no laboratório. Agradeço à Universidade de Brasília (UnB) pela oportunidade de cursar o doutorado em Ecologia.

Muito obrigado Vânia por me receber como aluno e me orientar nesta área, que eu não dominava; imagino que foi um desafio enorme.

Muito obrigado Aldicir por me receber sem nem pestanejar nessa minha nova fase aqui em Brasília. Pela paciência, pela luta para financiar este projeto e pelas correções do trabalho.

Agradeço meus pais por terem me apoiado a vir para Brasília encarar esse desafio. Agradeço muito o meu pai pelos conselhos na área acadêmica. Agradeço minha mãe pelo apoio de mãe, sempre dizendo que vai dar tudo certo.

Agradeço a minha mulher Dani pela paciência aguentando o meu mau humor tentando terminar essa tese. Você foi muito companheira e parceira, obrigado!

RECURSOS FINANCEIROS

Este trabalho está inserido dentro do projeto “Manejo de Plantas do Cerrado: Subsídios Técnicos às Políticas Públicas de Uso Sustentável e Conservação da Biodiversidade” que recebe o apoio financeiro do Fundo Brasileiro para a Biodiversidade (FUNBIO) e Embrapa Recursos Genéticos e Biotecnologia (Cenargen). Este trabalho também foi financiado pelos recursos da Capes (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) me apoiando com bolsa de estudos nos dois primeiros anos de doutorado.

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INTRODUÇÃO GERAL

Os hábitats naturais estão cada vez mais fragmentados e boa parte dos hábitats remanescentes está sujeito a queimadas, derrubada de árvores e presença de gado. Esta redução populacional leva a gargalos genéticos, efeito fundador, deriva genética e redução no fluxo gênico. Tais efeitos levam consequentemente à depressão endogâmica e fixação de alelos deletérios, afetando assim o *fitness*, vigor, mortalidade e capacidade de adaptação das espécies. O Cerrado brasileiro é a mais rica savana do mundo mas já perdeu 50% da sua vegetação original e apenas 3,1% de sua área está inserida em áreas de proteção integral. Dentre as espécies arbóreas endêmicas do Cerrado, encontram-se o araticum (*Annona crassiflora* Mart.) e a cagaita (*Eugenia dysenterica* DC.). Tais espécies se destacam pelo seu potencial de exploração econômica através do extrativismo de seus frutos para consumo na forma de doces, sorvetes, sucos ou *in natura*. As espécies possuem algumas características que podem fazer com que respondam de forma diferente aos impactos antrópicos. *Annona crassiflora* ocorre em baixa densidade, é dispersa principalmente pela anta que está em risco de extinção e polinizada por besouros de baixa mobilidade. Já *E. dysenterica* ocorre em maior densidade, é dispersa por aves e mamíferos e possui um alto potencial de regeneração através de rebrota.

Assim, o objetivo dessa tese é determinar os efeitos dos impactos antrópicos na diversidade genética de araticum e cagaita. A presente tese está dividida em duas partes, na primeira parte, representada pelo primeiro capítulo, trato dos efeitos da fragmentação de hábitat na diversidade genética de araticum. Na segunda parte, capítulos 2 e 3, trato dos efeitos da fragmentação e distúrbio de hábitat na diversidade genética e reprodução

clonal de cagaita. Sendo que o segundo capítulo trata das técnicas utilizadas para desenvolver os marcadores moleculares de cagaita utilizados no terceiro capítulo.

No primeiro capítulo, testamos os efeitos da fragmentação na estrutura genética espacial, heterozigosidade e endogamia em *Annona crassiflora* Mart. (Annonaceae) baseado no polimorfismo de dez pares de microssatélites. No segundo capítulo, desenvolvemos marcadores moleculares para estudos de diversidade genética de cagaita. O objetivo foi de desenhar, validar e otimizar pares de marcadores microssatélites de *Eugenia dysenterica* DC. (Myrtaceae). Para isso produzimos uma biblioteca enriquecida de microssatélites. No terceiro capítulo investigamos os efeitos da fragmentação e distúrbios antrópicos na estrutura genética espacial, heterozigosidade, endogamia e reprodução clonal de *E. dysenterica* utilizando 13 microssatélites desenvolvidos no segundo capítulo.

CHAPTER 1 THE IMPACT OF HABITAT FRAGMENTATION ON THE GENETIC DIVERSITY OF *Annona crassiflora* Mart., A NEOTROPICAL SAVANNA TREE

ABSTRACT

In this study, we evaluated the effects of habitat fragmentation and disturbance on the spatial genetic structure, heterozygosity and inbreeding in *Annona crassiflora* Mart. (Annonaceae), a Brazilian savanna tree, based on the polymorphism of ten microsatellite loci. Leaves were collected from 180 adult individuals in six populations with markedly distinct histories of disturbance and fragmentation: three continuous populations (C1, C2 and C3), with no history of recent disturbance and three small and isolated populations (F1, F2 and F3), with history of disturbance due logging, fire and cattle. All populations showed high levels of polymorphism and genetic diversity but isolated sites showed a higher fixation index. No spatial genetic structure was detected for populations. Structure analyses showed three important scenarios K = 5, 3 and 2. Isolated populations may not have been affected from genetic effects of fragmentation due to the natural high genetic diversity of the species, the long lived cycle of trees and/or because there is gene flow among fragments. Our results suggest that isolated populations of *A. crassiflora* have high levels of polymorphism and genetic diversity but the high inbreeding is a matter of concern. The high genetic diversity of *A. crassiflora* found in isolated sites indicates that currently it is possible to conserve this species *in situ* in fragments. Populations with high genetic diversity also enable the collection of propagules with broad genetic base necessary for ecological restoration, reintroductions, or enrichment conservation programs.

Keywords: microsatellite, genetic diversity, conservation, Cerrado and araticum.

1. Introduction

Deforestation and habitat fragmentation are the leading causes of biodiversity loss (Sala et al. 2000; Pereira et al. 2010; Rands et al. 2010). Seventy per cent of remaining forest is within 1 km of the forest's edge, subject to the degrading effects of fragmentation (Haddad et al 2015) and associated with anthropic disturbances, mainly burning, logging, and cattle grazing (Nepstad et al. 1999; Ellis and Ramankutty 2008). Such disturbances are causing a decrease in effective population sizes and disrupting many ecological and genetic processes (Ewers and Didham 2006; Aguilar et al. 2008).

Drastic reductions in population sizes often lead to a genetic bottleneck (Oostermeijer et al. 2003; Lowe et al. 2005; Aguilar et al. 2008). The isolation of habitat fragments can lead to founder effects, genetic drift and restricted gene flow, which may further increase population genetic isolation and divergence (Freeland 2005; Allendorf et al. 2008; Dawson et al. 2014). Ultimately this can increase inbreeding and the fixation of deleterious alleles, which may affect species persistence by reducing individual fitness, its vigor and capacity of adaptation, and increases mortality (Freeland 2005; Lowe et al. 2005; Vranckx et al. 2012).

Habitat fragmentation often has negative effects on plant reproduction as a result of reduced pollination rates (Lowe et al. 2005; Aguilar et al. 2006). Similarly, the abundance and diversity of seed dispersers is often reduced in habitat fragments, thus affecting seed dispersal (Finger et al. 2011; Sebbenn et al. 2011). The disruption of pollination and dispersal services in habitat fragments can result in spatial aggregation of similar genotypes, increasing spatial genetic structure (Nason and Hamrick 1997; Moreira et al. 2009).

The Brazilian Cerrado is the richest savanna of the world and originally covered an area of approximately two million square kilometers. It is home for about 12 thousand plant species, of which one third are endemic (Mendonça et al. 2008). The Cerrado, one of the world´s biodiversity hotspots (Myers et al. 2000), has already lost 50% of its original vegetation to agriculture and cattle ranching (MMA 2011) and by 2050 will lose possibly about 13% of the remaining areas (Ferreira et al. 2012). In addition, only 3.1% of the Cerrado is within protected areas (CNUC 2016). Much of the remaining vegetation occurs as small isolated patches within private lands. These natural habitat patches can constitute important refuges of biodiversity, although most of them are quite small and often ignored in terms of their conservation value (Viana et al. 1998). Studies investigating the genetic variability of species that survive in these small patches may provide essential information to enhance their conservation value (Sebbenn et al. 2003).

Among the typical plant species of the Cerrado, araticum (*Annona crassiflora* Mart.) stands out due to its potential for commercial exploitation. Its fruits are marketed locally and the pulp is consumed in the form of sweets, juices, popsicles and ice cream (Ribeiro et al. 2000). Depending on the population density the income can reach two local minimum salaries per hectare per year (EMATER 2007). *A. crassiflora* is an arboreal species and it is found in every Cerrado physiognomy (Ribeiro et al. 2000), but mostly in the cerrado *sensu stricto*, which occupies 70% of the Cerrado biome (Eiten 1994).

The flowers of the *A. crassiflora* are pollinated by the beetles *Cyclocephala atricapilla* Mannerheim, *C. latericia* Hohne and *C. octopunctata* Burmeister (Cavalcante et al. 2009). *Cyclocephala* beetles usually pollinate plants near each other

and cover short distances (García-Robledo et al. 2004). The seeds of the *A. crassiflora* are dispersed mainly by tapirs (*Tapirus terrestris*) (Golin et al. 2011), a species that is threatened by hunting and habitat loss and which is classified as "vulnerable" according to IUCN criteria (Medici et al. 2012).

Some studies found high rates of inbreeding and an excess of homozygotes in populations of *A. crassiflora*, but the authors did not describe the conditions of the sampled areas (Telles et al. 2003; Pereira et al. 2008). Here we evaluated the potential effects of habitat fragmentation on the genetic structure of *A. crassiflora* comparing populations in large (> 4,000 ha), undisturbed sites with those found in small (< 100 ha), isolated fragments. Our hypothesis is that populations in small isolated sites have lower genetic diversity and high endogamy rates because of a higher cross between closely related individuals (i.e., with some degree of partnership). We also tested if the spatial genetic structure of populations in isolated sites is stronger due to distance (isolation) effects as well as a result of disrupted pollination and dispersion services.

2. Methods

2.1. Study sites

We sampled six populations in central Brazil (Figure 1) in the east of the State of Goiás and the Federal District. Three populations were located in large and protected Cerrado reserves (hereafter control populations), in the *Estação Ecológica de Águas Emendadas* (10,500 ha), the *Parque Nacional de Brasília* (42,000 ha), and the reserve of the University of Brasília at *Fazenda Água Limpa* (4,340 ha; Figure 1; Table 1), hereinafter named EEAE, PNB and FAL. The *Fazenda Água Limpa* is contiguous with two other reserves: *Reserva Ecológica do IBGE* (1,300 ha) and *Jardim Botânico* (5,000 ha) forming a large area of more than 10,000 hectares. The other three populations are

located in small (< 205 ha) and isolated Cerrado fragments (hereafter isolated populations). The three isolated populations are at least three kilometers from any other patches of Cerrado vegetation (Figure 1). These isolated sites have a low density of trees and shrubs and a high density of invasive grasses.

