Genetics and Molecular Research

A comparative framework of the *Erythrina velutina* tree species in reforested land and native populations

E.M.S. Souza[1], G.S. Pereira[2], R. Silva-Mann[3], S.V. Álvares-Carvalho[4] and R.A. Ferreira[5]

[1]Programa de Pós-Graduação em Agricultura e Biodiversidade, Universidade Federal de Sergipe, São Cristóvão, SE, Brasil
[2]Programa de Pós-Graduação em Biotecnologia em Recursos Naturais, Universidade Federal de Sergipe, São Cristóvão, SE, Brasil
[3]Departamento de Agronomia, Universidade Federal de Sergipe, São Cristóvão, SE, Brasil
[4]Programa de Pós-Graduação PNPD-CAPES, Universidade Federal de Sergipe, São Cristóvão, SE, Brasil
[5]Departamento de Ciências Florestais, Universidade Federal de Sergipe, São Cristóvão, SE, Brasil

Corresponding author: E.M.S. Souza
E-mail: ericafloresta@yahoo.com.br

Genet. Mol. Res. 15 (2): gmr.15028534
Received February 4, 2016
Accepted March 11, 2016
Published June 17, 2016
DOI http://dx.doi.org/10.4238/gmr.15028534

ABSTRACT. *Erythrina velutina* Willd. (Fabaceae: Papillionoideae) is a pioneer species found in tropical and subtropical regions of the world that has medicinal properties and that is used in reforestation projects. This species is rare in some areas of northeastern Brazil. This study aimed to characterize and compare genetic structures of natural and restored populations of *E. velutina*, with a focus on the selection of tree seeds. A total of 108 individuals from five natural populations and one restored population were analyzed using ISSR markers, resulting in 407 polymorphic fragments. A high rate of polymorphism was

observed in the restored population. The highest genetic variability was identified within populations (82%). Genetic bottleneck tests were significant for the Carmópolis/Rosário do Catete and Laranjeiras natural populations along with the Laranjeiras restored population. Genetic distances significantly correlated with spatial distance. Only the restored population retained unique alleles. Similarly, increased genetic distance was observed in individuals of the restored populations compared to the other populations. Observed genetic variation in both natural and restored populations of *E. velutina* was moderate, thus enabling selection of divergent trees from those trees supplying seeds. Environmental protection and management of these areas is necessary for the maintenance of these individuals and subsequent reproduction. We recommend suggestions for *E. velutina* conservation, since the restoration model adopted in this study did not promote the development of the specimens until the reproductive stage in a fashion that aims to augment the soil seed bank supply, as is suggested for pioneer species.

Key words: *Erythrina velutina*; Genetic diversity; Reforestation; ISSR

INTRODUCTION

The effects of climate change include consequences such as vegetation loss. One of the derivatives of the impacts of environmental change on plant species is the reduction of genetic diversity in plant populations due to directional selection, which may affect ecosystem functioning and resilience (Bellard et al., 2012). This scenario leads to the need to adopt alternatives that minimize the impact caused by deforestation; the alternatives include forest restoration projects. In reforestation programs using native species, knowledge of the genetic origin of the seed is crucial (Sebbenn, 2002) since a portion of the self-sustainability of an implanted population is imparted by introduced genetic diversity (Aitken et al., 2008; Pautasso, 2009). However, the genetic material to be implemented is rarely considered in restorative practices. Forest fragmentation complicates the selection of tree seed suppliers, given that reducing the size of the population could ease crossing between closely related individuals, and result in immediate loss of alleles (Young et al., 1996).

Genetic studies on tree populations guide the selection of tree seed producers since these studies allow the identification of genetically diverse trees for seed collection. In reforested areas, genetic studies provide important information for management and conservation measures of a given restored area, making that area able to remain conserved over generations. Inter-simple sequence repeat (ISSR) molecular markers assist these studies due to identification of substantial polymorphism. Their use in genetic studies for native populations has been effective (Gonçalves et al., 2014; Silva et al., 2014). On the other hand, there are few genetic studies comparing restored and natural populations.

