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Populations of *Erythrina velutina* Willd. at risk of extinction

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ABSTRACT. The goal of this study was to characterize the structure of two natural populations of the coral tree using RAPD and ISSR markers. The study evaluated all individuals in two different areas in the northeastern region of Brazil: the first was in the riparian area, 10 km x 100 m along the edge of the lower São Francisco River, and the second was in the municipality of Pinhão, in a semiarid region between the municipalities of Neópolis and Santana do São Francisco. We used all the coral trees present in those two areas (37 individuals). The results of the RAPD and ISSR markers were highly congruent, supporting the reliability of the techniques

used. Similarity was estimated using the Jaccard arithmetic complement index. A dendrogram was constructed using the unweighted pair group method with arithmetic mean cluster algorithm, and the robustness of the data was bootstrapped with 5000 replicates. A principal coordinate analysis was performed on the basis of Jaccard coefficients. The total genetic variation observed was 21%, corresponding to the variation between the populations, and 79% of the variation was observed within the populations.

Key words: RAPD; ISSR; Endangered species; Genetic variability; Molecular markers

INTRODUCTION

Numerous species of plants in Brazil and other regions with tropical climatic conditions have been listed as priorities for conservation, due to their high degree of endemism (Kageyama et al., 2003; Bertoni et al., 2010). *Erythrina velutina* Willd. is commonly known as the coral tree or mulungu tree, and it is of a great interest due to its importance as a medicinal plant (Silva et al., 2011). The State of Sergipe has regions with a high incidence of degradation. The study of genetic diversity permits the study of tree populations, as well as determining the history of genetic drift, which promotes the loss of genotypes, before knowing about their ecological and human benefits (Gois et al., 2009).

Especially in the riparian region of the São Francisco River and the semiarid region in the Pinhão municipality, there is suppressed vegetation, since these places are only a few kilometers away from urban areas (Santana et al., 2008; Rabbani et al., 2012). A small population size, isolation, and habitat disturbance are factors frequently associated with the risk of extinction for a species. The mean consequence of habitat fragmentation can promote the reduction of distinct ecological areas, and the decrease of inter- and intrapopulation genetic variability; consequently, endogamy over the years and the reduction of the capacity for such species to adapt to environmental changes have proven detrimental (Yeh et al., 2000; Liu et al., 2006; Domingues et al., 2011). The coral tree in the Sergipe region, as a consequence, showed a small number of individuals and seeds in the riparian region of our study, compromising the establishment of seed banks.

Molecular tools are useful in studies of natural populations aimed at conservation. RAPD (random amplified polymorphic DNA) and ISSR (inter-simple sequence repeat) techniques are valuable in studies aimed at genetic mapping, population genetics, molecular systematics, genotype fingerprinting, and marker-assisted selection in plant and animal breeding (Lacerda et al., 2001; Pinheiro et al., 2012). Among the advantages are low-cost markers that provide an unlimited number of polymorphic fragments, which nearly cover the entire genomic DNA (Bertoni et al., 2010).

The combination of RAPD and ISSR markers provide a quick, reliable, and highly informative system, and have been used for DNA fingerprinting and genetic study (Fernández et al., 2002; Pinheiro et al., 2012). These tools were utilized in genetic and ecological studies of *Cattleya labiata* (Pinheiro et al., 2012), wild rice (*Oryza granulata*) (Qian et al., 2001), endangered rare *Dalbergia oliveri* (Phong et al., 2011), and *Polygonum* species (Soodabeh et al., 2011).

The objective of our study was to determine the genetic diversity and genetic structure of two natural populations of coral tree, under constant anthropic pressure, using RAPD and ISSR markers.