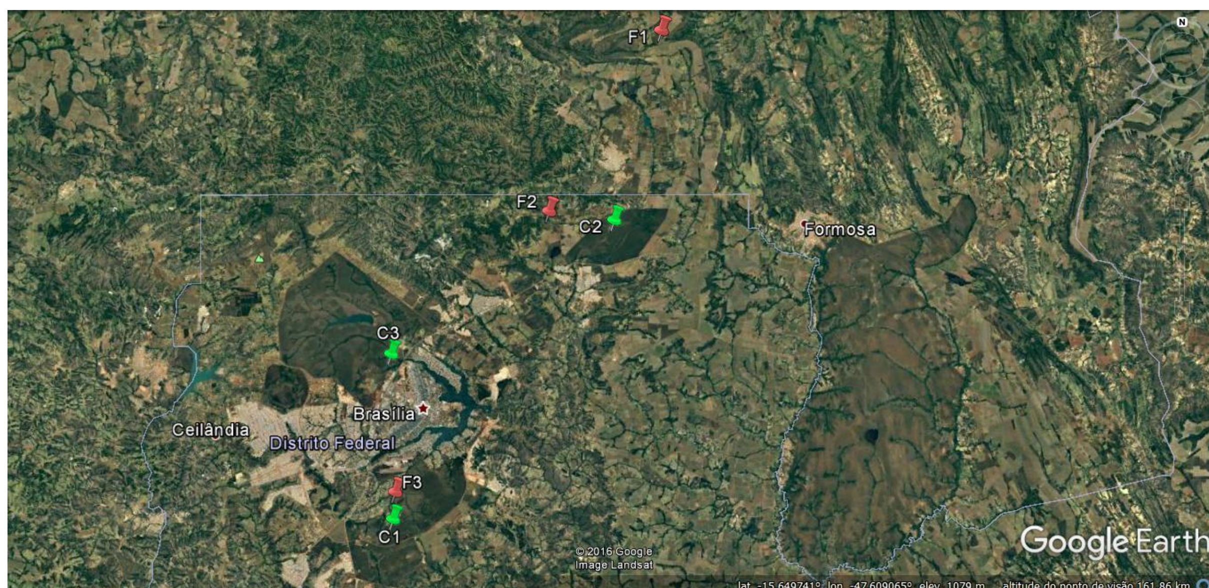

Figure 1. Google Earth image showing the location of the six *Annona crassiflora* populations studied.

Table 1. Characteristics, name, area and coordinates of the six sites where *Annona crassiflora* was sampled.

Site	Site characteristics	Site name	Area (ha)	Coordinates
F1	Fragmented/Isolated	São Gabriel	100	-15.286556 -47.550483
F2	Fragmented/Isolated	Monjolo	120	-15.539032 -47.706514
F3	Fragmented/Isolated	Fazenda Água Limpa degraded	205	-15.922122 -47.924832
C1	Continuous	Fazenda Água limpa preserved (FAL)	10,600	-15.973291 -47.925046
C2	Continuous	Estação Ecológica Águas Emendadas (EEAE)	10,500	-15.552583 -47.614932
C3	Continuous	Parque Nacional de Brasília (PNB)	30,000	-15.732769 -47.937516

The Cerrado region is characterized by a rainy summer and a dry winter (Eiten 1972). The precipitation ranges from 700 to 2000 mm yearly, with 70% of the rains occurring between November and March (Danelichen et al. 2013). The average monthly temperature is 22 °C (Inmet 2012). The predominant soils are poor in nutrient availability, with strong to moderate acidity (Lopes and Cox 1977).

In each site we collected newly mature leaves from 30 reproductive trees (diameter at breast high > 5 cm; as proposed by Araújo et al. (2008) for sampling reproductive individuals), to ensure consistency to estimate the genetic variability of a population (Berg and Hamrick 1997). We sampled trees at least 50 m apart from each other to sample greater genetic diversity (Wright 1943), and recorded the geographic coordinates of all individuals using a GPS (Global Positioning System) receptor. To keep the humidity low and thus increase the time conservancy of the leaves the harvested leaves were stored in paper bags in a box with silica.

2.2. DNA extraction and Genotyping

We extracted the genomic DNA from the leaves using the CTAB 2% protocol (Cetyltrimethylammonium bromide) (Doyle and Doyle 1987). We macerated pieces of the leaves with two beads in a tube of 2mL using a Mini Beadbeater 96 (Biospec). To quantify the DNA we compared a standard DNA (λ DNA) in 1% agarose gel colored with ethidium bromide. We diluted the extracted DNA to 1.0 ng/µL.

We amplified 10 microsatellites loci (Pereira et al. 2008) using Polymerase Chain Reaction (PCR) according to the following protocol: 3 µl of DNA, 4.17 µl of H_2O distillate, 0.8 µl of buffer (10 mM Tris-HCL pH 8.3 with 50 mM KCl, 1.5 mM $MgCl_2$), 0.8 µl of each dNTP (0.25 mM), 0.8 µl of Bovine Serum Albumin (2.5mg/mL), 0.2 µl of Taq DNA polymerase (5U/µl) and 0.23 µl of each pair of primers (0.25µM). We carried out amplifications using the Thermal Cycler 9700 (Applied Biosystems) and Veriti with the following conditions: 95 °C for 5 min, 35 cycles at 95 °C for 1 min, 1 min at specific annealing temperature for each primer pair (Table 2), 72 °C for 1 min. The products of the amplifications were visualized by electrophoresis in 3.5% agarose

gel stained with ethidium bromide. To compare the sizes of the amplified fragments we used the 1Kb DNA ladder from Invitrogen.

PCR reactions were performed for each locus. Using multiplex systems, all products of each locus were analyzed together in the electrophoresis with the purpose of detect alleles by its fluorescence in specific fragments, applying the automatic analyzer ABI 3730 (Applied Biosystems). For this we used a mixture of Hidi (10 µl), an internal marker (Rox; Brondani and Grattapaglia 2001) (1 µl) and PCR reaction (1 µl) denatured for five minutes at 95 °C.

The detection of the fluorescence peaks and genotyping were performed using the 4.1 GeneMapper software (Applied Biosystems). From the detection of the amplified fragment sizes with each pair of primers for each individual, a spreadsheet used for analysis and estimates was generated. The rounding of allelic size was made using the software AlleloBin (Prasanth et al. 2006). To check for the presence of null alleles and their interference in the estimates we used the micro-checker software (Van Oosterhout et al. 2004). The extracted DNA was stored in the Bank of Genetics of Embrapa Genetic Resources and Biotechnology, located in Brasília, Brazil, and cataloged in Allele System of Information of this institution.

2.3. Data analysis

We characterized the number of alleles, private alleles, observed and the expected heterozygosity under Hardy-Weinberg expectation (HWE) for 10 polymorphic microsatellite loci. To test for differences of this index between control and isolated populations a two-sample permutation test was performed using the “coin” package in R (Hothorn et al. 2008). We also performed two-sample permutation tests to evaluate differences in HWE and two-locus linkage disequilibrium. We estimated Wright`s

fixation index (*f*) based on all individuals genotyped and the apparent outcrossing rate (Ta = 1 - *f*). We calculated the consistence of the estimates by a bootstrap resampling using 10,000 permutations. The genetic analyses were carried out using the software Genetic Data Analysis (GDA; Lewis 2001). To check if all individuals sampled were from a single genetic pool or if they belonged to different populations we used a Bayesian analyses of the structure of the six populations with the software Structure 2.3.4 (Pritchard et al. 2000). In order to define the genetic relationships among populations, we also performed the neighbor-joining analysis (Saitou and Nei 1987) using GDA.

We used the geographic coordinates of the sampled trees to analyze the intra-population spatial genetic structure. All the possible pairwise combinations of trees were considered for each one of the pre-defined distance classes. This estimate was obtained by calculating the Loiselle et al. (1995) co-ancestry coefficient (xy θ) for each distance class and locus and for the overall locus mean. For each distance class we built 95% confidence interval of the medium co-ancestry coefficient based on the standard deviation of the average of the estimates, obtained by 10,000 jackknifed permutations among loci. We calculated the spatial genetic structure (SGS) with the software SPAGeDI 1.3 (Hardy and Vekemans 2002).

3. Results

A total of 35 alleles at ten microsatellite loci were found across 180 individuals from six populations of *A. crassiflora* (Table 2). Across the ten loci analyzed we found an average of 22.2 alleles (Table 2). Null alleles were detected in *Monjolo* (F2) and *FAL degradado* (F3) populations in marker ACR10 (Oosterhout index > 0.30).

All pairs of microsatellite loci were in linkage equilibrium (all P > 0.05, adjusted to a nominal 5% level with Bonferroni correction). For most loci, except ACR 37 and ACR44, the observed heterozygosity was lower than the expected under Hardy–Weinberg equilibrium, with fixation indexes significantly different from zero (Table 2).

Table 2. Genetic characterization of 10 microsatellite markers in *Annona crassiflora* Mart. screened in 180 individuals. Ta, annealing temperature (°C); n, number of individuals successfully genotyped; A, number of alleles; Ho, observed heterozygosity; He, expected heterozygosity; *f*, intra-population fixation index; P value, significance levels associated to the deviates from HWE proportion.

Locus	Size (bp)	N	Ta	A	He	Ho	*f*	P value
ACR01	203-263	174	60	24	0.885	0.879	0.006	
ACR10	130-183	133	60	32	0.840	0.488	0.419	
ACR19	146-174	178	60	13	0.816	0.685	0.160	
ACR20	161-205	163	60	15	0.703	0.607	0.136	
ACR22	183-239	180	60	22	0.844	0.733	0.132	
ACR26	150-156	180	58	4	0.619	0.794	0.283	
ACR33	181-231	175	58	24	0.907	0.891	0.017	
ACR34	132-186	178	58	24	0.852	0.803	0.057	
ACR37	231-299	167	60	35	0.947	0.574	0.393	*
ACR44	97-155	180	60	29	0.948	0.661	0.303	*

* P < 0.05 significance under Bootstrapping 10,000 permutations.

The genetic diversity was slightly higher in the isolated sites (He = 0.844 ± 0.0138 SD vs. 0.791 ± 0.0139 SD) than in the control sites, although this difference was only marginally significant (Z = -2.0596, P = 0.03944). The observed heterozygosity was the same in both sites (isolated 0.703 ± 0.02 vs. 0.716 ± 0.02 control sites; Z = 0.71297, P = 0.4759). Polymorphism was high in both sites but not different (12.8 ± 0.8 SD vs. 11 ± 0.5 SD allele; Z = -1.8926, P = 0.0584). We found more private alleles in isolated sites than in control sites (12.3 ± 2.8 vs 6.3 ± 2.8; Table 3).

We detected an excess of homozygotes in all isolated sites and in one control site. Fixation index was higher than zero in all populations studied, and significantly different in all the isolated sites and in one control site (Table 3). The species showed a mixed system of reproduction, with crossing between individuals and self-fecundation.

Table 3. Descriptive statistics of *Annona crassiflora* sampled populations. N, Mean number of individuals analyzed per locus; A, number of alleles; Ap, number of private alleles; He, expected heterozygosity; Ho, observed heterozygosity; *f*, intra-population fixation index and t_a, apparent outcrossing rate (1-*f*).