In regions of severe fragmentation, seed collection for forest restoration becomes difficult given that fragment size can influence the reduction of genetic variability, and introduce the need to identify additional seed producers. In Sergipe, a state in northeastern Brazil, there is territorial land extension of 21,918,493 km^2, and more than 89% of the total forest fragments are smaller than 50 hectares.

Erythrina velutina Willd. is a widely known species belonging to the *Erythrina* (Fabaceae - Papillionoideae) genus that is popularly known as mulungu. It is most common in tropical and subtropical world regions (Vasconcelos et al., 2003). Currently, there are more than 130 *Erythrina* spp, of which at least 70 are native to the Americas (Oliveira et al., 2012). It is widely used in reforesting projects in northeastern Brazil because it is a pioneer species that is capable of nitrogen fixation and drought resistance (Lorenzi, 2008). The species has significant economic value due to medicinal properties (Oliveira et al., 2012; Saraiva et al., 2015). However, it is rare in some regions of Brazil due to intense exploitation and suppression of natural occurrence areas. Studies conducted in the state of Sergipe identified 20 individuals in a protected area of 100 ha (10 km and 100 m of extension) (Gonçalves et al., 2014; Melo et al., 2015).

Few studies evaluate the genetic diversity of individuals within restored and natural population areas. This is the first study carried out in Sergipe. *E. velutina* was selected for the study as it is being often used in reforestation projects, it has significant medicinal value, and it is rare in some regions of the state. Therefore, the objective of this study was to measure and compare the genetic variation of *E. velutina* populations in natural and restored areas in order to set management and conservation strategies as well as to identify genetically superior individuals for seed collection.

MATERIAL AND METHODS

Areas of study and plant materials

E. velutina individuals (N = 108) were sampled at one restored population and five natural populations (Table 1; Figure 1). The Laranjeiras restored population (RP) is surrounded by sugarcane production, a cement factory, grasslands and fragmented riparian forest, and the restoration area was established in 2005. The Laranjeiras (LA) natural population is located within a private farm surrounded by grasslands. The Carmópolis/Rosário do Catete (CA) natural population is located along a road and adjacent to sugarcane production fields and potassium factories. The Pinhão (PI) and Lagarto (LR) natural populations are close to pasture fields. Finally, the Santana do São Francisco (SF) natural population is located along the edges of the São Francisco River Permanent Preservation Area near pastures and agricultural land.

Table 1. Number of individuals (N), geographic location and altitude of *Erythrina velutina* Willd. populations. Sergipe, Brazil.

Code	Municipality	Land use	N	Latitude and longitude	Elevation (m)
Natural population					
LA	Laranjeiras	Grasslands	22	10°50'00.8"S, 37°11'29.1"W	60
CA	Carmópolis/Rosário do Catete	Sugarcane crop	21	10°40'23.9"S, 37°01'42.3"W	23
PI	Pinhão	Grasslands	21	10°32'36.2"S, 37°38'33.4"W	215
SF	Santana do São Francisco	PPA*	12	10°18'16.8"S, 36°35'25.7"W	9
LR	Lagarto	Grasslands	9	10°55'11.3"S, 37°39'17.5"W	168
Restored population					
RP	Laranjeiras	Sugarcane crop	23	10°49'10.0"S, 37°09'36.0"W	16
Total			108		

*Permanent preservation area.

