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GENETIC VARIABILITY OF *SOTOL (DASYLIRION CEDROSANUM)* POPULATIONS IN THE MEXICAN COAHUILA SOUTHERN AREA

ABSTRACT

In the Chihuahuan desert area, in Mexico, exist an endemic plant denominated Sotol (*Dasylirion* spp); which is used by rural producers to manufacture a liqueur named Sotol. This beverage has origin denomination but it industrial application has lack scientific reports, as well as the plant genetic information. This is the first study about ecological distribution of sotol plants and, may be, a first step to continue the scientific investigation of a Mexican endemic plant, with many potential industrial applications. In this paper, we used AFLP molecular markers to calculate population genetics parameters as genetic diversity, a conglomerate analyses, poblational structure based in Wright´s statistics and analysis of molecular variance. Differences in soil composition and weather conditions, of the sampling region, may affect sotol plants growth and their morphologic characteristics; as consequence the organisms generate ample genetic variability, which ensures the species permanence. Genetic diversity values were similar to the average for cross-pollinated plants, while the heterozygosis detection was deficient; Wright statistics suggest plants genetics interchange in the subpopulation; AMOVA found significant differences within and between collections. These results help us to maintenance the genetic diversity, in order to prevent potential extinction and guarantee sustainable development; the preservation of the endemic plants is necessary to the ecosystems conservation and after Sotol origin denomination, by the Mexican government, the importance of *D. cedrosanum* plants was increased.

Key words: AFLP, Genetic Diversity, Sotol, Dasylirion cedrosanum.

INTRODUCTION

Dasylirion spp. is a genus with 16 species of fibrous, prickly-leaved rosette plants which occur in arid mountainous regions of Mexico and the

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Southwestern United States (Bogler 1998). In Mexico Dasylirion duranguense, D. cedrosanum and D. wheeleri are used to produce a fermented -alcoholic beverage, with origin denomination, known as Sotol; but the most abundant species in Coahuila state is D. cedrosanum and the common name of these plants is sotol, like the alcoholic beverage.

D. cedrosanum are short stemmed green plants, height from 1.0 to 1.5 m, leaves has 20 mm wide and yellow spines; inflorescences had height average of 5 m; fruits are elliptic, narrow (4-5 mm and 7-9 mm). One application is in human and animal feed, but the principal product obtained since ancient times is the Sotol beverage; the steams of adult plants (pineapple or head) (30-50 cm diameter and 30-45 kg weight) are used to obtain fermentable sugars (De la Garza *et al.* 2008). Plants culture is without legislation and the farmers harvest the plants in unbalance way. This plant is a very vulnerable resource, because the only one process to species conservation is selecting the bigger sotol plants to be cut. Although is estimated that a plant need at least six years to reach the recommended cutting size, in addition 1. - sexual reproduction is the only way of this species preservation, 2.- Sotol requires cross pollination because it is a dioecious plant, 3.- Pollination is mainly by wind, 4.- Sotol seeds present dormancy-latency and presence of insects damaging mature seeds. All of these factors reduce availability of viable sotol seeds. Also, there is a generalized myth that more and better liqueur is obtained from female plants, by this reason females plants are preferably cut. This practice made the female plant frequency to go down and there is concern that this species may disappear if no conservation practices are taken place (Cruz-Requena *et al.* 2007).

In recent years, some Mexican institutions have been developed some alternatives to protect this natural resource. These alternatives include: Inventory of sotol populations mainly in the Mexican State of Coahuila, where it is reported that the Coahuila southern region is that has the biggest population density of this species (Zarate 2003). In addition, sotol *in vitro* propagation (Palma 2000), seed scarification (Arce-Gonzalez *et al.* 2003) and physicochemical studies of sotol plants (De la Garza-Toledo *et al.* 2008b; De la Garza-Toledo *et al.* 2010) and liqueur produced from females and male plants had been performed (Cruz-Requena 2007). However, still there is a lack of knowledge of this species in order to prevent the extinction of the sotol plants; is necessary to establish a germplasm conservation program for the reason that sotol has a greater future, because it may have national and international popularity, as tequila, given by its large history of good liqueur. First of all, is indispensable, make a determination of some population genetic parameters from *D. cedrosanum* plants.