Population	N	A	Ap	He	Ho	*F*	t_a
F1	27.7	11.9	9	0.828	0.688	0.171*	0.828
F2	27.4	13.2	14	0.852	0.689	0.193*	0.806
F3	27.4	13.4	14	0.852	0.731	0.143*	0.856
Mean		12.83	12.3	0.844	0.703	0.169	
SD		0.8	2.8	0.01	0.02	0.02	
C1	29.2	10.5	3	0.803	0.715	0.111	0.888
C2	29.5	11.2	8	0.792	0.692	0.128*	0.871
C3	29.6	11.5	8	0.776	0.741	0.045	0.954
Mean		11.06	6.3	0.790	0.716	0.095	
SD		0.5	2.8	0.01	0.02	0.04	

* P < 0.05 significance under Bootstrapping 10,000 permutations.

The neighbor-joining tree created two groups, separating control from isolated sites (Figure 2) and aggregating sites with greater genetic similarity. The gene pool analysis suggests that the six populations studied form two, three or five clusters (Delta K respectively 11.67, 13.15 and 14.91; Figure 3). The two clusters scenario indicated a trend towards to a group with the control sites and other with the isolated sites. The scenario with three clusters may indicate a third group only with the *FAL degraded* (F3), the less isolated site of the three isolated sites, located near (5km) the *FAL preserved* (C1) site. The scenario with five groups was the most significant but it did not form a clear cluster. The intra-spatial genetic structure was not significant for any of the populations in isolated and control sites (Figure 4).

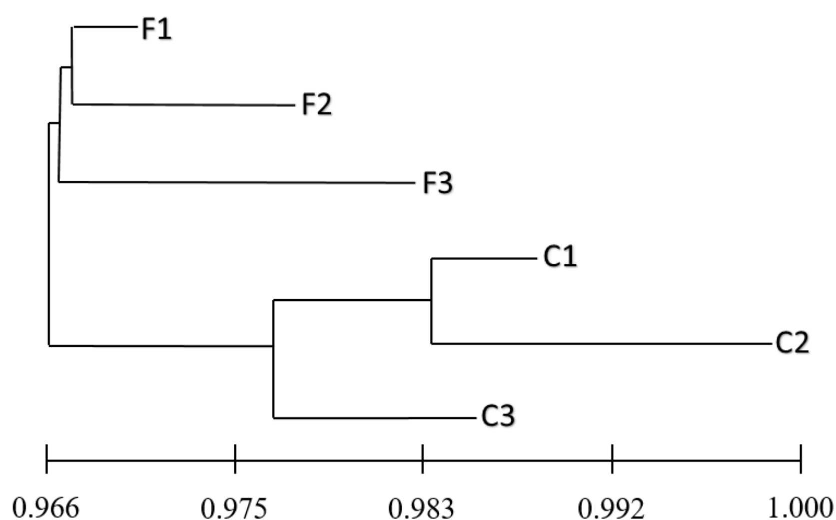

Fig 2 Neighbor-joining tree showing the genetic similarity among six populations of *A. crassiflora*. Control (C1, C2 and C3) and isolated sites (F1, F2 and F3)

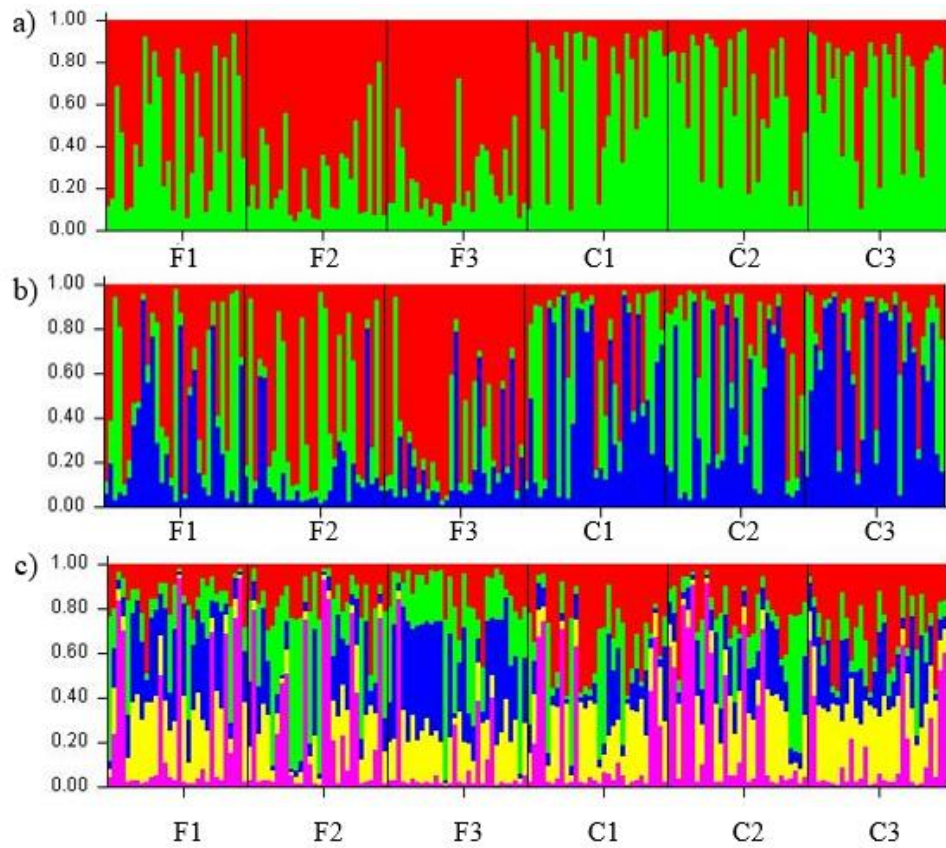

Fig 3 Clustering of six populations of *A. crassiflora* by Structure software. Each population is represented by a vertical bar that is partitioned into colored segments that represent the individual's estimated membership fractions. Same color in different populations indicates that they belong to the same cluster. Showing above are two (a) three (b) and five clusters (c).

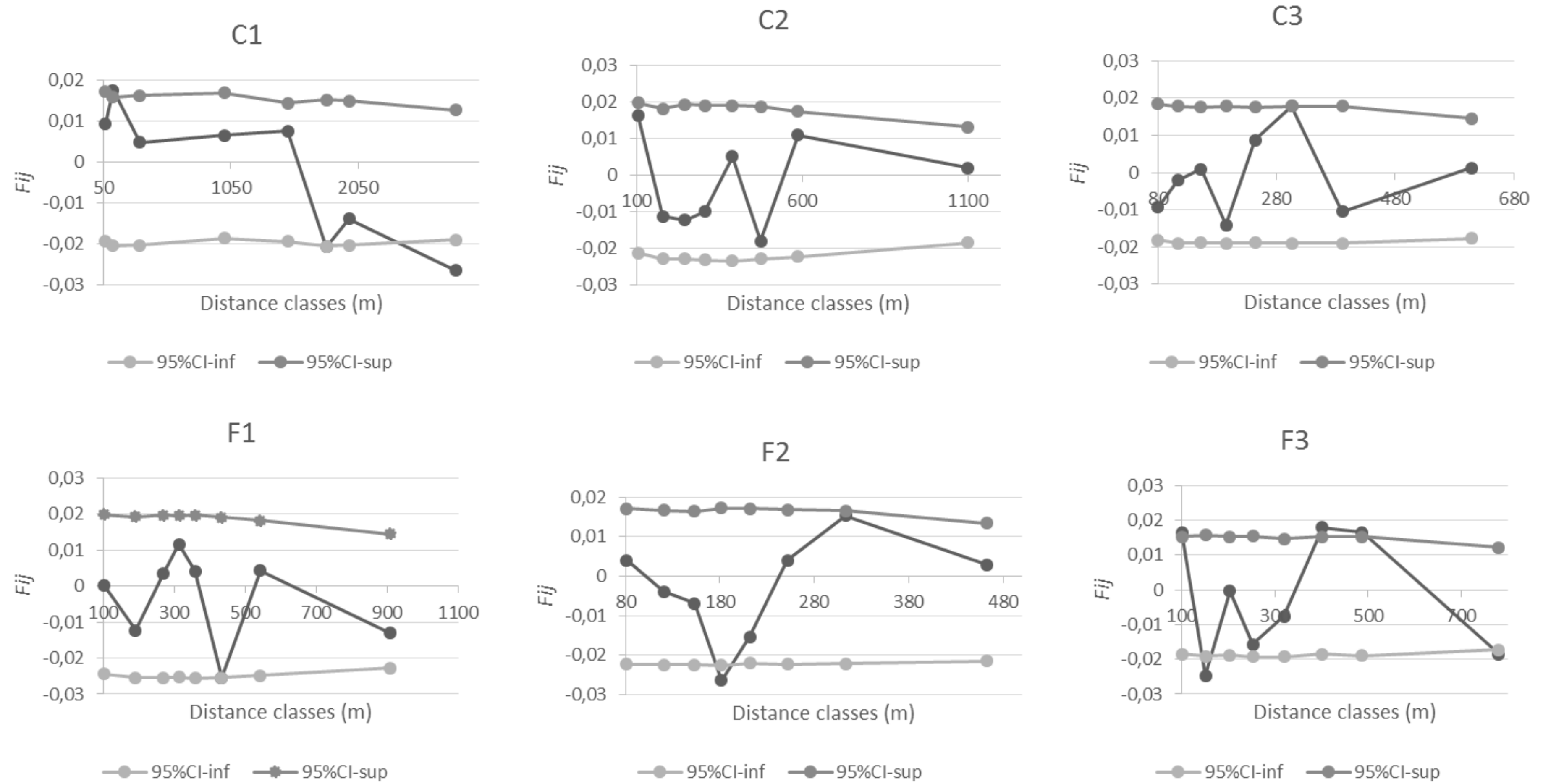

Fig 4 Kinship coefficients (*Fij*) per distance classes in six populations of *Annona crassiflora* sampled in Brazilian savanna. Control sites (C1, C2 and C3) and isolated sites (F1, F2 and F3). Confidence intervals around each *Fij* value were obtained through a 10,000 jackknifed permutations.

4. Discussion

Contrary to our expectations *A. crassiflora* populations in small isolated habitat fragments did not have a lower genetic diversity than those in large undisturbed areas. In fact, the polymorphism, the number of private alleles and expected heterozygosity were slightly higher in the isolated than in the control populations. In addition, we did not find evidence that the spatial genetic structure of the populations differs between small fragmented and large undisturbed areas. The polymorphism and genetic diversity found in *A. crassiflora*, both in control and isolated sites are similar to those found in other savanna tree species (Collevatti et al. 2001; Moreira et al. 2009; Antiqueira et al. 2014; Antiqueira and Kageyama 2014).

Previous studies in the Cerrado also found no differences in genetic diversity between isolated sites and continuous sites in populations of *Caryocar brasiliense* Camb. (Collevatti et al. 2001), *Qualea grandiflora* Mart. (Antiqueira and Kageyama 2014), *Copaifera langsdorffii* Desf. (Antiqueira et al. 2014) and *Annona crassiflora* Mart. (Collevatti et al. 2014).

