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Assessment of Genetic Diversity in Five Nicaraguan Populations of *Cedrela odorata* L. (Meliaceae) using RAPD Markers

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The goal of this study was to assess the genetic diversity of Nicaraguan populations of *Cedrela odorata* using Random Amplified Polymorphic DNA (RAPD) markers. Thus, genomic DNA was isolated from leaf samples collected from ninety-two trees belonging to five Nicaraguan natural populations of *C. odorata*. The mean number of alleles per locus, effective number of alleles per locus, percentage of polymorphic loci, genetic diversity (H_e) of Nei and diversity index (H_o) of Shannon were estimated for each population assuming that the populations were in Hardy-Weinberg equilibrium. Total genetic diversity was partitioned in intrapopulational and interpopulational diversity using Nei's genetic differentiation (G_{ST}) and through an Analysis of Molecular Variance (AMOVA). The Φ_{ST} matrix was used to construct a dendrogram by the neighbor-joining method. According to values of both H_e and H_o, Esquipulas (Deparment of Matagalpa) presented the lowest diversity level; while La Trinidad (Department of Estelí) showed the highest diversity level. Genetic differentiation was calculated obtaining a G_{ST} value of 13.36%. AMOVA also showed a similar differentiation value Φ_{ST} =13.81%). Neighbour-joining dendrogram clustered the five populations in two groups, where the group formed by La Trinidad and El

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Refugio (Department of Granada) presented the biggest differentiation. Correlation between genetic and geographical distances was not found.

Keywords: *Cedrela odorata*, Conservation, Genetic Diversity, Nicaragua, RAPD

Abstract in Spanish:

Evaluación de la diversidad genética de cinco poblaciones nicaragüenses de *Cedrela odorata* L. (Meliaceae) usando marcadores RAPDs

El propósito de este estudio fue evaluar la diversidad genética de poblaciones nicaragüenses de *Cedrela odorata* usando marcadores RAPD (ADN polimórfico amplificado al azar). Así, se aisló ADN genómico de hojas recolectadas de noventa y dos árboles pertenecientes a cinco poblaciones naturales de *C. odorata*. Se estimó para cada población el número de alelos por locus, número efectivo de alelos por locus, porcentaje de loci polimórficos, diversidad genética de Nei (H_e) e índice de diversidad de Shannon (H_o) asumiendo equilibrio de Hardy-Weinberg. La diversidad genética total fue particionada en diversidad intrapoblacional e interpoblacional usando la diferenciación genética de Nei (G_{ST}) y el Análisis de Varianza Molecular (AMOVA). La matriz Φ_{ST} se usó para construir un dendrograma con el método de *neighbor-joining*. De acuerdo a los valores de H_e y H_o, la población de Esquipulas (Departamento de Matagalpa) presentó el nivel de diversidad genética más bajo; mientras que La Trinidad (Departamento de Estelí) mostró el nivel de diversidad más alto. La diferenciación genética fue calculada, obteniendo un G_{ST}=13.36%. El análisis de varianza molecular (AMOVA) también mostró un valor de diferenciación similar (Φ_{ST}=13.81%). El dendrograma *neighbour-joining* agrupó a las cinco poblaciones en dos grupos, donde el grupo formado por La Trinidad y El Refugio (departamento de Granada) presentó la diferenciación más grande. No se encontró correlación entre distancias genéticas y geográficas.

