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Norma Paola Meléndez Rentería[1], Ramón Silva-Vázquez[2], Guadalupe Virginia Nevárez Morrillón[3], Cristóbal Noé Aguilar[1], Raúl Rodríguez Herrera[1]*

[1]Food Research Department. School of Chemistry, Universidad Autónoma de Coahuila. Saltillo, Coahuila, México. [2]Centro de Investigación de Recursos Naturales (CIReNa). Salaices, Mpio. de López, Chihuahua, México. [3]School of Chemistry, Universidad Autónoma de Chihuahua. Circuito Universitario S/N Chihuahua, Chihuahua, México.
*Corresponding author: raul.rodriguez@mail.uadec.mx

GENETIC DIVERSITY OF MEXICAN OREGANO *LIPPIA BERLANDIERI* SCHAUER, FROM THE CHIHUAHUAN DESERT AREA

ABSTRACT

The Mexican oregano *Lippia berlandieri* Schauer is widespread in Mexico. Several studies have involved the characterization of its essential oil in order to use it as food additive and for medical aims, as well as its antimicrobial and antioxidant capacity, although there is a lack of information about genetic diversity of this species. In order to increase *Lippia berlandieri* Schauer application, knowledge of the genetic characteristics of the species and creation of a program for germplasm conservation seem to be essential. This study was carrying out in order to understand the population genetics of *Lippia berlandieri* Schauer. Oregano seeds were collected from 8 locations of the Chihuahua Southeastern desert area. The results showed high genetic variability among oregano populations suggesting that this species is mainly allogamous, pollinated mainly by insects because of the attractive flower color; AMOVA showed genetic differences within and among populations, the last one was found approximately 5 times higher than the genetic differences within populations. The analysis of the Wright´s statistics, especifically F_{IS}, supported the hypothesis that plants are not self-pollinating. On the other hand, it was found that the number of plants per a sample recommended for further genetic analysis of this specie is 13 or more.

Key words: AFLP, Genetic Diversity, *Lippia berlandieri* Schauer, Mexican oregano, Wright´s statistics.

INTRODUCTION

There are several plant species known as oregano, and the most commonly investigated around the world is *Origanum vulgare*, known as European oregano. One of the least studied is *Lippia berlandieri* Schauer known also as *Lippia graveolens* H.B.K., which is the most widespread oregano species in México and has more intensive smell and taste than the European representative (Silva-Vázquez *et al.* 2008), what causes that it is exported to USA. *Origanum* belongs to the family *Labiatae* while *Lippia* belongs to the family *Verbenaceae*. The chemical composition of Mexican

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oregano is complex and it depends on the collection season, plant phenology, soil fertility conditions and geographical characteristics of the growth locality (Pascual *et al.* 2001; Silva-Vázquez *et al.* 2008). Most of the studies on *L. berlandieri* Schauer reffer to agronomic conditions of oregano growth (Dunford & Silva-Vázquez 2005), chemical content characteristics (Martínez-Rocha *et al.* 2008), microbiological (Portillo-Ruiz *et al.* 2005) and antioxidant activity of oregano essential oils (Chaquilla-Quilca *et al.* 2008), but there is a lack of information on Mexican oregano genetic characterization. Recently, there has been a need to industrialize specific genotypes of this species, given its agronomical and economical importance. Therefore, it is important to develop a Mexican oregano germplasm conservation program.

The state of Chihuahua, México, has three locations where oregano is grown commercially and used for essential oil extraction, which is later exported. The three locations are situated in the central and south part of the state (Delicias, Naica and Jimenez-Parral). Oregano from the Delicias region has carvacrol as major component (40-45 % of total), the one collected in Jimenez-Parral region have the highest thymol content (50-55 % of total) and oregano from the Naica region has thymol and carvacrol – as main compounds with similar concentrations (Jacinto-Soto *et al.* 2007). These compounds, thymol and carvacrol, are used as antimicrobial agent in the food and pharmaceutical industries (Ultee *et al.* 2002; Nostro *et al.* 2007).

AFLP´s molecular markers are useful tools for genetic identification of microorganisms and plants. AFLP's have been used to study the population genetics of agave (Sánchez-Teyer *et al.* 2009), sesame (Laurentin *et al.* 2008), black pepper (Joy *et al.* 2007) and other species of oregano (Ayanog *et al.* 2006) giving satisfying results in reliability and reproducibility. This technique is based on the amplification by PCR (Polymerase Chain Reaction) of polymorphic fragments, obtained by restriction enzymes, from whole DNA genomes (Vos *et al.* 1995). AFLP's help on the identification of polymorphisms in DNA without previous knowledge of the organism´s DNA sequence. These molecular markers were 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). The aim of this paper was investigation of the population genetics of eight wild populations of *Lippia berlandieri* Schauer from the state of Chihuahua, México using AFLP's.