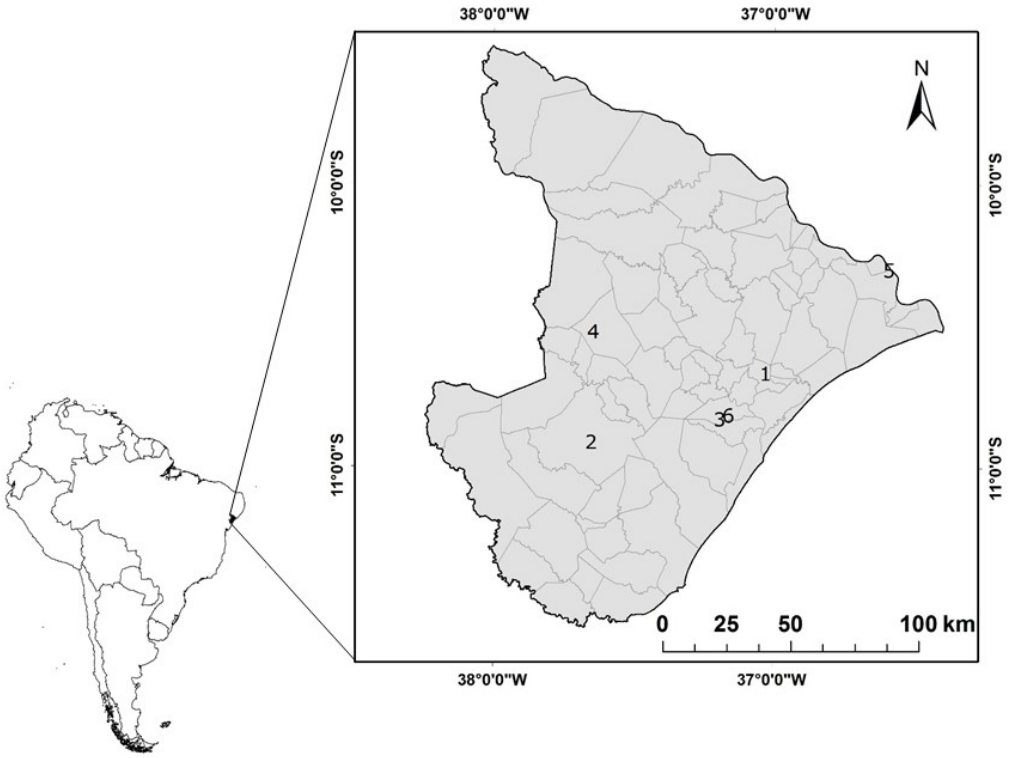

Figure 1. Location map of *Erythrina velutina* Willd. populations (Natural: 1. Carmópolis; 2. Lagarto; 3. Laranjeiras; 4. Pinhão; 5. Santana do São Francisco, and 6. Restored). Sergipe, Brazil.

Shoot material of adult individuals from each population was collected for subsequent extraction of genomic DNA as described by Nienhuis et al. (1995) with modifications.

Sampling in the restored area was performed in 30 plots (20 m x 30 m each), each separated by 127 m. Only twelve plots contained *E. velutina* trees. In natural areas, the collection was randomized in accordance with Kageyama and Gandara (1999).

PCR amplification

Eight ISSR primers (Table 2) were used. PCR amplification was conducted in a PTC-100 Thermocycler (MJ Research Inc., Quebec, Canada) as follows: initial denaturation at 94°C for 90 s; 35 denaturation cycles at 94°C for 45 s; annealing at the temperature indicated for each primer for 30 s; extension at 72°C for 90 s; and a final extension at 72°C for 5 min. Fragments were visualized on 1.5% agarose gel (1X TBE: 89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.3) in a horizontal electrophoresis system (Loccus Biotecnologia LCH 20 x 25) at 120 V for 2 h. The gel was stained with GelRed® dye (Biotium) and amplification products were visualized under UV light.

Table 2. Primer annealing temperature (T_m), number of fragments (NF), number of polymorphic fragments (NFP) and percentage of polymorphism generated by each primer for *Erythrina velutina* Willd.

Primer	Sequence (5'- 3')	Tm (°C)	NF	NFP	P%
UBC 807	(AG) 8-T	43	48	34	70.833
UBC 808	(AG) 8-C	47	48	43	89.583
UBC 809	(AG) 8-G	48	60	53	88.333
UBC 811	(GA) 8-C	45	64	59	92.187
UBC 825	(AC) 8-T	47	53	45	84.905
GOOFY	(GT) 7-YG	48	60	46	76.666
MAO	(CTC) 4-RC	45	78	78	100.000
OMAR	(GAG) 4-RC	47	68	49	72.058
Total			479	407	
Mean			59.875	50.875	

R = purine (A or G) e Y = pyrimidine (C or T).