Palabras clave: *Cedrela odorata*, conservación, diversidad genética, Nicaragua, RAPD

1. Introduction

Cedrela odorata L. (Spanish cedar or *cedro real*) is a Neotropical tree belonging to the Meliaceae family found in the subtropical and tropical humid or seasonal dry forests (Holdridge, Poveda Alvarez & Jiménez Madrigal, 1997; Cordero & Boshier, 2003). Its natural range goes from latitude 26° N in the Pacific Coast of Mexico to latitude 28° S in Argentina, being also present in the Caribbean islands (Niembro Rocas, 2002), and it has been introduced in Ethiopia, Fiji Islands, Ghana, Nigeria, Sierra Leone, Southern Florida, and Tanzania (Webb, Wood, & Smith, 1980; Gezahgne, Coetzee, Wingfield, Wingfield & Roux, 2004). It is an economically important timber tree producing high quality wood whose uses include construction, furniture and boat building (Niembro Rocas, 2002; Cordero & Boshier, 2003). Its wood is resistant to

attacks by fungi and insects, and its fragrance lasts for many years (Niembro Rocas, 2002). As a result, its natural populations have been severely reduced to the point that the species is now classified as "vulnerable" by the International Union for Conservation of Nature (Americas Regional Workshop, 1998), appearing in the CITES (Convention on International Trade of Endangered Species of Wild Fauna and Flora) Appendix III for the countries of Brazil, Guatemala, Colombia, Bolivia and Peru.

C. odorata is commonly found in Nicaragua with latitudes between 0 to 1200 m. It flowers from April to September, and it fruits from June to March (Pennington & Styles, 2001). This species, as in the rest of its natural range, has experienced a big population reduction in recent years. The size reduction of any population has a direct consequence in the reduction of its genetic variation (Frankham, Ballou & Briscoe, 2002). For this reason, it is necessary to detect and evaluate the genetic diversity of the populations in order to maintain their variation level and avoid extinction. There are no studies of population structure in Nicaraguan provenances of *C. odorata*. Thus, the conservation and plantation management of the species is done without this information. The only genetic data that exist are from phylogenetic studies carried out in Mesoamerica (Cavers, Navarro & Lowe, 2003b) and its distribution range (Cavers *et al.*, 2013), which can give us slight insights about how to manage the species in Nicaragua. Some research about the genetic structure of *C. odorata* has been carried out in Costa Rica (Gillies *et al.*, 1997; Cavers, Navarro & Lowe, 2003a) which can be taken as reference, since some Costa Rican populations could share the same kind of ecosystems and evolutionary history than the Nicaraguan populations.

The use of molecular techniques, especially the DNA based markers, has permitted access to any information in the genome. Among the molecular techniques that already exist, the Random Amplified Polymorphic DNA (RAPD) (Williams, Kubelik, Livak, Rafalski & Tingey, 1990) is a very common technique used due to its simplicity and low cost. This technique is based in the random amplification of different DNA fragments with an only 10-nucleotides length primer that identify polymorphisms used as markers (Williams *et al.*, 1990). RAPD has been successfully used by many researchers to assess the genetic diversity of several forestry species, e.g. *C. odorata* (Gillies *et al.*, 1997), *Swietenia macrophylla* (Gillies *et al.*, 1999), *Prunus mahaleb* (Jordano & Godoy, 2000), *Pinus oocarpa* (Diaz, Muniz, & Ferrer, 2001), and *Pinus tecunumanii* (Cerda Granados, 2007).

In this study, RAPD markers were used (i) to assess the genetic differentiation between populations and (ii) to estimate the levels of genetic diversity within populations of Nicaraguan *C. odorata* populations. This information will be useful in planning conservation and management programs of *C. odorata* in Nicaragua.

2. Materials and Methods

2.1 Plant Material

Leaf samples were collected from 92 trees distributed in five Nicaraguan natural populations of C. *odorata* (Figure 1): (1) Volcán Casita (12° 40' 27.0'' N, 86° 57' 50.6'' W) in Department of Chinandega, (2) Esquipulas (12° 42' 19.5'' N, 85° 47' 15.9'' W) in Department of Matagalpa, (3) Masatepe (11° 54' 12.1'' N, 86° 09' 20.7''W) in Department of Masaya, (4) El Refugio (11° 49' 45.0'' N, 86° 00' 34.0'' W) in Department of Granada and (5) La Trinidad (12° 54' 36.4'' N, 86° 14' 11.4'' W) in Department of Estelí. The minimum distance between trees sampled was 100 m. The number of trees from each population varied from 14 to 22. Field-collected material was dehydrated in silica gel during 3—5 days, grinded in lab conditions, and stored at –20° C prior to DNA isolation.