Amplified Fragment Length Polymorphisms (A*FLP's)* are one of the most used molecular markers for population genetics studies, these markers helps on identification of DNA polymorphisms without previous knowledge of the organism´s DNA sequence and the obtained results are highly reproducible. AFLP's have been used for analysis of genetic diversity, local marker saturation, and construction of genetic maps as well as quantification of specific loci and genetic mapping in

fungi, plants, insects and animals (Brugmans et al. 2003). This technique is based on amplification by PCR (Polymerase Chain Reaction) of polymorphic fragments, obtained by restriction enzymes, from whole DNA genomes *(Vos et al. 1995)*. AFLP's have been used to study the population genetics of agave (Demey *et al.* 2004), apricot (Hagen *et al.* 2002), black pepper (Joy *et al.* 2007), Mexican oregano (Meléndez-Rentería *et al.* 2010) and others crops (Ibiza *et al.* 2011), giving satisfactory results in reliability and reproducibility. The aim of this work was to generate an approach to the genetic variability among and within eight *Dasylirion cedrosanum* populations at the Mexican Coahuila Southern Area, determined by AFLP markers.

MATERIALS AND METHODS

Vegetal material

Table 1

Regions and locations from Coahuila state, where sotol plants were collected

Region 1	Region 2	Region 3	Region 4
Carneros	Palmas altas	Presa Palo Blanco	Yucatán
Bañuelos	Palmas altas (2)	Puente Ing. Quiroz	Yucatán (2)
India	Santa Victoria	Hacienda Palo Blanco	San Juan Sauceda
Natilla y Tinajera	Santa Victoria (2)		
Astillero	El Cinco	San Miguel	La Paloma
	El Cinco (2)		
Region 5	Region 6	Region 7	Region 8
Teresitas	Tanque de emergencia 3	Santa María	Ojo Caliente
La Tosca	Tanque de emergencia 2	Meson del Norte	Ojo Caliente (2)
El Puerto	Gómez Farías	Sertuche	Puente Yayo
Chorros	Zacatera	Sertuche (2)	Higuera
Chorros (2)	Guadalupe Victoria	Higo Mesillas	Higuera (2)

Sotol plants were collected in 40 locations, which were grouped in eight regions (populations) because its differences in weather and soil composition. All locations belong to the Mexican Coahuila Southern area which includes the Arteaga, General Cepeda, Parras, Ramos Arizpe and Saltillo counties (Fig. 1, 1-8 are the number identification of the collection sites); collection sites and regions are showed in Table 1. The distance between each location of collection was at least 5 kilometers. At each location 20

plants were selected and 1 leaf without damages was collected per plant. Leaves disinfection was made in three steps, the first step consisted in soap and sterile distilled water washing, for the second step leaves were submerged in 70 % ethanol for 2 minutes, after alcohol evaporation, the leaves were submerged in 10 % chloride for 5 minutes and washed with sterile distilled water. After that, the leaves were stored in sterile bags at -20°C until its analysis.