Data analysis

The generated ISSR fragments were used to obtain a binary matrix of presence (1) or absence (0). Fragments with inferior staining and low resolution were not included in the analysis. The optimal number of fragments was estimated by the GENES software (Cruz, 2013) using the population with the smallest number of individuals in order to obtain the stress value.

POPGENE software (version 1.32) (Yeh et al., 1999) was used to characterize genetic variability. Nei genetic diversity (*H*), the percentage of polymorphic loci (P%), and Shannon Index (*I*) were estimated for each population and for the set of populations. The same software was used to analyze the gene flow (N_m), with 1000 permutations. Bottleneck 1.2.02 software (Cornuet and Luikart, 1996) was used to determine the effect of genetic bottlenecks in the natural and restored populations, using the infinite alleles model (IAM) with 1000 replications.

Genetic variation of individuals within and among populations was evaluated by analysis of molecular variance (AMOVA) with GenAlEx software version 6.5 (Peakall and Smouse, 2012) with a resampling value of 9,999. The same software was used to analyze genetic distance (F_{ST}), the number of unique alleles, and the principal coordinates (PCoA) within populations.

NTSYS software 2.1 pc (Rohlf, 2002) was used for construction of a dendrogram by the UPGMA clustering method for simplified representation of genetic distances among the six *E. velutina* populations (F_{ST}). The Mantel test was conducted in order to identify correlation between genetic and spatial distance.

RESULTS

Polymorphism detected by ISSR analysis

The stress value for the smallest population (LR) was 0.0123 with a correlation of 0.995, thus confirming stability among the number of primers and the number of fragments obtained.

The use of eight ISSR primers resulted in relatively high polymorphism rates for 108 individuals (Table 2). P% ranged from 70.833 to 100%. A total of 479 fragments was obtained, of which 407 were polymorphic (84.968%). The number of polymorphic fragments ranged from 34 to 78. The primer MAO generated the highest number of fragments (78), all of which were polymorphic. The primer UBC 807 primer generated the fewest fragments and the least polymorphism when compared to the other primers (Table 2).

Genetic diversity and structure

Table 3 shows genetic parameters at the population level. Four parameters showed moderate genetic variability for populations. RP presented the highest rates (N_A = 1.9651; N_E = 1.6176; H = 0.3559, I = 0.5277 and P = 96.51%) whereas the SF natural population had the lowest rates (N_A = 1.4767; N_E = 1.2834; H = 0.1633; I = 0.2440 and P = 47.67%) of genetic variation. Unique alleles were only identified in RP. The genetic bottleneck test for infinite alleles (IAM) was significant (P < 0.01) for CA, LA, and RP, with 52 (P = 0.00076) 54 (P = 0.00030) and 66 (P = 0.00000) loci (respectively) with excess heterozygosity.

Table 3. Number of samples (N), polymorphic loci (*K*), number of alleles (N_A), number of effective alleles (N_E), Nei's genetic diversity (*H*), Shannon Index (*I*) and percentage of polymorphic loci (*P%*) in *Erythrina velutina* Willd. populations.

Population	N	*K*	N_A	N_E	*H*	*I*	*P%*
Natural							
LA	22	73	1.8488	1.5262	0.3026	0.4496	84.88
CA	21	70	1.8140	1.5111	0.2921	0.4336	81.40
PI	21	62	1.7209	1.4365	0.2499	0.3716	72.09
SF	12	41	1.4767	1.2834	0.1633	0.2440	47.67
LR	9	66	1.7674	1.5020	0.2827	0.4169	76.74
Restored							
RP	23	83	1.9651	1.6176	0.3559	0.5277	96.51
Total	108		1.7654	1.6247	0.3573	0.5308	76.55

Ranking of multivariate data (PCoA; Figure 2) for the first two principal coordinates explained 18.27% of the total variability. Individuals LA7, LA8, and LA10 had the highest genetic distance.