MATERIAL AND METHODS

Area, plant material and DNA extraction

Prospecting was carried out in two wild populations of coral tree located in the north-eastern region of Brazil: the first was located in the region of the lower São Francisco River (ST) and the second in the municipality of Pinhão (PH) (10º33'50'' S and 37°42'47" W). The ST forest fragment is an area of 100 ha (10 km x 100 m along the edge of the river), between the cities of Neópolis (10º18'39''S and 36º34'36''W) and Santana do São Francisco (10º15'55'' S and 36º38'15'' W). We used all of the individuals present in those areas, totaling 37 genotypes, 20 in the first population, and 17 in the second, confirming the degradation in this location due to the rarity of the species. Our DNA samples were isolated from young leaves as described by Nienhuis et al. (1995).

Molecular markers

RAPD - Twenty primers (IDT01, IDT02, IDT03, IDT04, IDT05, IDT06, IDT07, IDT08, IDT09, IDT10, IDT11, IDT12, IDT13, IDT14, IDT15, IDT16, IDT17, IDT18, IDT19, and IDT20) from IDT Integrated DNA Technologies (Germany) were used to screen for polymorphisms (Table 1). PCR amplifications were performed using a PTC-100 Thermocycler (MJ Research Inc., Quebec, Canada), with reactions consisting of an initial denaturation at 94°C for 5 min, followed by 45 cycles of denaturation at 94°C for 1 min, primer annealing at 36°C for 2 min, and extension at 72°C for 1 min. A final extension step was performed at 72°C for 5 min. Fragments were visualized on a 1% agarose gel (1X TBE - 89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.3) in a horizontal electrophoresis system (Sunrise, Gibco BRL). The electrophoresis was carried out at a constant voltage of 100 V for 90 min. The gel was stained with 5 mg/mL ethidium bromide for 15 min.

Eleven ISSR primers (5, 17, 898, 899, 902, AW3, ISSR3, ISSR4, ISSR6, M2, and MANNY) were obtained from IDT and were used to screen for polymorphisms (Table 1). PCR amplification was performed as follows: 94°C for 90 s for the initial denaturation, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at temperature indicated for each primer, and 72°C for 90 s for extension, and one final extension at 72°C for 5 min in a MyCycler ThermalCycler System. Fragments were visualized on a 1.4% 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 20X25) at a constant voltage of 120 V for 4 h. The gel was stained with BlueGreen Loading Dye I (LGC Biotecnologia). ISSR and RAPD amplification products were visualized under ultraviolet light.

For RAPD, the fragments were visualized on a 1% agarose gel in a horizontal electrophoresis system. Electrophoresis was carried out at 100 V for 90 min. The gel was developed with 5 mg/mL ethidium bromide for 15 min. For ISSR, PCR fragments were visualized on a 1.4% agarose gel in a horizontal electrophoresis system at 120 V for 4 h. The gel was stained with BlueGreen Loading Dye I. ISSR and RAPD amplification products were visualized under ultraviolet light.

Data analysis

Bands that were intensely stained, unambiguous, and clear were used for statistical

analysis. Consensus profiles were recorded on the basis of the presence (1) or absence (0) of bands. The results of RAPD and ISSR were highly congruent, supporting the reliability of the techniques of our study. All data obtained were combined and used to analyze the genetic parameters (Awasthi et al., 2004; Bhattacharya et al., 2005).

Similarity was estimated using the Jaccard arithmetic complement index (Jaccard, 1908). A dendrogram was constructed using the unweighted pair group method with arithmetic mean cluster algorithm, and the robustness of the data was bootstrapped with 5000 replicates using the FreeTree software. Principal coordinate analysis (PCoA) based on Jaccard coefficients was performed using the package XLSTAT software.

For our analysis, each genotype class was treated as haploid alleles (Oliveira et al., 2010). We estimated K number of reconstructed panmictic populations (RPP) using values from 1 to 10, and the sampled genotypes were from anonymous plants of unknown origin (usepopinfo and popflag set to 0). We set up runs with a 15,000-iteration burn-in period, and a Monte Carlo Markov chain (MCMC) of 100,000 iterations with five replications. The structure estimates the most likely number of clusters (K), by calculating the log probability of data for each value of K (Santos et al., 2011; Pinheiro et al., 2012). We assessed the best K-value, supported by the data, according to Evanno et al. (2005).