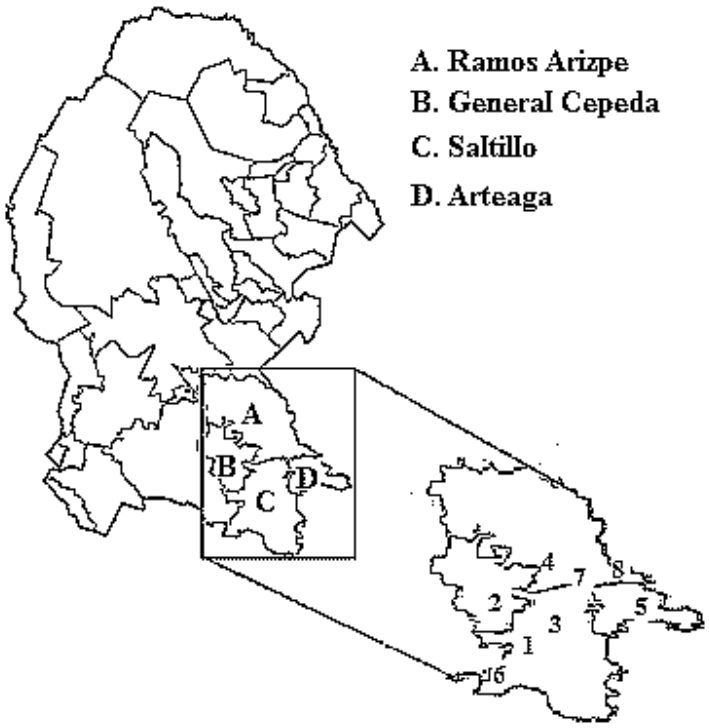

Fig.1. Geographic localization of analyzed sotol (*D.cedrosanum*) collections in South Coahuila State, Mexico. A, B, C, D is the identification letter to counties; 1-8 are the identification number of the collection sites

DNA isolation

DNA was isolated from sotol leaves following the protocol reported by Dellaporta *et al.* (1983). DNA from each location came from twenty individual plants, which were treated individually in order to make an analysis within a single region. Thus, plant leaves from each location were cut in small pieces and 0.25 g of these leaf pieces were freezing in liquid nitrogen, then ground and added to a tube with 1 ml extraction buffer, vortex mixed and then incubated at 65°C for 12 minutes. After that, 330 µl of potassium acetate 5 M were added and the samples were incubated for 40 minutes in ice, then a centrifugation step (20 min, 13,200 g, 4°C) was performed. The

supernatant was separated into new tube with 1 ml of isopropyl alcohol; the followed step was all night incubation at -20 °C and a centrifugation (10 min, 12,000 g, 4°C). The precipitate was resuspended with 200 µl of dilution solution. After that, 5 µl of RNAase (10 mg/ml) were added and incubated 40 min a 37°C to mixed in vortex step the sample with 400 µl of phenol:chloroform:isoamyl alcohol (25:24:1) and then it was centrifugated (5 min, 12,000 rpm). The supernatant was separated into new tube with 75 µl of sodium acetate 3 M and 500 µl of isopropyl alcohol; it was softly mixed and incubated at -20 °C for 1 h. Finally the tubes were centrifugated (10 min, 10,000 rpm) and the precipitate was washed with 70 % ethanol and resuspended in 60 µl of TE 0.1 X buffer. Integrity of the isolated DNA was evaluated using agarose (1%) gel electrophoresis. DNA quantification was performed using a spectrophotometer at 260 and 280 nm in a sample dilution (1:200) and the following equation:

$$DNA\ concentration_{[\mu g/ml]} = \frac{Abs_{260\ nm} \times 50 \times dilution\ factor}{1000\ ml}$$

AFLP's analyses

The AFLP procedure was carried out following the suggestions of the LICOR® kit manual. The technique consists of a restriction digestion with two restriction enzymes (*Eco*R1 and *Mse*I) to generate small DNA fragments; following by a ligation of double-stranded DNA adapters to the ends of the restricted DNA. Then DNA fragments were amplified by PCR with two primers (*Mse*I 5´-GACGATGAGTCCTGAG-3´; 3´-TACTCAGGACTCAT-5´; *Eco*R1 5´-CTCGTAGACTGCGTACC-3´; 3´-CTGACGGATGGTTAA-5´), complementary to the adapter-ligated ends. These fragments (pre-amplified) were amplified again, this time the primers (selective primers) M-CTG/E-AGC and M-CTG/E-ACT for 700 and 800 nm panels respectively were selected from a previous screening. The fragments were separates in a 6% polyacrylamide gel (1500 V, power to 40 W, current to 40 mA and temperature to 45°C).

Genetic analyses

A binary matrix (1 = presence, 0 = absence) was prepared based on the band patterns. Data were introduced in the Info-Gen software (Balzarini and Di-Rienzo 2004) to determine genetic diversity; a conglomerate analyses, based on similarty data Positive Matching and appliyng the UPGMA method (the equations are in Table 2) and the poblational structure based in Wright´s statistics. PCA analysis and analysis of molecular variance

(AMOVA) were applied; this last analysis considered of 400 interactions (permutations) to calculate ***p*** value.