Figure 1. Nicaraguan populations of C. *odorata* (■) sampled for this study

2.2 DNA Isolation

The genomic DNA isolation was carried out as described by Möller, Bahnweg, Sandermann and Geiger (1992) with the following modifications: 2 µl of β-mercaptoethanol 14.4 M was added to the extraction buffer, while proteinase K was not included during extraction. The first incubation was done at 65° C for 30 min. We did a second extraction with chloroform:isomyl alcohol. The RNse treatment was carried out at the end of the procedure.

2.3 RAPD amplification

RAPD reactions were carried out in 25 µl volumes containing 25 ng genomic DNA, 1X Buffer A (10 mM Tris-HCl (pH 9.0), 50 mM KCl, and 1.5 mM $MgCl_2$), 0.2 µM of primer, 200 µM dNTPs and 1U of Taq polymerase (Fisher Scientific) (Williams et al. (1990) with slight modifications). Amplifications were performed in a termocycler

(Amplitron® II Thermolyne) with the following program: 3 min at 92° C (initial denaturation); 45 cycles of 1 min at 92° C (denaturation), 2 min at 37° C (annealing), and 1 min at 72° C (extension); all followed by a final 8 min extension at 72° C. Nine primers (OPF-01, OPG-10, OPN-09, OPR-07, OPS-18, OPT-08, OPU-16, UBC-228, and UBC-411) were selected from initial tests. Amplification products were separated by electrophoresis in 2.0% agarose gels with 1X TAE, stained with ethidium bromide, and then photographed under UV light.

2.3 Data Analysis

RAPD bands were scored as either present or absent in each DNA sample. Analysis was restricted to polymorphic bands, *i.e.* reliable scored bands with a frequency of less than 95% for the most common phenotype. The data file was investigated for nonrandom associations between individual pairs for bands using the correlation test of the SIMIT program (NTSYSpc 2.20 software) (Rohlf, 2005). The mean number of alleles per locus (N), effective number of alleles per locus (N_e) (Kimura & Crow, 1964), percentage of polymorphism over total bands (P), the genetic diversity (H_e) of Nei (1973) and the diversity index (H_o) of Shannon (Lewontin, 1972) were estimated for each population assuming Hardy-Weinberg equilibrium. An analysis of simple correlation was carried out to detect possible relationships between diversity indexes. Total genetic diversity was partitioned in intrapopulational and interpopulational diversity using the Nei (1973)'s genetic diversity according to (Nei, 1987). Gene flow (Nm) was calculated using the Nei (1987)'s coefficient of gene differentiation (G_{ST}) according to McDermott and McDonald (1993). These analyses were done using the POPGENE ver. 1.32 program (Yeh & Yang, 2000).

An Analysis Molecular of Variance (AMOVA) was carried out to obtain variance components within populations and between populations. Variance components were tested statistically by nonparametric permutational procedures using 1000 permutations. Genetic differentiation (Φ_{ST}) between pairs of the *C. odorata* populations, and levels of significance were also calculated. All analyses were performed using Arlequin 3.01 software (Excoffier, Laval & Schneider, 2005). The Mantel test (Mantel, 1967) was used to estimate the possible association between the geographical distance matrix and the Φ_{ST} pairwise matrix using the MXCOMP program (NTSYSpc 2.20). Significance was determined using 1000 permutations.

The Φ_{ST} matrix was used to construct a dendrogram by the neighbor-joining method (Saitou & Nei, 1987) using the TREE program (NTSYSpc 2.20). A cophenetic value matrix was produced from the tree matrix using the COPH program (NTSYSpc 2.20). This matrix was then employed to check the goodness of fit of the cluster analysis by comparing it to the Φ_{ST} matrix (Sneath & Sokal, 1973) using a Mantel test (Mantel, 1967) with 1000 permutations.