The lack of differences between isolated and non-isolated populations of *A. crassiflora* may be attributed to its naturally high intra-population genetic diversity (as observed in the control sites) and as a result of ecological and demographic factors (Noreen and Webb 2013). All of which can counteract the effects of fragmentation and help the species to maintain its genetic diversity in small fragments (Hamrick 2004).

The high genetic diversity in isolated sites could be explained by gene flow between fragments, through pollen flow and/or seed dispersal, which would then counteract genetic erosion (Van Geert et al. 2008; Ismail et al. 2014). Several lines of evidence give support to this idea. *A. crassiflora* is preferably allogamous, as showed by

our results and an experimental study (Cavalcante et al. 2009). Genetic diversity is usually low for species with restricted distribution in the Cerrado, as is the case, for example, of *Tibouchina papyrus* (Pohl) Toledo (Collevatti et al. 2012) and *Lychnophora ericoides* Less. (Collevatti et al. 2009). *A. crassiflora*, however, has a wide distribution throughout the Cerrado biome (Ribeiro et al. 2000). Furthermore, *A. crassiflora* is not restricted to undisturbed Cerrado areas. In fact, it is commonly found along road verges (Vasconcelos et al. 2014) or as scattered trees in cattle pastures, in small rural properties, or even within urban areas. Brasília has 15,200 trees of 162 different species in its urban area, and *A. crassiflora* is one of these species (Silva Júnior and Lima 2010). These scattered trees of *A. crassiflora* are likely to have a pivotal role in maintaining gene flow between fragments, as also noted for other tree species (Lander et al. 2010).

The effects of habitat fragmentation on genetic diversity are less likely to be detected in areas where the process of habitat fragmentation has occurred over a short period of time relative to the generation time of the plant (Farwig et al. 2008; Kramer et al. 2008; Sebbenn et al. 2011; Finger et al. 2012). This may well be the case for *A. crassiflora* both because it is a long-lived species, and because habitat fragmentation in our study region started only at about 80 years ago. In fact, studies conducted with other long-lived Cerrado trees (Moreira et al. 2009; Collevatti et al. 2001; Collevatti et al. 2014) have also not detected an effect of habitat fragmentation on genetic diversity. In contrast, studies with the short-lived shrub *Solanum lycocarpum* A. St. Hill. showed that populations in large undisturbed areas contain more alleles than in fragmented populations (Moura et al 2012).

Despite the high genetic diversity observed in isolated populations of *A. crassiflora*, these populations showed significant higher fixation indexes, which may be

the outcome of inbreeding, most likely due to recent population size reduction (Lowe et al 2005). A similar result was found by Collevatti et al. (2014) in populations of *A. crassiflora* located in an isolated site compared to a control site. *A. crassiflora* is pollinated by *Cyclocephala* beetles (Cavalcante et al. 2009), that cover short distances and usually pollinate neighbor plants (García-Robledo et al. 2004), increasing the chances of inbreeding in smaller populations.

We did not find intra-spatial genetic structure in the control sites as expected given the presence of high mobility dispersal agents (Golin et al. 2011) in these sites, and the low density of tree individuals, factors which often weaken the SGS (Caron et al. 2000; Degen et al 2001). However, we also did not find SGS in the isolated sites where seed dispersal was expected to be limited (Nason and Hamrick 1997; Moreira et al. 2009). This suggests that seed dispersal is still taking place in isolated sites thus maintaining the SGS as weak as in the control sites. However, earlier studies showed that in distance classes smaller than the ones we studied (50 m), SGS in *A. crassiflora* is stronger in isolated than in non-isolated sites (Collevatti et al. 2014).

In the present study we could not determine the impact of fragmentation on seedlings because we could not find enough number of seedlings for robust analyzes. Many savannah species are long-lived (Hay and Barreto 1988; Alves 1994; Alves et al. 2013), we lack a study about longevity of *A. crassiflora*, but a study found that *A. crassiflora* grows 0.3 cm in diameter per year (Lima 2016). Thus, the possible deleterious effects of human impacts on genetic variability may take more time to appear in the adult class (Lowe et al. 2005; Aguilar et al. 2008; Farwig et al. 2008; Sebbenn et al. 2011; Finger et al. 2012), but the effect of fragmentation may be found in the seedlings.

Half of the Brazilian native vegetation occurs on private properties (Soares-Filho et al. 2014) as isolated fragments, given that the Brazilian law requires that 20% of the area of each rural property to be set aside as "legal reserves" (LR; Federal Law number 12,651; MMA). Vegetation on these lands store 105 ± 21 $GtCO_2$ eq. and play a vital role in maintaining a broad range of ecosystem services (Millennium eco 2005). The high genetic diversity of *A. crassiflora* found in isolated sites indicates that currently it is possible to conserve this species *in situ* in fragments. Populations with high genetic diversity also enable the collection of propagules with broad genetic base necessary for ecological restoration, reintroductions, enrichments and economic plantations (Falk et al. 2001). Thus, individuals of *A. crassiflora* in fragments could serve as a source of propagules for the restoration and recovery of deforested areas and based on the results from SGS, the seeds can be collected from trees at least 50 meters apart from each other.

In conclusion, this study found a high genetic diversity in isolated and control sites. The combination of high intra-population genetic diversity, individual long life cycle and the potential for high pollen flow rates appears to make *A. crassiflora* resistant to the negative effects of fragmentation. However, in the absence of dispersing and pollination agents it is expected a reduction in gene flow between isolated populations of *A. crassiflora*. Years of isolation and consequent reduction in gene flow may increase the genetic divergence between populations leading to a higher inbreeding depression and low germination or seedling survival (Spielman et al. 2004).

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CHAPTER 2 DEVELOPMENT AND CHARACTERIZATION OF MICROSATELLITE MARKERS IN *Eugenia dysenterica* DC.

ABSTRACT

The objective of this study was to design, validate, and optimize pairs of SSR microsatellite primers for the wild fruit-bearing tree, *Eugenia dysenterica* DC (Myrtaceae), a typical species of the Brazilian Cerrado with a high agronomic potential. We produced a library enriched for microsatellites by capture using two different dinucleotide repeat oligonucleotides; $(TG)_{13}$ and $(AG)_{14}$. A total of 95 primer pairs were designed, synthesized, and optimized. From the 95 pairs designed, 20 pairs produced clear polymorphic PCR products. From these 20 pairs, we selected 13 that showed the highest reliable data to test across six *E. dysenterica* populations. Among the 13 microsatellite loci, a high allele number was observed, ranging from 10 to 39. The mean genetic diversity was also high among all loci. Expected heterozygosity ranged from 0.631 to 0.948, while the observed heterozygosity varied between 0.467 and 0.994. The reported set of markers are highly informative and constitute a powerful tool for genetic characterization studies in *E. dysenterica*.

Keywords: microsatellite, genetic diversity, conservation, cagaiteira and Cerrado.

1. Introduction

Microsatellites, or Simple Sequence Repeats (SSR), are tracts of repetitive DNA in which certain DNA motifs, of between one and six nucleotides, are repeated (Tautz 1989). SSRs have been detected in the genomes of numerous organisms and are distributed throughout the genome in both coding and non-coding regions (Li et al. 2002; Wu et al. 2007). With a wide genomic distribution, co-dominant inheritance, a multi-allelic nature, and a high level of polymorphisms, SSRs are highly favored molecular markers for characterization of wild species (Baraket et al. 2011; Campoy et al. 2011; Swapna et al. 2011). Microsatellites are also a very effective tool for the study of genetic structure of populations, gene flow, parenthood, clone detection, and population viability as well for analysis of the effects of habitat fragmentation effects and the definition of conservation strategies (Collevatti et al. 1999; Arnaud-Haond et al. 2007; Mason 2015).

Eugenia dysenterica DC. is a member of the Myrtaceae family and is popularly known as “cagaiteira” in Brazil due to its laxative properties. As is also the case for most of the native Cerrado species, it is not yet domesticated. This wild fruit-bearing tree species is typical to the Brazilian Cerrado (which is the largest savanna in South America), where it occurs at high densities on latosols (Naves 1999). The fruits of *E. dysenterica* are highly appreciated by local people that consume it *in natura* or in ice creams, juices or sweets. *E. dysenterica* has medicinal proprieties and is used in folk medicine for treating diarrhea, heart problems (Brandão 1991), diabetes, and jaundice (Silva 1999). The fruits are rich in polyunsaturated fats and present a linolenic acid

content (10.5%) higher than olive oil. *E. dysenterica* has an elevated productive potential, producing *ca*. 1500 fruits per tree annually (Scariot and Ribeiro 2015).

In spite of its high economic importance, so far, only five pairs of SSR primers have been developed for *E. dysenterica* (Telles et al. 2013) with an additional seven pairs of primers transferred from *Eucalyptus* spp. (Zucchi et al. 2002). The development of additional specific molecular markers can be important to clarify the mechanisms of genetic variability in this species. In addition, the development of new markers for *E. dysenterica* can be useful for programs of domestication, genetic improvement, or genetic conservation (*in situ* and *ex situu*). Previous studies on the population genetics of *E. dysenterica* indicated a low genetic diversity within populations and a high differentiation among different populations (Telles et al. 2003; Zucchi et al. 2005). In this paper, we report the development of 20 new polymorphic SSR markers and used 13 of them to characterize 180 individuals from six distinct *E. dysenterica* populations.

2. Methods

2.1. Microsatellite marker development

We produced a library enriched for microsatellites by hybridization capture, based on the method of Refseth *et al.* (1997) with modifications. *E. dysenterica* genomic DNA (20 µg) was digested overnight in a volume of 200 µl with 50U of the restriction enzyme *Sau*3AI. We stopped the reaction by incubation at 65°C for 20 min. The DNA fragments were resolved on a 1.5% agarose gel in TAE buffer, stained with ethidium bromide and the region between 400 and 800 bp excised. The fragments were purified using the QIAquick Gel Extraction Kit (Qiagen) and linked to 1.2 nmol of an adapter with overhang compatible with *Sau*3AI (Short: 5' CAGCCTAGAGCCGAATTCACC; Long: 5'

GATCGCTGAATTCGGCTCTAGAGCTG) at 4°C overnight using 20U T4 DNA ligase.

Fragments containing SSRs were captured using a probe composed of a mixture containing 100 pmol each of the oligonucleotides $(TG)_{13}$ and $(AG)_{14}$, which were previously end-labelled with biotin using 40 U Terminal deoxynucleotidyl transferase (TdT) and 50 nmol of Biotina 16 ddUTP and bound to magnetic beads (Dynabeads Streptavidin M-280; Dynal Inc). The size-selected DNA was heat-denatured and hybridized to the probes at 55°C for two hours. We washed the dynabeads five times at 65°C and the captured DNA eluted in water at 98°C for 10 min. We amplified the selected fragments by PCR using a primer specific to the adapter (*Sau*3AI PCR primer: 5' CAGCCTAGAGCCGAATTCACCGATC). PCR products were then cloned using the TOPO-TA kit (pCR™4-TOPO vector; Invitrogen) and the resultant library was transformed into *E. coli* by electroporation. Cloned inserts were directly amplified from bacterial colonies by PCR using cloning vector-specific primers. The inserts were sequenced using the BigDye 3.1 kit (Applied Biosystems) and an ABI Prism model 3730 Genetic Analyzer (Applied Biosystems). We sequenced the reverse strand of all SSR-positive clones and contigs were assembled in Genious (v5.5.9; Biomatters Inc.). SSR screening and primer design was done using msatcommander (v0.8.2; Faircloth 2008).