Table 2

Equations to calculate genetic parameters

Parameter	Equation	Abbreviations
Genetic diversity	$D = 1 - \sum_{i=1}^{1} p_i^2$	D = genetic diversity, p = allele frequency in the i locus
F_{ST}	$F_{ST} = 1 - \frac{Nei's unbiased heterozygosis from the collection}{Total Nei's unbiased heterozygosis}$	
F_{IS}	$F_{IS} = 1 - \frac{Genetic diversity from the collection}{Nei's unbiased heterozygosis from the collection}$	
F_{IT}	$F_{IT} = 1 - \frac{Genetic diversity from the collection}{Total Nei's unbiased heterozygosis}$	
Distance Positive Matching	$\sqrt{1-S}$	S is the similarity
Coordinate principal analysis	$Q_{ij} = A_{ij} - \bar{A}_{i.} - \bar{A}_{.j} - \bar{A}_{..}$	A_{ij} is the similarity between the observations i and j, $\bar{A}_{i.}$ is the average of similarity from i row, $\bar{A}_{.j}$ is the average of similarity from j row, $\bar{A}_{..}$ is the general average of similarity in A

RESULTS

The sotol regions classification was based on plant morphology and geographic localization. All samples regions belong to the Coahuila Southern area which is formed by four counties (Arteaga, Saltillo, Ramos Arizpe and General Cepeda) (INEGI 2012); at each region, plants from several locations were collected.

Weather conditions in the South of Coahuila state were investigated to determine correlations between plants phenotype and genotype. The sampling region has temperatures between 12-22 °C but in winter it has freezing temperatures about 0-60 days per year, while the average of hail was 0-2 days per year. In rainy season the average precipitation was 200-600 nm per year (SEMARNAT 2012).

The wheatear conditions above mentioned are for the general sampling area, but in each region the characteristics are little different. Region 1 is a semi-desert area (approximately 1700 m above sea level); region 2 is temperate with pines; region 3 raised about 1500-1600 m above sea level; region 4 is the most desert area and as

consequence very dry; region 5 as well region 2 is temperate, but the altitude is different (1700-2000 m above sea level); region 6 has about 40 km distance from region 1 and is an arid plateau; between region 7 and region 4 exists a difference of about 40 km and finally the region 8 is located at about 1200-1500 m above sea level (SEMARNAT 2012).

In this study the AFLP analysis included 40 samples of Sotol (*Daysilirion cedrosanum*) from 8 regions. After polyacrylamide electrophoresis, were obtained 56 polymorphic bands. The polymorphic loci showed a range from 0.286 (Region 6) to 0.696 (Region 1 and 2) while total value of polymorphic loci was 0.929. The results are summarized in Table 3.

Table 3

Genetic values from *D. cedrosanum* plants with raw data and bootstrap analysis

Genetic Diversity Indicator	Region	Value	Bootstrap Value	Bootstrap Standard Error	Confidence Intervals
Polymorphic loci	1	0.696	0.693	0.062	0.571 – 0.804
	2	0.696	0.699	0.063	0.571 – 0.821
	3	0.554	0.553	0.067	0.411 – 0.696
	4	0.571	0.569	0.067	0.446 – 0.714
	5	0.679	0.679	0.062	0.554 – 0.804
	6	0.286	0.285	0.061	0.179 – 0.411
	7	0.429	0.430	0.067	0.304 – 0.571
	8	0.393	0.394	0.065	0.268 – 0.518
	Total	0.929	1.000	0.000	1.000 – 1.000
Genetic Diversity	1	0.269	0.346	0.031	0.286 – 0.402
	2	0.267	0.350	0.032	0.286 – 0.411
	3	0.219	0.276	0.034	0.205 – 0.348
	4	0.241	0.285	0.034	0.223 – 0.357
	5	0.249	0.340	0.031	0.277 – 0.402
	6	0.117	0.142	0.030	0.089 – 0.205
	7	0.148	0.215	0.033	0.152 – 0.286
	8	0.171	0.197	0.033	0.134 – 0.259
	Total	0.300	0.500	0.000	0.500 – 0.500
Unbiased Heterozygosis	1	0.298	0.462	0.041	0.381 – 0.536
	2	0.291	0.466	0.042	0.381 – 0.548
	3	0.250	0.368	0.045	0.274 – 0.464
	4	0.276	0.380	0.045	0.298 – 0.476
	5	0.276	0.453	0.041	0.369 – 0.536
	6	0.130	0.190	0.040	0.119 – 0.274
	7	0.161	0.287	0.044	0.202 – 0.381
	8	0.190	0.263	0.043	0.179 – 0.345
	Total	0.303	0.667	0.000	0.667 – 0.667