The Shannon index (I) (Brown and Weir, 1983) and genetic diversity (H) were estimated using Genalex v.6.3 (Lynch and Milligan, 1994; Maguire et al., 2002). The same software was used for analysis of molecular variance (AMOVA) (Excoffier et al., 1992; Michalakis and Excoffier, 1996).

To examine the spatial structure in the coral trees, a proportion of genetic differentiation, explained by geographical distance, was estimated using the Mantel test. The genetic distance between each pair of individuals, as well as their geographical distance, was estimated using Genalex v.6.3.

RESULTS

A total of 283 DNA fragments (149 ISSR and 134 RAPD) were obtained using the 31 primers (Table 1).

We found a high correlation between markers (Awasthi et al., 2004; Bhattacharya et al., 2005) allowing for the RAPD and ISSR data to be combined to estimate the genetic parameters ($r = 0.19$; $P < 0.01$).

Genetic similarity

Jaccard coefficients (JC) ranged from 0.28 (ST14 and PH3) to 0.84 (ST9 and ST11) with an average similarity of 0.54 for the first group (ST); while the second group (PH), within each population, accounted for an average JC of 0.67, ranging from 0.45 (ST7 and ST17) to 0.84 (ST9 and ST11) for the lower São Francisco River, and from 0.44 (PH3 and PH19) to 0.76 (PH1 and PH2) for the Pinhão region with an average JC of 0.57.

The genotypes were clustered (Figure 1A) and the distribution of the genotypes in the dendrogram showed groups, as well as the high divergence of genotypes for the populations (PH14 - 0.58 JC, ST5 - 0.64 JC, and PH9 - 0.62 JC). One large group comprised genotypes from ST; another cluster grouped more than half of the genotypes from PH. Two genotypes from ST (ST7 and ST4 - JC = 0.54) were set apart from others, showing a high divergence for ST.

Table 1. Number of fragments (NF) and number of polymorphic fragments (NPF) generated by each primer for the mulungu trees (*Erythrina velutina* Willd.) collected from the northeastern region of Brazil.

Primers	Sequence	NF	NPF
ISSRs		149	146
5	$(CAG)_5$	14	13
17	$(GTG)_4$	19	19
898	$(CA)_6$ AT	14	14
899	$(CA)_6$ AG	15	15
902	$(GT)_6$ AT	15	15
AW3	$(GT)_6$ GG	9	9
ISSR3	$(CTC)_4$ RC	18	18
ISSR4	$(AG)_8$ YC	10	10
ISSR6	$(CA)_8$ GC	8	8
M2	$GGGC(GA)_8$	16	16
MANNY	$(CAC)_4$ AC	11	9
RAPD		134	115
IDT 1	CAG GCC CTT C	10	7
IDT 2	TGC CGA GCT G	6	6
IDT 3	GTT TCG CTC C	7	6
IDT 4	TGA TCC CTG G	6	4
IDT 5	TTC GAG CCA G	8	8
IDT 6	GTG AGG CGT C	1	1
IDT 7	ACC GCG AAG G	5	5
IDT 8	GGA CCC AAC C	6	1
IDT 9	CCC AAG GTC C	11	7
IDT 10	GGT GCG GGA A	5	2
IDT 11	ACG GAT CCT G	10	8
IDT 12	GAG GAT CCC T	7	6
IDT 13	CTA CGG AGG A	4	4
IDT 14	CTA CGG AGG A	8	8
IDT 15	GGC ACT GAG G	16	16
IDT 16	GGT CGG AGA A	9	9
IDT 17	ACC TGG ACA C	6	6
IDT 18	GGA GGA GAG G	9	8
IDT 19	CCC GGC ATA A	6	6
IDT 20	AAA GTT GGG A	4	4
Total		283	261

Genetic structure and diversity

Bayesian analysis was performed to determine the genetic structure between the genotypes. The ln[Pr(X/K)] values increased up to K = 2, which corresponded to the differentiation between the two localities (Figure 1B and Table 1). A smaller peak at K = 3 was found when the posterior ΔK statistics of Evanno et al. (2005) were applied (Figure 1C). Similar results were also observed by Barnaud et al. (2007), Pereira-Lorenzo et al. (2010, 2011), and Santos et al. (2011).