3. Results

3.1 RAPD Profile

The number of bands per primer varied from 10 for primers OPT-08 and OPU-16 to 15 for primer UBC-228. The mean total bands per primer were 12. Thus, a total of 108 bands with a molecular weight from 270 bp to 3700 bp were generated. Seventy eight bands were polymorphic (mean of 8.76 polymorphic bands per primer, ranging from 4 for OPN-09 to 14 for UBC-228).

3.2 Genetic Diversity

Genetic parameters calculated per population are presented in Table 1. N varied from 1.821 (Volcán Casita) to 1.910 (El Refugio) with an overall mean of 2.000. N_e varied from 1.489 (Masatepe) to 1.559 (La Trinidad) with an overall mean of 1.579. P varied from 56.5% (La Trinidad) to 68.5% (Esquipulas) with an overall mean of 63.3%. H_e varied from 0.289 to 0.323 with an overall mean of 0.347. H_o varied from 0.436 to 0.479 with an overall mean of 0.524. A strong and significant correlation was found between H_e and H_o (Pearson, r = 0.999, P < 0.0001). Both diversity values showed that the least diverse population was Esquipulas, while the most diverse population was La Trinidad.

Table 1. Genetic variation parameters, genetic diversity of Nei (H_e) and Shannon's information index (H_o) for five Nicaraguan populations of *C. odorata* using RAPD markers.

Population	N	N_e	P	H_e	H_o
Volcán Casita	1.821	1.499	59.3	0.294	0.440
Esquipulas	1.872	1.490	68.5	0.289	0.436
Masatepe	1.910	1.489	67.4	0.292	0.442
El Refugio	1.910	1.541	64.8	0.316	0.474
La Trinidad	1.885	1.559	56.5	0.323	0.479
All populations	2.000	1.579	63.3	0.347	0.524

Note: N, number of alleles per locus; N_e, efective number of alleles per locus (Kimura & Crow, 1964); P, percentage of polymorphic loci over total bands; H_e, Nei (1973)'s gene diversity; H_o, Shannon's information index (Lewontin, 1972) over loci.

3.3 Partitioning of Variation per Population

Total genetic diversity (H_T) was 0.350, and the genetic diversity within populations (H_S) was 0.303. Thus, Nei (1993)'s genetic diversity index assigned a 13.3% (G_{ST} = 0.133) of the total variation to the intrapopulational component, while 86.7% of the total variation was caused by differences between individuals within populations. Nm was 3.251 migrants per generation.

3.4 AMOVA Analysis

In agreement with the G_{ST} value, AMOVA (Table 2) found that most of the total variation was within populations (86.19%) rather that between populations (Φ_{ST} =13.81%). This value was statistically significant (P < 0.001). All pairwise Φ_{ST} values derived from AMOVA were statistically significant (P < 0.001). The lowest genetic differentiation was found between Esquipulas and Masatepe (Φ_{ST} =0.062), while the highest differentiation was present between Volcán Casita and La Trinidad (Φ_{ST} =0.241). There was no correlation between genetic and geographical distances. This shows that Nicaraguan populations of *C. odorata* do not follow the isolation by distance model.

Table 2. AMOVA analysis in five Nicaraguan populations of C. *odorata* using RAPD markers.

Source of variation	d.f.	Sum of squares	Components of variance	Percentage of variation	Φ statistic
Between populations	4	209.855	2.13930	13.81*	$\Phi_{ST} = 0.1381$
Within populations	87	1161.591	13.35162	86.19*	
Total	91	1371.446	15.49092		

* P<0.001
Significance test after 1000 permutations.

3.5 Phylogenetic Relationships

The dendrogram constructed with the Φ_{ST} pairwise matrix (Figure 2) shows the formation of two clusters. One group formed by the populations of Volcán Casita, Esquipulas and Masatepe and the other group by the remaining populations, El Refugio and La Trinidad. The dendrogram obtained was consistent with the Φ_{ST} pairwise matrix as demonstrated with correlation coefficient between the Φ_{ST} pairwise matrix and the cophenetic matrix from the dendrogram (Mantel test, r = 0.990, P = 0.018).