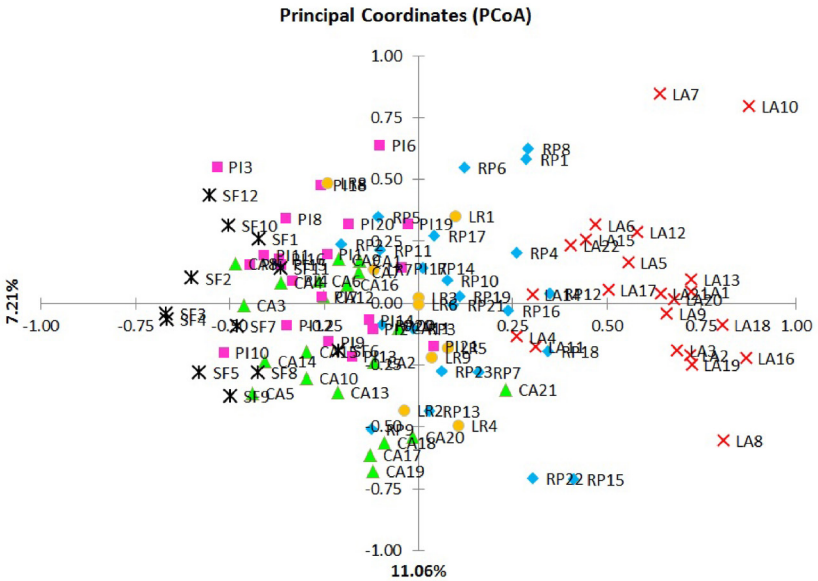

Figure 2. Coordinate means of 108 individuals of six *Erythrina velutina* Willd. populations (LA: Laranjeiras, CA: Carmópolis, PI: Pinhão, SF: Santana do São Francisco, LR: Lagarto and RP: Restored), Sergipe, Brazil.

Analysis of molecular variance (AMOVA) revealed significant genetic variance ($P < 0.01$) among and within *E. velutina* populations (Table 4). Genetic variation within populations accounted for 82% of total variation. The genetic diversity index among populations (F_{ST}) was 0.184, indicating moderate genetic differentiation. N_m, was estimated at 1.7893, suggesting high gene flow among *E. velutina* populations.

Table 4. Analysis of molecular variance (AMOVA) of 108 *Erythrina velutina* Willd. individuals.

Variation	d.f.	SS	MS	%	F_{ST}	P
Among populations	5	303.488	60.698	18	0.184	0.000
Within populations	102	1240.512	12.162	82		
Total	107	1544.000		100		

d.f. = degrees of freedom; SS = sum of squares; MS = mean of squares.

Genetic similarity

E. velutina population pairwise comparisons of genetic differentiation (F_{ST}) (Table 5) was conducted using the UPGMA method (Figure 3). RP and CA populations were the most similar with 11.2% genetic differentiation, whereas LA and SF natural populations were the most divergent (35.8% genetic differentiation). LA natural population presented the highest genetic differentiation.

Table 5. Genetic distance (F_{ST}) values (below the diagonal) and geographic distance (km) (above the diagonal) between pairs of populations of *Erythrina velutina* Willd.

Population	RP	LA	CA	PI	SF	LR
RP	-	3.760	21.830	62.230	62.020	54.520
LA	0.139	-	25.290	60.160	59.960	50.580
CA	0.112	0.230	-	69.330	69.110	73.100
PI	0.155	0.267	0.133	-	117.760	42.970
SF	0.191	0.358	0.138	0.205	-	134.050
LR	0.114	0.184	0.117	0.150	0.249	-