2.2. Optimization

To test the developed microsatellite markers, we performed PCRs with the DNA of four individuals in a temperature gradient (ranging from 50 to 60°C at two °C intervals) to determine the optimum annealing temperature. To extract the DNA we macerated approximately 100 mg of leaf tissue (fresh or stored dry in silica gel) using a

Mini Beadbeater 96 (Biospec). We extracted genomic DNA from leaves using a 2% CTAB protocol (Doyle 1987). To quantify the DNA we compared a standard DNA (λ) in a 1% agarose gel stained with ethidium bromide. We diluted the extracted DNA to 1.0 ng μL^{-1}.

We amplified the regions with microsatellites using a reaction mix containing 3 µl of DNA, 4.17 µl of H_2O distillate, 0.8 µl of buffer (10 mM Tris-HCL pH 8.3 with 50 mM KCl, 1.5 mM $MgCl_2$), 0.8 µl of each dNTP (0.25 mM), 0.8 µl of Bovine Serum Albumin (2.5mg/mL), 0.2 µl of Taq DNA polymerase (5U/µl) and 0.23 µl of each pair of primers (0.25µM). An annealing temperature gradient was used for optimization of PCR cycling, which comprised an initial 95°C for 5 min, then 35 cycles of 95°C for 1 min, 1 min at a temperature gradient ranging from 50 to 60°C with 2°C intervals for primer annealing and 72°C for 1 min. The PCR products were visualized by electrophoresis using a 3.5% agarose gel stained with ethidium bromide. To compare the sizes of the amplified fragments we used a 1 Kb DNA ladder (Invitrogen). We verified the optimum temperature at which the primer amplified, where we always chose the highest temperature at which a band clearly appeared.

From the initial 95 pairs of primers, 60 primer pairs resulted in clear products. These primers were tested in 12 adults of *E. dysenterica* from the *Estação Ecológica Águas Emendadas* conservation reserve in Distrito Federal, Brazil, to select those polymorphic and more informative. We synthesized one primer from each selected primer pair with a 5´ fluorescent tag. Using multiplex PCR or pool-plexing, products of up to three loci were analyzed together by capillary electrophoresis in an ABI 3730 Genetic Analyzer (Applied Biosystems). Samples (one µl each) were denatured for five min in a 10 µl mixture containing Hidi formamide (Applied Biosystems) and an internal

size marker (ROX; Brondani & Grattapaglia, 2001). The sizing of the fluorescent peaks was performed using GeneMapper software (v.4.1; Applied Biosystems). Based on the degree of polymorphism, the loci that had fewer genotyping errors were selected for use in a genetic population study.

2.3. Genotyping

From the 20 polymorphic primers described we selected 13 that were the most informative. To test if the 13 selected markers were good descriptors of genetic diversity of *E. dysenterica*, we genotyped 180 individuals from six populations located in the District Federal and in the State of Goiás, Brazil. The sampling sites were separated by a maximum distance of 35 km from each other. In each population, we collected leaves from 30 adult trees of *E. dysenterica,* distant at least 50 m from each other. DNA was extracted from these population samples in the same way as the marker development test samples.

2.4. Data analysis

We used AlleloBin to round the allele size to intergers (Prasanth et al. 2006) and micro-checker software to detect the presence of null alleles and their interference with genetic estimates (Van Oosterhout et al. 2004). We calculated the number of alleles per locus, allele frequency, observed and expected heterozygosity under Hardy-Weinberg equilibrium (HWE) for the 13 polymorphic microsatellite loci. We also tested for HWE and two-locus linkage disequilibrium. We estimated the intra-population fixation index based on all genotyped individuals and calculated the consistency of the estimates by bootstrap resampling using 10,000 permutations. Genetic Data Analysis software (GDA; Lewis 2001) was used to run the genetic analyses. The extracted DNA samples

are stored in the Genetic Bank of Embrapa Genetic Resources and Biotechnology and recorded in the Allele program (http://alelo.cenargen.embrapa.br).

3. Results and Discussion

We sequenced 384 colonies from the SSR-enriched library, of which 50.8% contained microsatellites of at least eight dinucleotide repeats, indicating a very high enrichment efficiency. Of the 195 unique SSR-containing sequences following vector and adapter trimming and contig assembly, primer pairs were successfully designed for 95 clones using default parameters in msatcommander.

Of the 95 initial pairs of primers, 35 (36.84%) did not produce clear PCR bands in any of the annealing temperatures tested. From the 60 primer pairs that did amplify (63.16%), 52 produced clear bands at an annealing temperature of 60°C, six at 58°C, one at 54°C and another at 52°C. Twenty pairs (21.05%) produced polymorphic SSR products in the test population, of which 13 primer pairs were selected given that they produced fewer genotyping errors (less than 20%). All 13 markers had the same optimum annealing temperature of 60°C, which is an advantage for the assembly of multiplex PCR reactions.

The development efficiency of useable polymorphic SSR markers was 5.2% of the initially sequenced colonies (384) from the SSR-enriched library. This efficiency is high when compared to findings from other studies with species from the Brazilian Cerrado: *Eugenia dysenterica* (0.3%, Telles et al. 2013), *Caryocar brasiliense* (2.8%, Collevatti et al. 1999) and *Tibouchina papyrus* (0.14%, Telles et al. 2011).

We did not find any significant null alleles or private alleles among the loci and all of them were in linkage equilibrium. All populations deviated from HWE ($P < 0.05$)

suggesting that the loci used in this study were independent of each other. Some loci showed a high level of inbreeding ($f = 0.072 \pm 0.128$ SD; Table 1).

In all 13 selected microsatellite loci, the allele number was high (mean 18 ± 7.2 SD; Table 1) ranging from 10 at locus ED77 to 39 at locus ED68. Mean gene diversity was high among all loci (He: 0.847 ± 0.076 SD; Ho: 0.760 ± 0.130 SD). Expected heterozygosity ranged from 0.631 at locus ED77 to 0.948 at locus ED79, while the observed heterozygosity varied from 0.467 at locus ED77 to 0.994 at locus ED68.

The present study found higher genetic diversity in *E. dysenterica* compared to other studies using SSR markers. A previous study using five SSR primer pairs found between three and 11 alleles per locus, with a expected heterozygosity ranging from 0.309 to 0.884 and an observed heterozygosity ranging from 0 to 0.812 (Telles et al. 2013). Another study used seven primer pairs transferred from *Eucalyptus* spp. and found between three and 22 alleles per locus with an expected heterozygosity between 0.198 and 0.837 and an observed heterozygosity between 0.200 and 0.706 (Zucchi et al. 2002).

The reported set of markers proved to be highly informative and constitute a powerful tool for population genetic studies in *E. dysenterica*. We propose to extend their use by testing the possibility of their transfer to other species of the genus *Eugenia*.

Table 1. Primer sequence, range of fragment size and genetic characterization of 13 microsatellite markers in *Eugenia dysenterica* DC., screened in 180 individuals. Ta, annealing temperature (°C); N, number of individuals successfully genotyped; A, number of alleles; Ho, observed heterozygosity; He, expected heterozygosity; *f*, intra-population fixation index.

Locus	Initiator sequence		5´ Tag	Size range (bp)	N	Ta	A	He	Ho	*f*
	Forward	Reverse								
ED06	TCGGGCTGGAT AGATTGGTG	GGCCTCACGTT AGTTTGAGC	FAM	80-124	172	60	16	0.832	0.755	0.092
ED10	TCGGGCTGGAT AGATTGGTG	GGCCTCACGTT AGTTTGAGC	NED	260-312	168	60	16	0.845	0.666	0.212
ED22	CATTTGGAGTC CGTTCGGTC	CCTTTCCAACC GCCATCATC	FAM	230-262	133	60	14	0.834	0.729	0.126
ED36	GAAACAGGGA CAGGCTTTGG	CCGTTTGGTCT TTGTTCCCTC	VIC	102-148	178	60	13	0.828	0.870	-0.051
ED37	ACTCGCTGTTC CTCGTGATAG	AGACTTCCTTG TAAAGCCTCC	VIC	157-185	152	60	14	0.834	0.743	0.109
ED43	ACTAAATCTCA AACATGGCCTC	TCCGCCTAAAC TTACTGTCG	NED	234-264	169	60	15	0.898	0.739	0.176
ED60	AAACGCACATT CAAGCAAGC	GGCTCGTAGTG GAAATCGAC	VIC	40-62	170	60	12	0.837	0.652	0.220
ED65	ATCTCCGACCA TCTCAGACG	GTCCAATGGTG CTTCAACGG	NED	62-134	176	60	23	0.925	0.914	0.011
ED68	GCTGAGGCGA GTGAAATGG	ATGCACCTCGG GCCTATAAC	NED	401-499	175	60	39	0.829	0.994	-0.196
ED71	TCGCAGTCAAA GCTCAACAG	ATCGCCTCCTG AGCATGC	NED	161-229	173	60	23	0.865	0.797	0.078
ED76	CTTTGATGTTG GTTCTGCGTG	ACTTGATTGCC ACTTGTGGTC	NED	90-130	171	60	17	0.904	0.824	0.088
ED77	GGTCTGCCGAT AATTTCTCAGG	GGAGGAGCAA GAAAGGCAAC	NED	140-164	167	60	10	0.631	0.467	0.261
ED79	GGTGGACCGCT CTACTGTAC	TGCTGCTCTCT CCTAAGTGAC	VIC	97-143	163	60	22	0.948	0.723	0.237

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CHAPTER 3 EFFECTS OF ANTHROPOGENIC DISTURBANCE ON THE GENETIC DIVERSITY AND CLONAL REPRODUCTION OF A NEOTROPICAL SAVANNA TREE *Eugenia dysenterica* DC.

ABSTRACT

In this study, we investigate the effect of fragmentation and disturbance on the spatial genetic structure, heterozygosity, inbreeding and clonal reproduction of *Eugenia dysenterica* DC. (Myrtaceae) in a Brazilian savanna based on the polymorphism of 13 microsatellite loci. We sampled six populations with markedly distinct histories of disturbance and fragmentation: three continuous populations (C1, C2 and C3), with no history of recent disturbance and three small and isolated populations (D1, D2 and D3), with history of disturbance due logging, fire and cattle pasture. We found no individuals clone in any population. Degraded populations had similar genetic diversity than continuous populations. All populations showed high levels of polymorphism and genetic diversity and low levels of inbreeding. This could be explained by the high genetic diversity of the species that counteract the effects of degradation. In addition, no spatial genetic structure was detected for populations, probably because the pollinators and dispersal agents are playing their functional roles. Our results suggest that degraded populations can be used for *in situ* conservation of the species. Therefore, conservation efforts should include degraded areas as areas of seed source and genetic pool.