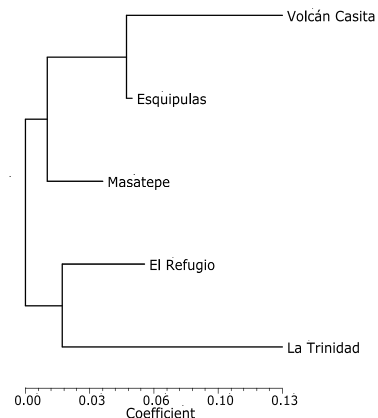

Figure 2. Neighbour-joining analysis showing the genetic relationships of five populations of C. *odorata* based in Φ_{ST} pairwise matrix generated by RAPD markers. The coefficient of cophenetic correlation was r=0.99

4. Discussion

The number of polymorphic bands (78) obtained in the present study is within the range that Nybom and Bartish (2000) recommended to have reliable RAPD data. Overall levels of genetic diversity (P = 63.3%, H_T = 0.350) are higher than the mean of plant species (H_{POP} = 0.214) reported by Nybom and Bartish (2000) and are comparable to other dicotyledonous tree species, *e.g.*, *Swietenia macrophylla* (Gillies *et al.*, 1999). They are also similar to values reported for other species of the *Cedrela* genus, such as *Cedrela balansae* in Northwestern Argentina using other molecular markers (expected heterozygosity = 0.643 for SSR, and P = 60.47% and expected heterozygosity = 0.222 for AFLP) (Soldati, Fornes, Van Zonneveld, Thomas & Zelener, 2013) and for *C. odorata* in Mesoamerica (P = 93.8%, H_T = 0.34 (Gilles *et al.*, 1997); P = 84.8%, H_T = 0.27 (Cavers *et al.*, 2003a)), and Peru (P = 98.8%, H_T = 0.22 (de la Torre, López, Yglesias & Cornelius, 2008)). The same general trend was found with the Shannon index, such as the findings in two Mexican red oaks, *Quercus affinis* (P = 54.98%, Shannon Index = 0.242) and *Quercus laurina* (P = 55.19%, Shannon Index = 0.249) using RAPD markers (González-Rodríguez, Arias & Oyama, 2005).

G_{ST} (0.133) and Φ_{ST} (0.138) values presented in the current study were lower than the mean of plant species (G_{ST} = 0.29 and Φ_{ST} = 0.35) found by Nybom and Bartish (2000), and the calculated gene flow (Nm = 1.224) is higher than the mean of plants. Most of the variation was found within populations as has been reported in several studies in forestry species with outcrossing pollination. The AMOVA analysis performed in Costa Rican *C. odorata* using RAPD (Gilles *et al.*, 1997) also showed that most of the variation was within populations (65.1%); although this value was much lower than the value found in our study (86.19%). On the other hand, G_{ST} value gave a different outcome. Most of the variation was between populations (55%). The authors explained this was due to the presence of two ecotypes (dry type and mesic type) in Costa Rica. The existence of these two ecotypes in Costa Rica has been demonstrated in later studies using cDNA analysis (Cavers *et al.*, 2003a) and morphological traits in an experiment with a common garden (Navarro, Ward & Hernández, 2002). In fact, two haplotypes (A and B) were found in the Costa Rican populations, each of them corresponding to an ecotype (Cavers *et al.*, 2003a). Haplotype A corresponds to dry ecotype; while haplotype B corresponds to the mesic ecotype. This may also be applicable to the Nicaraguan populations of *C. odorata*, since the populations from both Western Nicaragua and Northwestern Costa Rica belong to the same haplotype and ecotype (Cavers *et al.*, 2003b). An AMOVA analysis using AFLP showed that most of the variation of Costa Rican *C. odorata* was between groups (83.5%); while a 5.4% was between populations within groups and 11.2% was within populations (Cavers *et al.*, 2003a). However, they did a two-level AMOVA separating the groups according to the haplotypes identified through cDNA analysis (haplotypes A and B). The haplotype A, to which the Nicaraguan populations belong (haplotype 3 in Cavers et al. (2003b)), had a similar partition of variation to the one found in our study. They reported values of 79.8% within populations and 20.2% between populations; while we found values of 86.19% within populations and