For the first one, tree RPP (RPP1) included 17 genotypes of which 14 were from the lower São Francisco River presenting a probability of membership of (q*I*) > 80%; three genotypes were q*I* < 80% (ST14, ST15 and ST17). The second RPP (RPP2) included nine genotypes from Pinhão, eight for q*I* > 80%, and only PH11 showed q*I* < 80%. The third RPP (RPP3) clustered 11 genotypes, all with q*I* > 80 (Figure 1).

The ordering of multivariate data (PCoA; Figure 2) for the first two principal coordinates, explained 42.08% of the total variability.

The Shannon diversity index was 0.38 for the populations, according to origin, with an increase using the three reconstructed populations (0.40). A high value was obtained for RPP2 (K = 3, 0.44). The genetic diversity was 0.25 for the populations, according to origin, and 0.27 for the reconstructed populations. The original populations of coral tree showed the

maximum genetic diversity (I and H), and were associated with the Pinhão municipality and RPP2. The percentage of polymorphic loci among the populations was 76.37%, according to origin, and 76.90% according to RPP (Table 1).

AMOVA was performed with the 37 genotypes of coral tree, according to their original location, and showed a genetic differentiation of 27% ($P < 0.01$); when analyzed, according to the three RPPs, the genetic differentiation only accounted for 18% ($P < 0.01$) of the variation (Table 2).