Keywords: microsatellite, genetic diversity, conservation, savanna and cagaita.

1. Introduction

More than 75% of natural areas have suffered some kind of anthropogenic impact, such as hunting, extraction, livestock, fires, deforestation and selective logging (Broadbent et al. 2008; Ellis and Ramankutty 2008; Dornelas 2010). Moreover, populations are becoming isolated due to habitat fragmentation (Galeuchet et al. 2005; Hoffmeister et al. 2005; Haddad et al. 2015). Such impacts may affect plant species in different ways depending on their characteristics. Many species of plants combine sexual reproduction with regrowth, resulting in populations with two types of individuals: genets and ramets (Bond and Midgley 2001). A genet is an individual composed of tissues originating from a single zygote, while a ramet is an individual originating from a genet through vegetative growth and which has the potential to become independent of the genet (Maddox et al. 1989; Eriksson 1993). Population dynamics through clonal growth allows species to increase their chances of survival after disturbances (Eriksson 1994; Eriksson and Ehrle`n 2001; Peltzer 2002). This is because, compared to seedlings, sprouts have more vigorous and sturdier trunks (Bond and Midgley 2001), enabling a rapid re-colonization of degraded areas (Pennings and Callaway 2000; Cirne and Scarano 2001).

This survival strategy can reduce the genetic diversity and the spatial genetic structure (SGS) of populations (Namroud et al. 2005; Ohsako 2010). The increase in the density and grouping of ramets (Kleijn and Steinger 2002; Reisch et al. 2007) may concentrate pollinator agents on groups of ramets, transferring pollen among the same genotype. This condition, known as geitonogamy, reduces the frequency of crossovers between different genotypes (Charpentier 2002; Honnay and Jacquemyn 2008; Vallejo-Marín et al. 2010). With the reduction of genetic diversity, the population may be subject to inbreeding depression characterized by reduced productivity, fertility, seed

viability, adaptation and vitality (Keller and Waller 2002; Freeland 2005). This further reduces the potential of individuals to respond to climate change, thereby affecting the survival of the population and species (Primack 1998; Pauls et al. 2013).

The Savanna is a biome disturbed by fires and so their trees are adapted to disturbance through regrowth (Bond and Midgley 2001). Fire is an important driver of tree dynamics in savannas worldwide because it can limit seedling recruitment and prevent the transition of juvenile trees to the canopy (Bond 2008; Midgley et al. 2010; Murphy et al. 2010). The Brazilian Cerrado is the savanna with the greatest richness of the world's flora, occupying about two million square kilometers. It is characterized by great diversity of vegetation types that are home to a high diversity of plant species (more than 12,000) of which at least one third are endemic (Mendonça et al. 2008). As the seed dispersal in the Cerrado is limited (Salazar et al. 2012), vegetative reproduction is the main source of spread and persistence of many tree species (Hoffmann 1998; Bond and Midgley 2001; Gardner 2006; Salazar and Goldstein 2014).

The Cerrado has lost more than half of its original cover (MMA 2011) and if current trends of land change continue by 2050 will lose about 13% of the remaining areas (Ferreira et al. 2012). In addition, the remaining areas are subject to human impacts, as little more than 3.1% of the Cerrado is preserved in full protection conservation units (CNUC 2016). Besides, the areas with remaining vegetation suffer varying levels of human disturbance resulting from grazing, extraction, hunting, fires and invasion of alien species (Durigan et al. 2007). Therefore, genetic conservation in the Cerrado should be largely done under management, hence the importance of understanding the impact of land use and management in the genetic diversity of populations.

In the Cerrado, few studies have focused on the impact of human disturbances on proportion of clonal individuals (Moreno 2009; Antiqueira et al 2014; Antiqueira and Kageyama 2014). Two studies found no clonal genotypes in both control and degraded sites (Antiqueira et al 2014; Antiqueira and Kageyama 2014) and another found 68 individuals from 18 genotypes in a degraded site and 47 individuals all of different genotypes (Moreno 2009). Furthermore, some studies may not detect the presence of clones due to the type of molecular marker used. Population genetic studies using SSRs markers (Simple Sequence Repeats) were better in detecting the presence of clones (Arnaud-Haond et al. 2007; Allendorf et al. 2008). It is crucial to quantify vegetative reproduction in natural populations to better support the management, use and conservation of species.

Among the typical species of the savannah cagaita or cagaiteira, *Eugenia dysenterica* DC. (Myrtaceae) regenerates well after disturbance (Cortes 2012). The species name come from the laxative properties of its fruits which are marketed locally and the pulp is consumed in the form of sweets, juices, popsicles, and ice cream (Martinotto et al. 2008). In one hectare *E. dysenterica* can yield up to one and a half minimum salary per year (EMATER 2007). It is a medium-sized Myrtaceae from 4 to 10 m tall, twisted trunk with 20 to 40 cm in diameter and a thick bark that offers fire protection. *Eugenia dysenterica* can be found throughout the Cerrado, both in savannah areas and in the cerradão (Ratter et al. 2003), at densities of up to 160 individuals per hectare (Scariot and Ribeiro 2015).

This species is ideal to study the effects of anthropogenic disturbances because it is self-compatible (Proença and Gibbs 1994), and as such disturbances may affect not only the proportion of genetically identical individuals coming from sprouts, but also the emergence of individuals with similar genotypes. Previous studies with *E.*

dysenterica showed low genetic diversity in natural populations and high rates of inbreeding (Telles et al 2003; Zucchi et al 2005; Chaves et al. 2011, Telles et al 2013, Barbosa et al. 2015), which may already represent the effect of asexual reproduction by regrowth, self-fertilization, and/or geitonogamy.

The objective of this study was to determine the effect of human impacts on the genetic diversity of populations of *E. dysenterica* using SSRs markers. We compared the genetic diversity, proportion of clones, and spatial genetic structure (SGS) of *E. dysenterica* between preserved and degraded populations. Our hypothesis is that the disturbance stimulates the production of clonal individuals, with a higher proportion of clones and a lower genetic diversity in degraded populations than in preserved populations. In addition, we expected that SGS would be stronger in degraded populations due to the presence of clones growing next to each other.

2. Methods

2.1. Study sites

Six populations, located in the eastern region of Goiás state and in Federal District, in central Brazil, were selected. The study region is under anthropogenic pressure caused by deforestation resulting from agricultural expansion and urban growth. Three control populations were selected: *Estação Ecológica Águas Emendadas* (10,500 hectares), *Embrapa Cerrados* (700 hectares) and the *Instituto Federal de Brasília* (2,000 hectares) hereinafter named EEAE, EC and IFB. Such areas are large, have a high density of trees and shrub and no evidence of human impacts such as cut and burnt trees. We also selected three isolated populations, in sites where there was clear evidence of human impacts. These isolated populations were located in small remnants of natural vegetation (up to 36 hectares in area) and are distant at least 1 km

from other fragments. The areas are all unprotected, have low tree and shrub density and high presence of invasive grasses. They have signs of anthropogenic impacts as cut and burned trees and casual presence of cattle. They are also contiguous to roads, pastures and crops. To gather this information we made field observations, interviews with local residents, as well as searching the disturbance history with satellite imagery.

Table 1. Characteristic, name, area and coordinates of studied sites of *E. dysenterica*.

Site	Site characteristics	Site name	Area (ha)	Coordinates
D1	Degraded/Isolated	Embrapa beira de estrada	25	-15.616448 -47.705607
D2	Degraded/Isolated	Planaltina GO 1	25	-15.383020 -47.680821
D3	Degraded/Isolated	Planaltina GO 2	36	-15.396517 -47.634774
C1	Protected/Continuous	Embrapa Cerrados (EC)	1400	-15.594190 -47.740485
C2	Protected/Continuous	Estação Ecológica Águas Emendadas (EEAE)	10,500	-15.585397 -47.618057
C3	Protected/Continuous	Instituto Federal de Brasília (IFB)	2000	-15.655709 -47.704694

To estimate the genetic variability of a population it is necessary to analyze at least 30 individuals (Berg and Hamrick 1997). Thus, in each population leaf material was collected from 30 adults (more than 10 cm circumference at 30 cm above ground) of *E. dysenterica* distant at least 50 meters apart. Individuals had their geographical location recorded with a handheld GPS (Global Positioning System) with a maximum error of three meters. Previous studies in deciduous forests found that distance between genets and ramets varied from 0.6 to 14 meters (Rodrigues et al. 2004), values which are similar to those found for *Hymenaea stignocarpa* (Moreno 2009). Thus, to detect the presence of clones, leaf material was collected from all individuals found up to 10 meters away from each of the 30 adult trees found (Figure 1).

Fig 1 Pictures showing possible clonal individuals of *E. dysenterica* in site D2.

2.2. DNA extraction and genotyping

The extraction of genomic DNA and genotyping of individuals were conducted in the Plant Genetics Laboratory of EMBRAPA Genetic Resources and Biotechnology (CENARGEN), Brasília, DF. We extracted the DNA from newly emerged leaves of all individuals selected. We stored the leaves in paper bags containing silica gel to keep the leaves fresh. We insert one cm^2 of leaf fragment into 2 ml tubes containing two beads each. We insert the tubes into a Mini Beadbeater 96 (Biospec) so that the beads shattered the leaf material. Genomic DNA was extracted using the procedure 2% CTAB (cetyltrimethylammonium bromide) (Doyle and Doyle 1987). The extracted DNA was diluted to 1.0 ng/uL and then quantified by comparison with standard DNA (λ DNA) in 1% agarose gel stained with ethidium bromide. The extracted DNA was stored in the Bank of Genetics of Embrapa Genetic Resources and Biotechnology, located in Brasília, Brazil, and cataloged in Allele program of Embrapa Genetic resources information (http://alelo.cenargen.embrapa.br).

We amplified the regions with 13 microsatellites described in chapter two using Polymerase Chain Reaction (PCR) according to the following protocol: 3 μl of DNA, 4.17 μl of H_2O distillate, 0.8 μl of buffer (10 mM Tris-HCL pH 8.3 with 50 mM KCl, 1.5 mM $MgCl_2$), 0.8 μl of each dNTP (0.25 mM), 0.8 μl of Bovine Serum

Albumin (2.5 mg/mL), 0.2 µl of Taq DNA polymerase (5 U/µl) and 0.23 µl of each pair of primers (0.25 µM). Amplifications was carried out using the Thermal Cycler 9700 (Applied Biosystems) and Veriti with the following conditions: 95 °C for 5min, 35 cycles at 95 °C for 1min, 1min at specific annealing temperature for each primer pair, 72 °C for 1min. The product of the amplifications was visualized by electrophoresis in 3.5% agarose gel stained with ethidium bromide. To compare the sizes of the amplified fragments we used 1 Kb DNA ladder from Invitrogen.