Table 3

Continued

Genetic Diversity Indicator	Region	Value	Bootstrap Value	Bootstrap Standard Error	Confidence Intervals
Allele Average	1	1.696	1.693	0.062	1.571 – 1.804
	2	1.696	1.699	0.063	1.571 – 1.821
	3	1.554	1.553	0.067	1.411 – 1.696
	4	1.571	1.569	0.067	1.446 – 1.714
	5	1.679	1.679	0.062	1.554 – 1.804
	6	1.286	1.285	0.061	1.179 – 1.411
	7	1.429	1.430	0.067	1.304 – 1.571
	8	1.393	1.394	0.065	1.268 – 1.518
	Total	2.000	2.000	0.000	2.000 – 2.000

Region 1 had the highest value for genetic diversity, unbiased heterozygosis and allele average (0.269, 0.298 and 1.696 respectably), while Region 6 was that showed the lowest values (0.117, 0.130, 1.286 and 1.207 respectively).

Wright statistics contribute to structural population knowledge based in genetic diversity. The F values obtained in the present study are showed in Table 4. According to the F_{ST} obtained values, the Regions 6, 7 and 8 were classified in very big differences; Region 3 in big difference; Regions 4 and 5 had moderate difference and finally Regions 1 and 2 was classified with small differences.

Table 4

Wright statistics coefficient values from *D. cedrosanum* plants

Coefficient	1	2	3	4	5	6	7	8
F_{ST}	0.017	0.040	0.175	0.089	0.089	0.571	0.469	0.373
F_{IS}	0.112	0.119	0.277	0.205	0.178	0.614	0.512	0.436
F_{IT}	0.097	0.104	0.265	0.191	0.164	0.607	0.503	0.426

F_{IS} values ranged from 0.614 to 0.112 (Regions 6 and 1, respectively); as well as the F_{IT} values 0.607 to 0.097.

In the Analysis of Molecular Variance (AMOVA) was found that there are significant differences between (p-value < 0.0001) and within regions (p -value < 0.0001) (Table 5). Observed differences among regions were two-folds higher than those observed within regions.

Table 5

Analysis of Molecular Variance of sotol plants from eight regions of Coahuila Southern area

Variation Cause	SS	FD	MS	p-value	Number of inter-actions
Regions	203.32	7	29.05	< 0.0001	400
Within	467.93	32	14.62	< 0.0001	400
Total	671.25	39	17.21		

A dendrogram was done based on positive matching (highest cophenetic correlation value- 0.957) distance measures (Fig. 2). Two main groups (A and B) were yielded. A group was subdivided into 4 subgroups, one was formed by regions 1 and 2; region 3 binds them to form the second sub-group; the third subgroup was formed with the cluster previously described and region 5; finally the fourth subgroup was formed by addition of region 4. Group B was constituted by the 8, 6 and 7 regions.