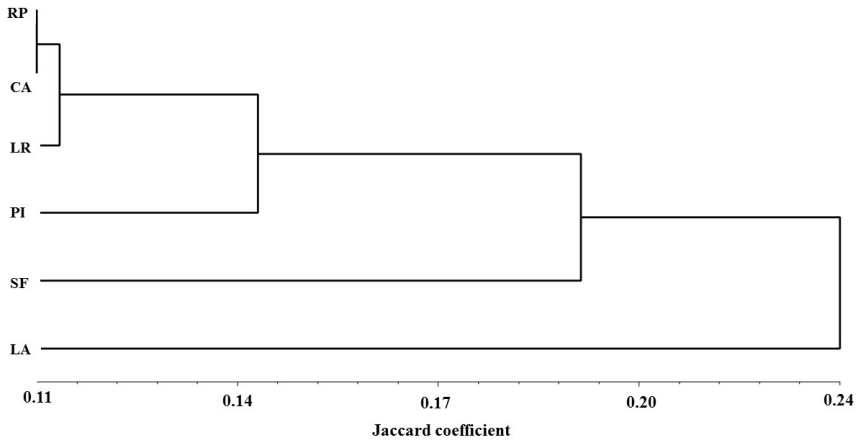

Figure 3. Dendrogram generated using the unweighted pair group method with arithmetic mean (UPGMA) for different *Erythrina velutina* Willd. populations based on F_{ST}.

Individuals in the RP population had the highest values of genetic distance (16 to 47%). The most genetically distant individuals were RP1 and RP15, whereas RP16 and RP18 were the most similar individuals in that population. The LA natural population also showed high levels of genetic distance among individuals (11 to 47%). The most distant pair of individuals were LA8 and LA22, and the most similar pairs were LA1 and LA2, and LA20 and LA21. The SF population presented the genetically most-similar individuals, and values ranged from 5% (SF 9 and SF5) to 28% (SF6 and SF12). Values in the PI population ranged from 8% (PI11 and PI16) to 31% (PI18 and PI21). In the CA population variation ranged from 9% (CA19 and CA20) to 36% (CA9 and CA19). Finally, in the LR population, values ranged from 15% (LR3 and LR6, and LR3 and LR7) to 40% (LR1 and LR5). There was no significant correlation between genetic and geographic distances among all populations ($r = 0.05791$; $t = 0.1498$; $P = 0.5595$).

DISCUSSION

Despite the knowledge of the importance of genetic restoration in self-sustainability of restored areas, there have been few studies conducted in this context (Dolan et al., 2008; Ritchie and Krauss, 2012; Cruz Neto et al., 2014). This is the first study to evaluate and compare the genetic diversity of *E. velutina* restored area and natural populations. In the present work, high polymorphism (479 ISSR markers, of which 407 were polymorphic) was observed. These values agree with the value reported by Colombo et al. (2000) that recommend 50 to 100 fragments as sufficient to estimate genetic relationships within and between populations of plant species. Shannon Index (*I*) values estimated in this study for the SF and PI natural populations were lower (0.2440 and 0.3716, respectively) than values obtained by Melo et al. (2015) (0.34 and 0.41).

The high polymorphism observed for the RP population in relation to the natural populations permit us to reach one of the goals for reforesting an area, which is increased genetic variability of the implanted individuals, in accordance with diverse seed sources used to establish plants in the area. The low values observed for SF is a consequence of the negative impact of human pressure on that population, which is located in a heavily fragmented area (Álvares-Carvalho et al., 2015) alongside the shores of the São Francisco River, itself a major Brazilian river. This river is extremely relevant as it has direct impact on food supply, including as a source of fish and agricultural irrigation water for five Brazilian states (Minas Gerais, Bahia, Pernambuco, Sergipe, and Alagoas). Restoration using *E. velutina* seedlings in the SF population (Santana do São Francisco) is recommended, given that *E. velutina* rarity in this area is due to the small population size and low genetic diversity.