Using multiplex systems, all products of each locus were analyzed together in the electrophoresis with the purpose of detect alleles by its fluorescence in certain (or specific) fragments, applying the automatic analyzer ABI 3730 (Applied Biosystems). We used a mixture of Hidi (10 µl), an internal marker (Rox) (1 µl) and PCR reaction (1 µl) denatured for five minutes at 95 °C. We detected the fluorescence peaks and genotyping using the 4.1 GeneMapper software (Applied Biosystems). From the detection of the amplified fragment sizes with each pair of primers for each individual, a spreadsheet used for analysis and estimates was generated. The rounding of allelic size was made using the software AlleloBin (Prasanth et al. 2006). We checked the presence of null alleles and their interference in the estimates using Micro-checker software (Van Oosterhout et al. 2004).

2.3. Data analysis

Genetic diversity

We characterized the number of alleles, private alleles, observed and expected heterozygosity under Hardy-Weinberg expectation (HWE) for all microsatellite loci. We also performed tests for HWE and two-locus linkage disequilibrium. We estimated the intra-population fixation index (*f*) based on all individuals genotyped. We calculated

the consistence of the estimates by a bootstrap resampling using 10,000 permutations. The genetic analyses were carried out using the software Genetic Data Analysis (GDA; (Lewis 2001). To test the difference of these indexes between control and degraded sites two-sample permutation tests were performed using the "coin" package in R (Hothorn et al. 2008). To check if all individuals sampled are from a single genetic pool or if they belong to different populations a Bayesian analyses of the populations structures was conducted using the software Structure 2.3.4 (Pritchard et al. 2000).

Spatial genetic structure

We recorded the geographic coordinates of the sampled trees to analyze the intra-population spatial genetic structure. The presence and extension of spatial genetic structure within the population was based on all 13 microsatellite loci and all 180 sampled adult trees. All the possible pairwise combinations of trees were considered for each one of the pre-defined distance classes. This estimate was obtained by calculating the Loiselle et al. (1995) co-ancestry coefficient (xy θ) for each distance class and locus and for the overall locus mean. Moran's I index values was used to test the significance of the deviations of the expected values from the null hypothesis of random distribution using a Bonferroni correction. The 95% confidence interval of the medium co-ancestry coefficient for each distance class was built based on the standard deviation of the average of the estimates, obtained by jackknifed permutation among loci. All the SGS data was calculated using SPAGeDI 1.4 (Hardy and Vekemans 2002).

Clones detection

The clonal diversity (CD), which describes the relationship between the amount of genets and ramets in a population, was calculated according to Dorken and Eckert (2001): CD = (L-1)/(N-1), in which G represents the number of genets found in the

sample and N is the number of sampled ramets. These analyzes were performed using the software GenClone 2.0 (Arnaud-Haond and Belkhir 2007).

3. Results

An average of 18 alleles at 13 microsatellite loci was found across 180 individuals from six populations of *E. dysenterica*. Null allele was detected in the *Águas Emendadas* (C2) population in ED76 (Oosterhout index > 0.30). No private allele was found. No loci combination showed linkage disequilibrium in more than two *E. dysenterica* populations, indicating that linkage disequilibrium in this case is actually related to specific populations and not to loci combination. We tested the diversity results with and without the linked locus and no difference was detected. For most loci, except ED36 and ED68, the observed heterozygosity was lower than the expected under Hardy–Weinberg equilibrium (Table 2).

Table 2. Genetic characterization of 13 microsatellite markers in *Eugenia dysenterica* DC. screened in 180 individuals from six populations. N, number of individuals successfully genotyped; A, number of alleles; He, expected heterozygosity; Ho, observed heterozygosity and *f*, intra-population fixation index.

Locus	Size range (bp)	N	A	He	Ho	*f*
ED06	80-124	172	16	0.832	0.755	0.092
ED10	260-312	168	16	0.845	0.666	0.212
ED22	230-262	133	14	0.834	0.729	0.126
ED36	102-148	178	13	0.828	0.870	-0.051
ED37	157-185	152	14	0.834	0.743	0.109
ED43	234-264	169	15	0.898	0.739	0.176
ED60	40-62	170	12	0.837	0.652	0.220
ED65	62-134	176	23	0.925	0.914	0.011
ED68	401-499	175	39	0.829	0.994	-0.196
ED71	161-229	173	23	0.865	0.797	0.078
ED76	90-130	171	17	0.904	0.824	0.088
ED77	140-164	167	10	0.631	0.467	0.261
ED79	97-143	163	22	0.948	0.723	0.237

There were no differences in genetic diversity between both sites (degraded vs control sites, He = 0.843 ± 0.017 SD vs. 0.797 ± 0.040 SD; Z = -1.4942; P = 0.1351; Table 3). The observed heterozygosity also did not show any differences (degraded vs

control, Ho = 0.770 ± 0.026 SD vs. 0.753 ± 0.024 SD; Z = -0.82805, P = 0.4076). Polymorphism was slightly higher in degraded sites (12.1 ± 0.1 SD vs. 10.3 ± 1.3 SD allele; Z = -1.7084, P = 0.08756). We found, on average, more private alleles in degraded sites (Ap = 8 ± 2 vs 6 ± 3). Fixation index was higher than zero in all degraded populations, but only significant in D1, showing an excess of homozygotes. In control sites C1 and C3 there was an excess of homozygotes but only significant in C3. C2 showed negative fixation index. The species have a hybrid system of reproduction, performing crossing between individuals and self-fecundation with an apparent outcrossing rate higher than 87% in all populations.

Table 3. Descriptive statistics by population of *E. dysenterica*. N, Number of individuals analyzed; A, number of alleles; Ap, private alleles; He, expected heterozygosity; Ho, observed heterozygosity; *f*, intra-population fixation index and t_a, apparent outcrossing rate (1-*f*).

Population	N	A	Ap	He	Ho	*f*	t_a
C1	28.76	9.4	3	0.794	0.724	0.088	0.911
C2	27.69	9.8	6	0.758	0.766	-0.011	1.000
C3	27.38	11.8	9	0.839	0.769	0.084*	0.915
Mean		10.3	6	0.797	0.753	0.038	
SD		1.3	3	0.040	0.024	0.070	
D1	26.76	12.1	8	0.843	0.741	0.123*	0.876
D2	28	12	6	0.825	0.776	0.060	0.939
D3	28.07	12.3	10	0.861	0.793	0.079	0.920
Mean		12.1	8	0.843	0.770	0.070	
SD		0.1	2	0.017	0.026	0.013	

f index, * P <0.05 significance under Bootstrapping 10000 permutations.

We found more individuals around matrix adult trees in degraded sites than in control sites (81.3 ± 87.6 SD in control sites versus 98.6 ± 57.3 SD in degraded sites). However, no clone genotype was detected in the 720 individuals analyzed using GenClone software, indicating that all individuals sampled belong to different genotypes.

The gene pool analyses using Structure identified three clusters (Delta K = 121.53; Figure 2). The analyses tend to group C1 with D1, C2 with D2 and C3 with D3. The neighbor joining tree analyses show a similar pattern (Figure 3). However this is

not a clear pattern, there are a lot of individuals that have a genetic pool similarity between populations.

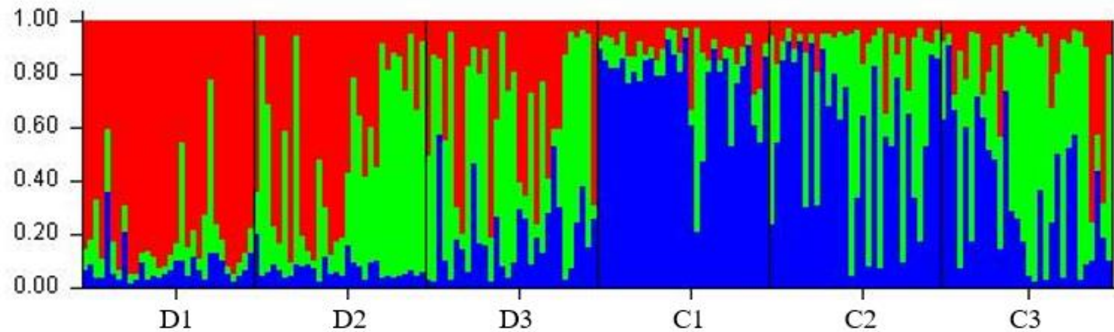

Fig 2 Clustering of six populations of *E. dysenterica* by Structure software. Each population is represented by a color. Each dot represents an individual and the position in the triangle represent its estimated membership to a cluster. Each corner represents one of the three clusters. Control sites (C1, C2 and C3) and degraded sites (D1, D2 and D3)

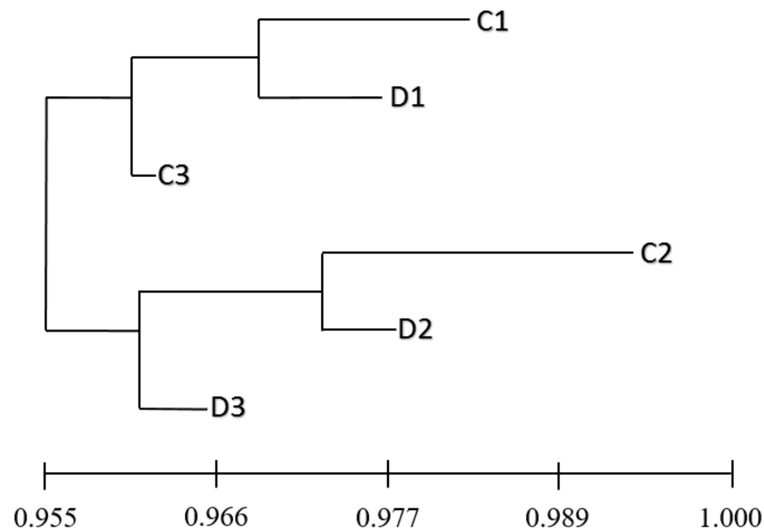

Fig 3 Neighbor-joining tree showing the genetic similarity between six populations of *Eugenia dysenterica* Mart. Control sites (C1, C2 and C3) and degraded sites (D1, D2 and D3)

The spatial genetic structure was weak in all populations (Figure 4). In control sites, there were more similar genotypes in the third class of distance for C1 (90m) and in the second class for C2 (150m). In degraded sites, the SGS was stronger only up to 80 meters in D3.