MATERIAL AND METHODS

Vegetal material recollection

Seeds of eight wild oregano populations were obtained from the central and south part of the state of Chihuahua, Mexico. Five oregano populations were collected in the Delicias region, two in Jimenez-Parral (specifically, Salaices) region and one in

Naica region; the number of samples was determined according to the oregano density of each area. Seeds collected in Delicias were identified with the letter D; those collected in Salaices were coded with the letter S; and that collected in Naica was identified with the letter N. Damaged seeds were eliminated using a blower (South Dakota with engine of 1/3 HP, 110 V, 50 Hz it works at 3,450 rpm). Additionally, seeds without embryo and small contaminants including dust and plant debris were separated; big contaminants as stones and mouse excreta were eliminated manually. Seeds were later disinfected with a chloride 3% solution, leaving seeds in the solution for 15 minutes followed by a rinsing step with distillated deionized sterile water; the process was repeated twice.

DNA isolation

DNA was isolated from seeds following the protocol reported by Kang *et al.* (1998). Each seed collection came from six individual plants, which were treated individually in order to make an analysis within a single collection, thus, DNA was isolated from each subsample (one plant). A total of 10-15 seeds per plant were added to a tube with 400 µL extraction buffer and 4 µl of K proteinase then the tube was incubated for an hour at 37 °C. Seeds were ground and 400 µl CTAB 2 % solution was added, vortex mixed and then incubated at 37 °C for 30 minutes. After that, a centrifugation step (5 min 12,000 rpm) was performed. The supernatant was separated into a new tube and washed with an equal volume of phenol-chloroform-isoamyl alcohol, next 2/3 volume of cold isopropanol to precipitate DNA were added. The precipitate was washed twice with 70 % ethanol and re-suspended in 50 µL of TE 1X buffer with 1 µL of RNAase. Integrity of the isolated DNA was evaluated using agarose (1 %) gels electrophoresis. DNA quantification was performed using a fluorometer Turner BioSystems (TBS-380).

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-CTC/E-AAG and M-CTC/E-AGG 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). 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 2003) to determine genetic diversity; F Wright´s statistics and a conglomerate analyses, based on similarty data Sokal&Sneath_3 and appliyng the UPGMA method (the equations are presented in Table 1). PCA analysis and an analysis of molecular variance (AMOVA) were applied. This last analysis considered 750 interactions (permutations) to calculate p-value. Finally the software gave the number of samples needed to future genetic analysis, the method is based on resampling of samples from 1, 2, 3,…,20 cases or locus alternately.

Table 1

Equations to calculate genetic parameters

Parameter	Equation	Abbreviations
Genetic diversity	$D = 1 - \sum_{i=1}^{l} p_i^2$	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 Sokal & Sneath_3	$(a.d) / \sqrt{[(a+b).(a+c).(d+b).(d+c)]}$	a, b, c, and d are the absolute frequencies for events (1,1), (1,0), (0,1) and (0,0) respectively
Coordenate 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

Genetic diversity

AFLP analysis in this study included 48 samples (6 per each of the 8 collects), with 121 polymorphic bands. The values of the polymorphic loci (Table 2) show values ranging from 0.413 (D5) to 0.620 (D3), while total value of polymorphic loci was 0.769. The D3 population showed the highest genetic diversity value

(0.218) (Table 2), while D5 showed the lowest genetic variability (0.147). The lowest score to unbiased heterozygosis was for D7 population with 0.168 (Table 2) and the highest value was for D3 (0.238).Allele average is in a range of 1.413 to 2.00 among the collections.

Table 2

Genetic variability descriptors of *Lippia berlandieri* Schauer

Statistic	D 8	D 7	D 5	D 3	D 2	S 2	S 3	N 2	Total
Polymorphic loci	0.463	0.438	0.413	0.620	0.570	0.512	0.595	0.562	0.769
Genetic diversity	0.161	0.154	0.147	0.218	0.214	0.169	0.209	0.202	0.217
Unbiased hetercygosis (Nei)	0.175	0.168	0.160	0.238	0.234	0.184	0.228	0.220	0.219
Allele average	1.463	1.438	1.413	1.620	1.570	1.512	1.595	1.562	2.000
Effective allele	1.260	1.251	1.241	1.358	1.366	1.265	1.342	1.336	1.321

D = Delicias; S = Salaices; N = Naica

Analysis of molecular variance

In the analysis of molecular variance (AMOVA, Table 3), it was found that significant differences between (p-value 0.001) and within populations (p-value 0.001).