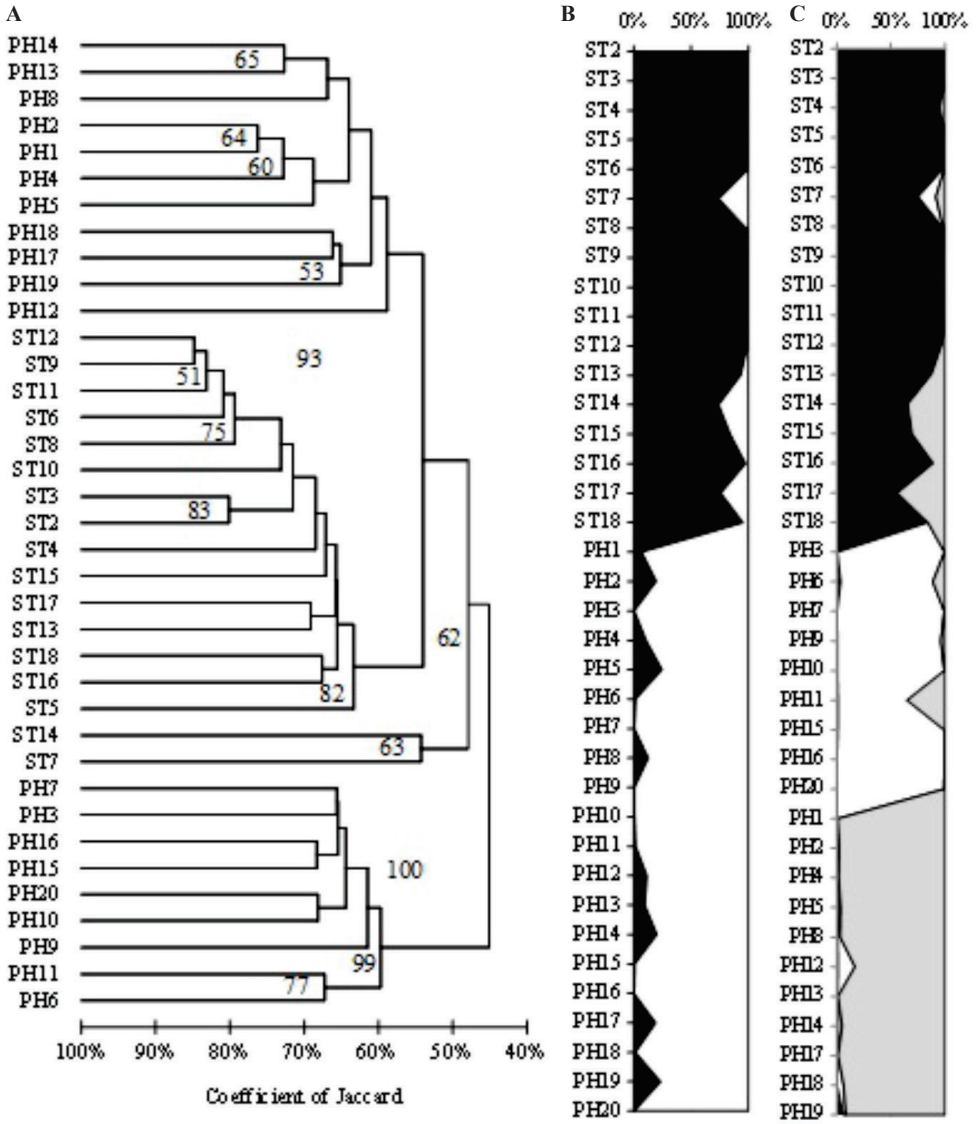

Figure 1. UPGMA dendrogram of genetic similarity generated from Jaccard coefficients (A) and reconstructed populations [RPP1 (gray), RPP2 (white) and RPP3 (black)] as defined by Structure (Pritchard et al. 2000) to two RPPs (B) and three RPPs (C) for 37 mulungu trees (*Erythrina velutina* Willd.) with the genotypes using RAPD and ISSR markers.

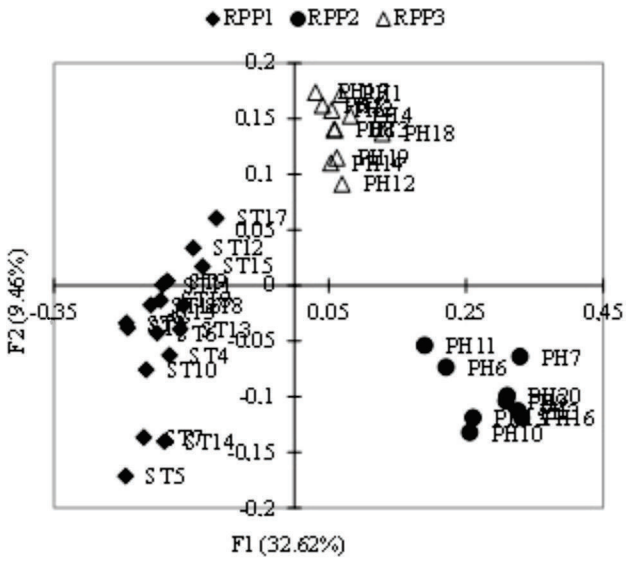

Figure 2. Principal coordinate analysis of 37 mulungu trees (*Erythrina velutina* Willd.) genotypes by origin and by a reconstructed population (RPP) as defined by the Structure software (RPP1 to 3) using RAPD and ISSR markers.

Table 2. Shannon index (I), genetic diversity (H), and the percentage of polymorphic loci (P%) in mulungu trees (*Erythrina velutina* Willd.) genotypes, by origin, and by a reconstructed population (RPP), as defined by the Structure (RPP1 to 3) software using RAPD and ISSR markers.

Origin	I (±SD)	H (±SD)	P%
Two RPPs			
LSFR (RPP1)	0.34 (±0.01)	0.23 (±0.01)	83.22
Pinhão (RPP2)	0.41 (±0.01)	0.27 (±0.01)	69.52
Total	0.38 (±0.01)	0.25 (±0.01)	76.37
Three RPPs			
RPP1	0.41 (±0.01)	0.27 (±0.01)	81.89
RPP2	0.44 (±0.01)	0.30 (±0.01)	79.53
RPP3	0.35 (±0.01)	0.24 (±0.01)	69.29
Total	0.40 (±0.01)	0.27 (±0.01)	76.90

SD - standard deviation; LSFR = lower São Francisco River.