13.81% between populations. A study in Peruvian populations of *C. odorata* using AFLP (de la Torre *et al.*, 2008) also showed this pattern (72.7% within populations, 16.5% between groups, and 10.8% between populations within groups) reporting a value of F_{ST} = 0.20. In recent findings, it has been detected that *C. odorata* has 3 or 4 cryptic species in Mesoamerica (Cavers et al., 2013). So probably, the haplotypes discovered in prior studies (Cavers *et al.*, 2003a; Cavers *et al.*, 2003b) should be elevated to species.

5. Implications for Conservation and Future Work

As mentioned before, knowledge of the level and distribution of the genetic variation of a species is necessary to assure the effectiveness and success of any conservation program (Ge, Hong, Wang, Liu & Zhang, 1998), inasmuch as the evolutionary potential of species and the diversity of their genepools can be maintained for current and future use (Butcher, Glaubitz & Moran 1999). For that reason, future decisions should be based on quantitative genetic variation as well as patterns of variation revealed from molecular markers. To craft an effective conservation strategy, it is necessary to understand the genetics of the developmental, adaptive and physiological strategies of the populations (Namkoong, 1992), as well as the reproductive biology of the species. Since *C. odorata* is an obligatory outcrossing species, its fecundity may be influenced by the level of fragmentation and anthropogenic perturbation of their habitats and availability of pollinators flying extensive areas (Hernández Sánchez, 2008). The same author also states that alterations in temperature due to climate change may influence the fecundity of the species by disturbing the pollinator behavior or augmenting fire occurrences.

In the case of the present study, only Western Nicaragua (dry ecotype) populations were taken into account, having most of the variation within populations suggest that sampling from a few populations, for either conservation or breeding activities, may capture a large proportion of the variation of the species in Western Nicaragua. Nevertheless, sampling from a wide range of populations is still advisable.

The presence of the ecotypes reported in Costa Rica is probably applicable for Nicaragua where there are humid ecosystems in the Caribbean Coast. Thus, it is advisable to keep separate both ecotypes for conservation and breeding purpose, especially now that they could be cryptic species (Cavers *et al.*, 2013). A study with more informative markers and including populations of the Caribbean Coast will be necessary to have a better knowledge of the genetic structure of the species in Nicaragua.

Due to the existence of a limited number of individuals of *C. odorata*, it is necessary to protect the entire existing subpopulations and individuals just as Navarro Pereira (2002) recommended, and taking into consideration minimum distance between trees to ensure gene flow (Hernández Sánchez, 2008). Sáenz-Romero, Snively, and Lindig-Cisneros (2003) proposed “the establishment of a network of *in situ* forest genetic resources conservation units to protect the genetic variation of *P. tecunumanii* against immediate-term treats like fragmentation and deforestation”. Since *C. odorata* has an even wider distribution range and also a great pressure as *P. tecunumanii*, this strategy might be a good approach. In fact, Cavers, Navarro,

and Lowe (2004) proposed to work in three regions based in the dominant cpDNA lineages that they found in their study with Mesoamerican populations (Cavers *et al.*, 2003b) and also taking into consideration the country boundaries. This regions would be (1) Mexico, Belize and Guatemala; (2) Honduras and Nicaragua; and (3) Costa Rica and Panama. This distribution is based on the fact that the populations sampled from Nicaragua and Honduras are from dry ecotypes. However, humid ecotype populations from Honduras and Nicaragua have to be sampled to have a better delimitation of the conservation units.

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