Table 3

AMOVA of *Lippia berlandieri* Schauer

Variation Cause	SS	FD	MS	p-value	Number of interactions
Population	503.57	7.0	71.94	< 0.0001	750
Within	1972.57	160.0	12.33	< 0.0001	750
Total	2476.14	167.0	14.83		

D = Delicias; S = Salaices; N = Naica

Wright statistics

F values obtained in the present study are showed in Table 4. F_{ST} value shows the extent of genetic variation in the sub population in relation to the total variation. The D3, D2, S3 and N2 populations were grouped although they are from different locations; S2 population was classified with moderate differences; D8 and D7 populations were grouped into big differences and finally D5 population was classified as much different.

F_{IS} values were close to 0; while F_{IT} results are in a wide range (0.0046 to 0.3288) which forms two groups: populations in HW equilibrium (D3, D2, S3 and N2) and populations with deficit of heterozygotes (D8, D7, D5 and S2).

Table 4

Wright´s F statistics values

Coeficient	D8	D7	D5	D3	D2	S2	S3	N2
F_{ST}	0.2009	0.2329	0.2694	0.0000	0.0000	0.1598	0.0000	0.0000
F_{IS}	0.0800	0.0833	0.0813	0.0840	0.0855	0.0815	0.0833	0.0818
F_{IT}	0.2648	0.2968	0.3288	0.0046	0.0228	0.2283	0.0457	0.0776

Conglomerates analysis

A dendogram was done with the distance measures (Fig. 1) where the cophenetic correlation value was 0.865.

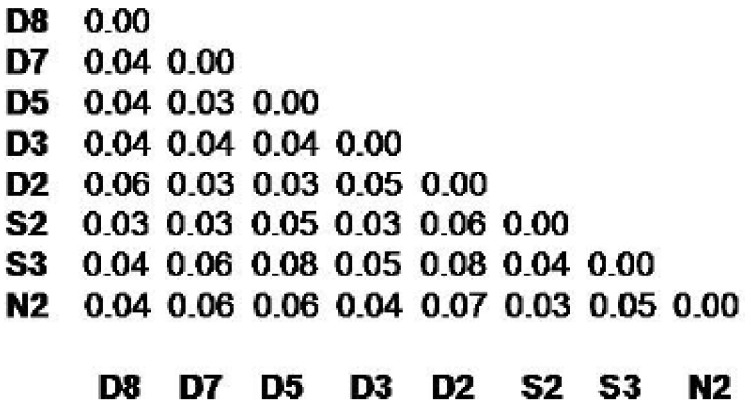

Fig. 1. Genetic distances between collections of *Lippia berlandier*i Schauer

The dendogram (Fig. 2) yielded three main groups A, B and C with two sub-divisions. The D2 and D5 collects were grouped in the sub divisions I, binds them D7 collection thus completing the group A, with a distance of 0.03 among them. In B group, the sub division II was formed by N2 and D3 collects, which bind to S2 to complete the group and the distance between them is 0.03. on the other hand S3 and D8 populations were grouped in group C with a distance of 0.04.

Principal coordinates analysis

The minimum path tree obtained by principal coordinates analysis (Fig. 3) based on AFLP, includes 39.4 % of the total variation of 8 collections of *Lippia berlandieri* Schauer made in Chihuahua State

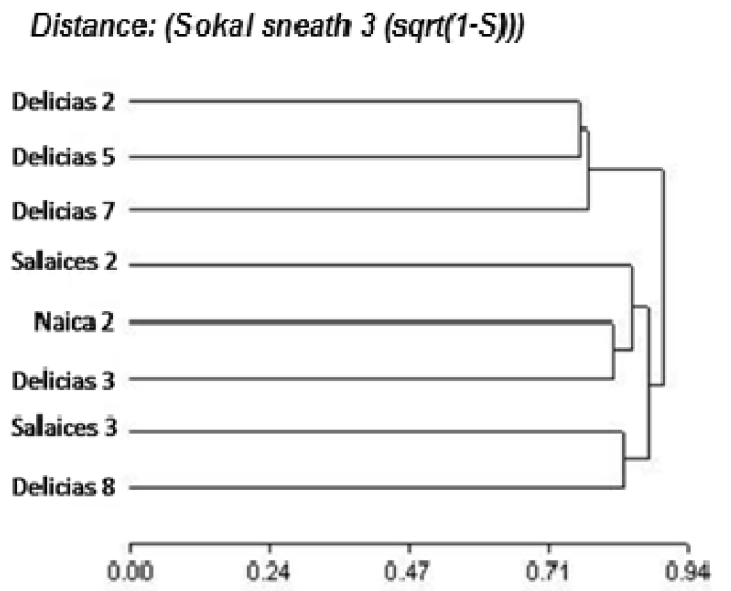

Fig. 2. Dendogram based in the distances obtained by AFLP´s in 8 collection of *Lippia berlandieri* Schauer made in Chihuahua State