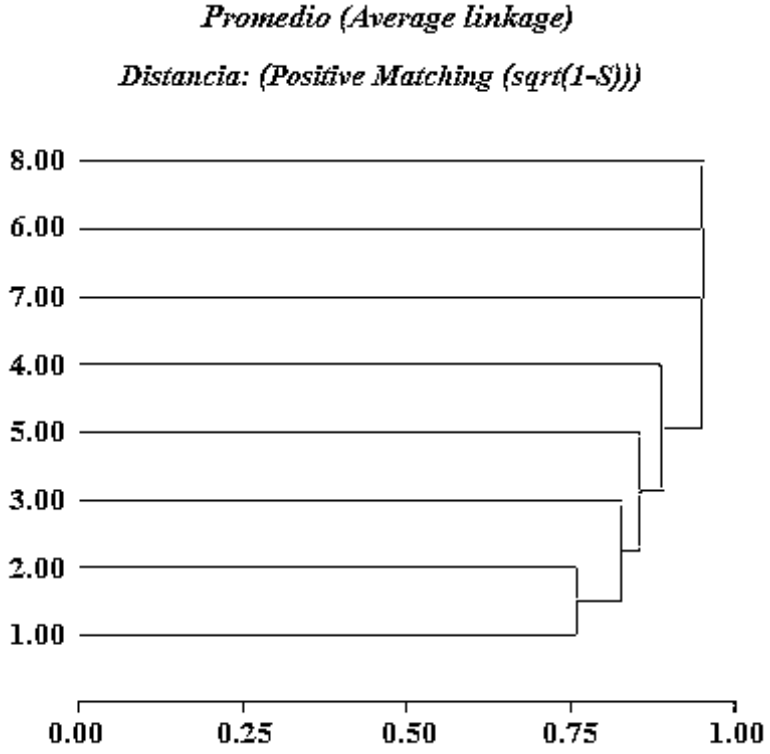

Fig. 2. Sotol samples dendogram, based in positive matching distance measures

DISCUSSION

Differences in soil composition and weather conditions of the sampling region may affect sotol plants growth, and their morphologic characteristics. However, semi-arid plants can growth because their adaptation capacity to extreme condition in temperature, nutrients content and water stress. As consequence of the weather conditions, the organisms generate ample genetic variability which ensures the species permanence (Rodriguez-Herrera and Reyes-Valdéz 2008).

In literature exists different methods to estimate genetic variation, the simplest descriptors are allelic and genotypic frequencies but resume measurements as heterozygosis and genetic diversity can be used too. In this study, the genetic variation of Sotol samples was determinate by the last two measurements. The statistics were made with the raw data and with resampling (bootstrap), because we did not have sample statistic distribution and the bootstrap can provide an empiric estimation of the genetic parameters and the standard error to construct a confidence interval (Balzarini *et al.* 2010). On the other hand, there is a lack of scientific information about sotol plants and for discussions, in this paper, all comparisons was done with agave and other semi-desert plants.

The genotypes diversity was measure in the sampled regions to known the intra and inter-genetic diversity of *D. cedrosanum*. This is the first study about ecological distribution of sotol plants and, may be, a first step to continue the scientific investigation of a Mexican endemic plant, with many potential industrial applications. Polymorphic loci determined for *D. cedrosanum* samples were higher than those reported for *Agave salmiana* plants (Alfaro-Rojas, Legaria-Solano and Rodriguez-Perez 2007) and *Agave angustifolia* Haw (Barraza-Morales *et al.* 2006). In addition, to genetic differences between these species, different levels of diversity among them may be attributed to the different procedure to estimate this measurement, diversity in *Agave salmiana* was determined using RAPD´s, in contrast in this study diversity of *D. cedrosanum* was estimated using AFLP´s. Polymorphism level detected with RAPD´s is lower than that detected with AFLP´s (Martinez, Helguera and Carrera, 2010).

Genetic diversity describes the variability of an inbreeding population, where there are few heterozygotes but there are many types of different alleles in homozygosis (Meléndez-Rentería *et al.* 2010). The obtained values in this study were similar to the average values (0.086) reported for cross-pollinated plants (Gottileb, 1981). The possible explanation to these results can be found in the pollination mechanism of sotol, which is a *dioecious* plant and without color flowers, by this reason it is believed that pollination is mainly by air, in this process the pollen remains on the air until a female plant catch it and the fertilization is done, thus one plant may had pollen

from several origins and the genetic diversity has a homogeneous distribution in the ecosystem. Unbiased heterozygosis was lower than that reported for *Agave angustifolia* Haw (Barraza-Morales *et al.* 2006, Sanchez-Teyer *et al.* 2009), *A. vitoriae-regina* and *A. lechuguilla* (Piñero *et al.* 2008); but higher than *A. cerulata, A. deserti* and *A. subsimplex* (Piñero *et al.* 2008).