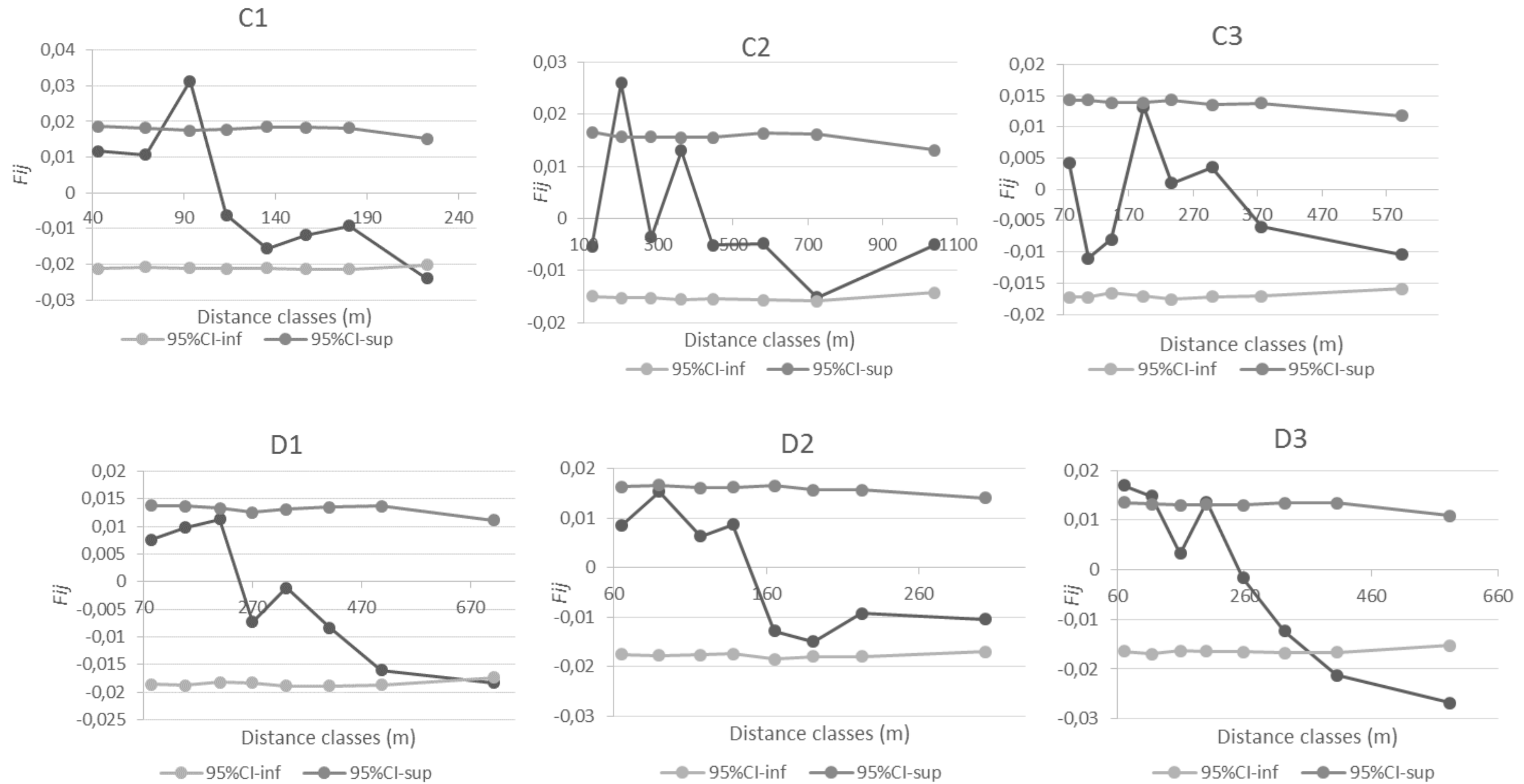

Fig 4 Kinship coefficients (*Fij*) per distance classes in six populations of *Eugenia dysenterica* DC. sampled in Brazilian savanna. Control sites (C1, C2 and C3) and degraded sites (D1, D2 and D3). Confidence intervals around each *Fij* value were obtained through a 10,000 jackknifed permutation.

4. Discussion

Contrary to our expectations, we did not find clones of *E. dysenterica* in the degraded sites. Our study stands out in contrast to predictions of genetic loss of species that have suffered rapid population declines and subsequent human impacts. There were no differences between sites in terms genetic diversity, observed heterozygosity and polymorphism. Actually, degraded sites showed more privates allelic than control sites. Fixation index was low in all populations studied. We also did not observe any significant spatial genetic structure in the populations studied.

Vegetative regeneration can be favored in situations involving environmental disturbances, such as herbivory, fire and logging (Bond and Midgley 2001; Bond and Midgley 2003; Aguilar et al. 2008). However, this study did not detect clones for *E. dysenterica* in degraded or control sites, and in this sense, our results are similar to those found for other Cerrado tree species (Antiqueira et al 2014; Antiqueira and Kageyama 2014). The absence of clonal individuals in all populations studied cannot be attributed by our sampling effort, which was higher than that in other studies with clonal species (Arnaud-Haond et al. 2007; Moreno 2009). It is unlikely that trees distant more than 10 meters would be clones as no clone was detected within the 10 m radius of adult tree. For the 27 species analyzed in semi deciduous forests the distance between genets and ramets varied from 0.6 to 14 meters in (Rodrigues et al. 2004), and these values are similar to those found for a Cerrado species (Moreno 2009).

Plants can present different adaptive traits to persist in degraded environments: some re-sprout and others rely on a massive production of seeds (Bond and Midgley 2001; Hoffmann and Moreira 2002; Klimešová and Klimeš 2007). Thus, there is a trade-off in allocation of resources between sprouter and non-sprouter strategies (Bellingham and Sparrow 2000; Bond and Midgley 2001). *E. dysenterica* presents reproductive traits that suggest the

existence of a non-sprouter strategy; the production of fruits is high -- varying 500 to 2000 fruits per plant (Martinotto et al. 2008; Scariot and Ribeiro 2015) -- and germination rates are also high (95% in experimental studies; Martinotto et al. 2008; Silva et al. 2015). Thus, the strategy of allocation of resources in *E. dysenterica* seems to be towards reproduction rather than sprouting, although this does not exclude the possibility of re-sprouting. In fact, plant regeneration includes three aspects: aerial regrowth capacity, regrowth of trunk base and of roots. The regrowth of roots is able to form new individuals (ramets) derived from genets roots while the aerial and the regrowth from the trunk base will not form a ramet. That may be the case for *E. dysenterica* in the degraded populations studied.

Studies involving human impacts and genetic diversity also did not find significant differences between populations in intact and degraded sites (Moraes et al. 2005; Gonçalves et al. 2010; Moura et al. 2012; Collevatti et al. 2014). In the case of *E. dysenterica*, the lack of differences between control and degraded sites can be explained by the high genetic diversity and high density of individuals, and perhaps because trees that were sampled were already present in the degraded sites well before the disturbance events. The polymorphism and genetic diversity found in our study are similar to those found for other savanna tree species (Collevatti et al. 2001; Moreira et al. 2009; Antiqueira et al. 2014; Antiqueira and Kageyama 2014) and higher than another study with *E. dysenterica* (Telles et al. 2013). Demographically, the naturally high density of trees of *E. dysenterica* (ca 150 per hectare; Scariot and Ribeiro 2015) may explain the genetic resistance of the species to degradation. In addition, *E. dysenterica* is a long-lived species, so a larger number of pre-degradation trees than expected may remain in the present adult population. In contrast, a study found lower polymorphism in a short cycle species S*olanum lycocarpum* on degraded sites (Moura et al. 2012).

In terms of ecology, pollinators and dispersal agents may be playing their functional roles in degraded sites. A good indicator of this scenario is the low inbreeding found for adults and weak SGS we found in these sites. Our study also indicates that *E. dysenterica* have low self-pollination rates, usually performing crossing between individuals with an apparent outcrossing rate higher than 87%. The strength of SGS is determined at a fine spatial scale by seed and pollen dispersal (Hardy et al. 2006; Born et al. 2008; Kettle et al. 2011). Other studies found a stronger SGS on degraded sites than on control sites (Moraes et al. 2005; Gonçalves et al. 2010; Collevatti et al. 2014) and a higher inbreeding (Collevatti et al. 2014) given the disruption of the pollination and dispersal functions in degraded sites.

There are no studies about the dispersal system of *E. dysenterica* (Scariot and Ribeiro 2015). However, many Myrtaceae species are dispersed by birds and small mammals (Pizo 2002) and reported distances of seed dispersal mediated by animals are generally in the range of 100 m to 1 km (Westcott and Graham 2000; Russo et al. 2006). *E. dysenterica* is pollinated by bees of the genera *Scaptotrigona*, *Bombus*, *Ceratina* and *Apis* (Proença and Gibbs 1994; Scariot and Ribeiro 2015). The study region has a large number of honey producers, with 160 producers registered in the Brazilian Confederation of Beekeeping (CBA). In addition, pollen movement usually travels distances from 100 m to 14 km in tropical species (Nason et al. 1998; Dick et al. 2008).

Our results therefore provide evidence that pollinators and dispersal agents are probably increasing the resilience of the tree species in a degraded landscape. The low inbreeding and high heterozygosity found in this study are good indicators of short-term population viability (Frankham 2005). High allelic richness and private alleles in both degraded and control sites indicate long-term viability (Frankham 2005). However, SGS may be stronger in *E. dysenterica* in distance classes lower than we studied (50 m), as found by

Collevatti et al. (2014). Nevertheless, a study found strong SGS in an animal dispersed species of Cerrado at distances up to 300 meters in a degraded site (Gonçalves et al. 2010).

These results may not be representative of the majority of Neotropical savanna species but they show a lower than expected genetic vulnerability of a tree species in a highly urbanized landscape. Half of Brazilian native vegetation occurs on private properties (Soares-Filho et al. 2014) and most of them have records of some disturbance. For example, only in the state of São Paulo over 30% of the remaining cerrado fragments have cattle records and more than 20% suffer from selective logging (Durigan et al. 2007). In this sense, it is important to protect the rich sources of genetic diversity that will enable adaptation in the future (Moritz 2002; Bonin et al. 2007). In addition, when sampling for seeds it is also interesting to select sites with higher levels of human impact, thus ensuring that the unique alleles in those sites would be preserved (Falk et al. 2001). Degraded areas can be useful for *in situ* conservation and serve as a source of seeds with broad genetic base to restoration and others conservations strategies. Finally, the high rates of natural regeneration of *E. dysenterica* added to the high genetic diversity found in degraded sites make it a good choice to use in the recovery of degraded sites.

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CONCLUSÕES GERAIS

Capítulo 1: A fragmentação parece não afetar a diversidade genética de araticum. Todas as populações encontradas apresentaram altas taxas de polimorfismo e diversidade genética. Isso se deve possivelmente a alta diversidade genética natural encontrada, o ciclo de vida longo da espécie e/ou porque ainda há fluxo gênico entre populações fragmentadas. A alta diversidade genética encontrada indica que é possível conservar a espécie *in situ* nos fragmentos. Essas áreas podem também servir como fonte de propágulos para programas de restauração. Contudo, vale notar que é possível que os indivíduos amostrados sejam representantes de um cenário antes da fragmentação devido a sua longevidade.

Capítulo 2: Os protocolos utilizados para desenvolver marcadores moleculares SSR para cagaita demonstraram ser de alta eficiência. Dos 95 pares de primers desenhados, 20 produziram produtos de PCR polimórficos. Esses 20 primers foram então testados em uma população de cagaita e apresentaram informações confiáveis a respeito da diversidade genética de cagaita. Sendo assim, são primers recomendados para estudos de caracterização genética de *E. dysenterica.*

Capítulo 3: A fragmentação parece não afetar a diversidade genética de cagaita tão pouco estimulou a reprodução clonal da espécie. Isso poderia ser explicado pela alta diversidade genética encontrada o que poderia se contrapor aos efeitos negativos da degradação das áreas. Além disso, a ausência de estrutura genética espacial nas áreas degradadas sugere que os polinizadores e dispersores estão atuando nessas áreas. Nossos resultados sugerem que áreas degradadas podem ser utilizadas para conservação *in situ* da espécie.