Spatial genetic correlation

A moderate but significant correlation between the genetic and geographical distances was detected by the Mantel test applied to the RPPs ($r = 0.59$; $P < 0.01$). No spatial correlation was observed for the different localities.

DISCUSSION

This study was the first to use ISSR with RAPD markers in coral tree individuals. The individual plants were not genetically identical, with a resolution sufficient enough to distinguish all genotypes. Only genotypes from the lower São Francisco River were grouped by origin. These results were confirmed by bootstrap and Bayesian analyses, demonstrating the genetic structure of the species. PCoA confirmed the difference between the genotypes, even

for the estimated low variation (42.08%).

The values obtained by the Shannon index (Table 2), for both localities, allowed us to infer the genetic diversity within each population, especially for those from the Pinhão region with a lower genetic diversity (0.41). The level of polymorphism estimated in our study was high, particularity for the three RPPs (76.90%), especially in comparison to other native Brazilian species such as *Genipa americana* L. and *Aspidosperma polyneuron* Müll. Arg., which both have shown values of 50% polymorphism (Maltez, 1997; Sebbenn et al., 1998). The geographical distance was not explained by the divergence of the individuals for both populations, and probably genetic drift was the cause of the present distribution.

The genotypes are in disturbed areas, under pressure from such factors as the expansion of land use, even for those areas established as legally protected land. The removal of extensive forested areas causes a decrease in population size, which may result in an immediate loss of alleles (Young et al., 1996). The few species that are in rare occurrence in those areas could suggest a low genetic diversity. However, the high rate of polymorphism can probably be explained by the fact that the species is preferentially allogamous (Yanaka et al., 2005).

The genetic variability between the populations of *E. velutina* was low. This variation within the populations was higher in AMOVA, and increased further, when we considered the three RPPs. The individual abilities to exchange genes, associated with gene flow between the populations, reduce the differences between the populations by genetic drift and selection (Kageyama et al., 2003). The distribution of genetic variation can be influenced by various life-history traits, such as breeding system (Nybom and Bartish, 2000). There are reports of the pollination of *Erythrina* by birds and hummingbirds (Neill, 1988), honeybees, and bumblebees (Carvalho, 2008). We believe that wildlife is also suffering from human activities, because the genotype groups of coral trees are completely isolated in their geographical distribution, which suggests that fauna contributes somewhat little to seed dispersal and ecology.

If fragmented populations remain small, and isolated for many generations, there may be an increase in endogamy and genetic drift and a divergence between the populations. This is due to the occurrence of intersections between the few individuals remaining, and the limitation of gene flow via pollen and seeds and between fragments. The main consequence of these processes is the reduction in genetic diversity (Martins et al., 2008). In the latter case, the loss of genetic diversity may result in a decrease in suitability for the remaining populations, and consequently followed by species extinction (England et al., 2002).

Knowledge of the genetic variability of the coral tree may contribute to the development of a proper management system focused on increasing and supplementing the native forests. We recommend the establishment of strategic planning for seedling growing and for seed bank establishment, to provide an exchange of alleles between individuals in the same area. This combination permits an increase in population size and composition of different genotypes, aimed at the recovery of degraded environments, and thereby promoting a better future sustainability of the populations and the reappearance of fauna. Furthermore, we propose the development of further studies within these populations, to clarify the events involving the degradation process and genetic structure.

This study is the first report of such a low occurrence of *E. velutina* in northeastern Brazil, and contributes to the characterization of native genotypes from degraded areas promoted by human disturbances. The genetic variation and the genetic relationships between the coral trees were efficiently determined by dominant markers. The identification of coral tree distribution contributes to our knowledge of genetic relationships and the strategies required

for protecting natural populations and preserving genetic variability. For the coral tree, the appropriate strategy would be to protect the few remaining individuals, and to establish new individuals, from seeds, from other regions, to increase genetic diversity.

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