Among Wright statistics, F_{ST} is the most used for genetic comparisons; by definition it is the genetic differentiation degree between populations as function of allelic frequencies (Balzarini *et al.* 2010). Grouping of 6, 7 and 8 Regions is attributed to the distance between these zones and the other sampling regions; Region 3 can be isolated from the others for geographical accidents as hills or valleys; plants from regions 4 and 5 can interchange pollen and maintain the differentiation degree. In comparison with *Agave angustifolia* (Sanchez-Teyer *et al.* 2009), sampling regions of sotol plants have lower and higher values, but the higher did not achieve the unit, so we can say that it has genetic diversity but not sufficient to separate into species.

F_{IT} can be interpreted as deficiency or excess of average heterozygotes in a population, if the results are positive values it indicates a deficit (Balzarini *et al.* 2010). In this study all values are in agreement with the last sentence; possible explanation is due by the molecular markers used, because AFLP are dominant markers and the detection of heterozygotes can be not efficient (Vos *et al.* 1995).

The last Wright statistic to analyze was F_{IS}, which is as well as F_{IT} a deficiency or excess of average heterozygotes but only in subpopulations; also can give us an approximation of self-pollination and inbreeding degree among the studied populations (Melendez-Rentería *et al.* 2010). Samples from Region 1 with values close to 0 can be self-pollinizated, but *D. cedrosanum* plants are dioecious and need a male plant to be fertilized, so this condition are in disagreement with the assumption; instead other Regions as 6 are more close to 1, which confirms the plants genetics interchange in the subpopulation.

AMOVA results showed that the variance among populations is twice times higher than the variance within populations. These results are in agreement with the expected values, since plants were collects at different locations of Coahuila State and the plants could be able to develop adaptive characteristics to the growing environment (Ocampo-Velazquez *et al.* 2009), besides the pollen exchange between plants from the same area allows the groups formation in that region. These results are in agreement with Gil-Vega *et al.* (2006) whom reported that the diversity between plants of *Agave tequilana* (used for Tequila production) from Guanajuato state were different within a same plantation.

Differences within populations were interesting because there are some physical barriers (stones, hills, or long distance) to separate the sampling

locations in one region, but this was no observed in all sampling regions. The differences among regions can be attributed to vegetal adaptations to the environment or soil characteristics (Ocampo-Velazquez *et al.* 2009) meanwhile the differences within sotol regions may be consequence of plant genetic interchanges. In addition to the previously mentioned factors of genetic variability, the widespread distribution, high fecundity, allogamy and long time generation contribute to delay loss of genetic variability; due to genetic drift, besides these traits, promote new variants acquisition by genetic flux within and between population and generations (Lopez-Alonso *et al.* 1988).

The conglomerate analysis showed that *Dasylirion cedrosanum* plants from one county had not grouped in the same cluster to descriptive analysis, instead it share clusters with others counties; but to cluster analysis interpretation all the regions were grouped together because it was integrated above 75 % of all distances. Similar results were founded in other endemic plant from Mexican semi desert area, *Agave angustifolia* (Sanchez-Teyer *et al.* 2009), where the formed groups with the genetic similarity indices did not match completely with the geographic distances that separate each site; in that case the authors attributed the differences to the asexual reproduction, random pairing events and endogamy of the plants but in *D. cedrosanum* plants reproduction is mainly by sexual way, so the genetic differentiation can be attributed to the cross pollination, seed dispersion (De La Garza -Toledo *et al.* 2008a) or environmental characteristics.

In literature is reported that the main goal of nature conservation, is the maintenance of genetic diversity in order to prevent potential extinction and guarantee sustainable development (Liu *et al.* 2006); the preservation of the endemic plants is necessary to the ecosystems conservation and after Sotol origin denomination by the Mexican government (NOM-159-SCFI-2004), the importance of *D. cedrosanum* plants is crucial. One of the best strategies is *in situ* conservation to maintain the nutritional characteristics of the plants and avoid phytosanitary diseases (Bellon *et al.* 2009).

CONCLUSIONS

All the *D. cedrosanum* genetic information generated by AFLP molecular markers confirms that in the Coahuila Southern area is high genetic diversity of this species with and within populations. For species conservation is necessary more investigation about genetic diversity in other states to decide the best places for industrial cultivation.

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