Most of the observed genetic diversity was distributed within populations (82%). This pattern was expected given that the range of values within populations is generally higher in perennial intercrossing species (Hu et al., 2010). We also suggest that self-incompatibility, may contribute for this observation in populations of *E. velutina*, since in other species *E. Falcata* also was reported such incompatibility occurrence (Etcheverry and Alemán, 2005). Mean genetic divergence among *E. velutina* populations ($F_{ST} = 0.184$) was below that often obtained for allogamous and pioneer species (Nybom, 2004). However, this result was influenced by reduced diversity values for the RP, as that population derived from the CA natural population. Cluster analysis confirmed genetic similarity between RP and CA populations.

There was substantial gene flow among populations, although the observed value corresponds to apparent gene flow that occurred in the past (historic gene flow). Due to seed origins, allele identities and frequencies in RP were similar to natural populations and especially CA. This implies likely historical fragmentation of a single population or meta-population (Raposo et al., 2007).

The occurrence of unique alleles in the RP contributed to differentiation among populations (F_{ST}). However, disappearance of these unique alleles from the population is possible if the individuals with these alleles are removed from the area, an event that may be plausible given the fragility of the population. Another possibility for allelic extinction arises from the pioneering character of the species, which tends to naturally disappear from a system when climax species are established. For example, some individuals in the RP had died within 10 years of implementation, and thus did not add seeds to the soil seed bank. A template for future restoration using *E. velutina* needs to consider the self-sustainability of the species and to reverse the species' characteristics rareness.

Populations RP, LA, and CA exhibited a significant number of loci with excess heterozygosity, thus suggesting a genetic bottleneck. The detection of population bottlenecks is important for conservation. Previous studies have shown that inbreeding depression, loss of genetic diversity, and fixation of deleterious alleles can reduce likelihood of population persistence (Frankham, 1995). For the RP, a bottleneck effect may be related to founder effects, given the selection of founder individuals with relatively low genetic variation.

Sebbenn (2002) recommends seed collection from 25 individuals from one or more population fragments for reforestation of areas smaller than 100 hectares. However, this recommendation is often not applicable for a state as small as Sergipe, since there are few restored areas, most of which are small, isolated, and have low species density. However, the genetic study of individuals from restored and natural populations is a tool to assist the selection of genetically distinct trees, even from small fragments, in order to obtain seeds for future reforestation projects.

Ecological restoration makes use of multidisciplinary concepts including biodiversity and habitat heterogeneity, resilience, and sustainability. One ecological restoration goal is to reach ecological succession in a restored location. Although with similar aspects of structure, composition, and function, the restoration differs in scale and time. Succession is limited to a particular ecosystem and can last between 10 and 200 years. Restoration includes larger scales, such as adjacent ecosystems, watersheds, and landscapes, and focuses on periods of between 1 and 20 years, or the duration of the human activity in the project (Walker et al., 2007).

Factors such as seed rain and the soil seed bank can be used to examine the establishment of an implanted species and the ecological succession beginning with this species in a restored area. Species classified as pioneers form a soil seed bank and are found in areas with various climatic and soil conditions. The presence of these species is related to openings or gaps in the forest canopy, since light is essential for seed germination and seedling development (Budowski, 1965).

In this study, the area restored with native species of the Atlantic forest were distributed in a quincuncial system, in which a type of non-pioneer successional group is distributed among four pioneers in a 3 m x 3 m spacing. *E. velutina* trees that were outside of the plots were analyzed, whereas less-dense area inside the plots has continued to develop. Seeds occurring in the soil seed bank were not observed, and were unlikely to set in the soil

given a literature review of groups of early-successional species. Concerning the limitations of the study, it is possible that human interference in all study areas, even in Areas of Permanent Preservation, may alter observation.

Finally, for Atlantic forest restoration projects incorporating *E. velutina* we recommend that monitoring of the soil seed bank occur from 5 to 10 years following implantation, in order to ensure that the species has a seed stock in the soil. Moreover, and based on these results, management measures, such as selective thinning that encourages tree development and seed production, should be adopted.

ACKNOWLEDGMENTS

Research supported by the Post-Graduation Program in Agriculture and Biodiversity (Universidade Federal de Sergipe - UFS), the Instituto Federal de Sergipe (IFS), and Cimento Sergipe - Cimesa S/A Company.

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