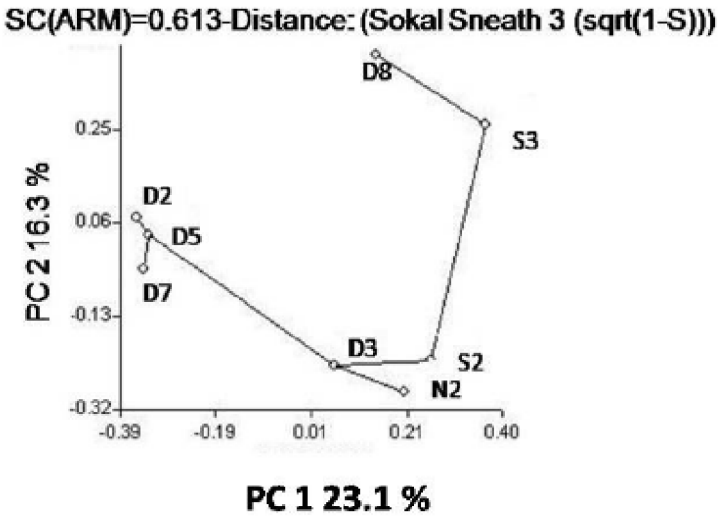

Fig. 3. Minimum path tree obtained by genetic distances

Size of the sample

Fig. 4 shows that with at least 13 plants, the genetic diversity do not change, so this is the minimum working sample for future genetic analysis of wild Mexican oregano (*Lippia berlandieri* Schauer) populations.

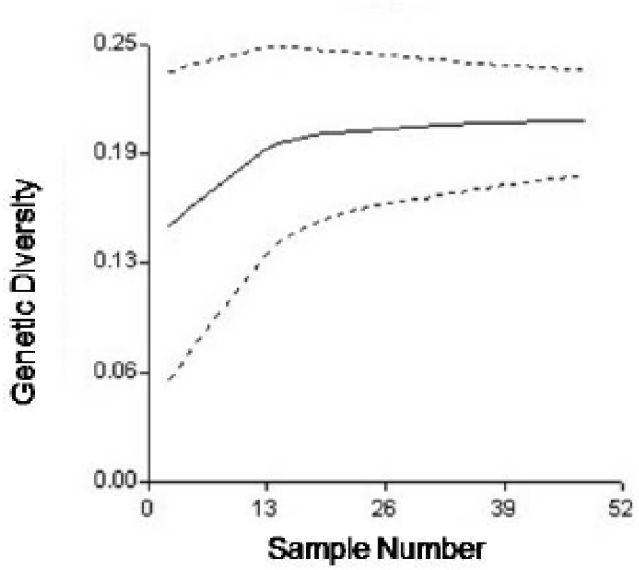

Fig. 4. Behavior of genetic diversity in relation with the number of repetions analyzed in 8 collections of Lippia berlandieri Schauer made in Chihuahua State

DISCUSSION

Genetic diversity

There are several ways to measure genetic variability, which are the summary of measures such as heterozygosis and genetic diversity. Polymorphic loci are when there is variation into the population and the frequency of the most frequent allele is lower than 0.99 or 0.95. When there are multiple loci and every locus is classified as polymorphic or not polymorphic, the percentage of polymorphic loci can be obtained. Cázares-Alonso *et al.* (2009) analyzed *Lippia berlandieri* Schauer populations collected in the State of Coahuila, México and used RAPD´s and SSR molecular markers to determine polymorphic loci values of 0.80 and 0.909 respectably. Results obtained in this study were slightly lower than those reported by Cázares-Alonso *et al.* (2009); this difference can be attributed to the specificity of the technique used in the present work.

Genetic diversity is a measure of the variability of an inbreeding population, where there are few heterozygotes but there are many types of different alleles in homozygosis. Our values are lower than those reported using RAPD (0.25) and SRR (0.26) in plant populations of the same specie but from different geographic locations (Cázares-Alonso *et al.* 2009). Gottieb (1981) reported that there are more differences between cross-pollinated (allogamous) than self-

pollinated (autogamous) herbaceous plants, being the average genetic diversity values of 0.086 versus 0.001. The values obtained in this analysis are similar to those reported for cross-pollinated plants.

The unbiased heterozygosis gives an estimate value of the population genetic variation in relation to expectations as allele frequencies. Reported values for self-pollinated are close to 0, with an average of 0.001; Mexican oregano plants differ from this condition supporting once more time the possibility of being allogamous. According to the results reported by Ocampo-Velazquez *et al.* (2009), *Lippia* flowers are open an average of 7 days before aging, which gives enough time for exchange of pollen from one plant to another. Likewise, the groupings of inflorescences make them attractive to insects that can conduct cross-pollination between plants.

Other measure to analyze the population genetic variation is the allele average. Values are similar to those reported by López-Alonso (1988) who mentioned that the herbaceous plants which have allogamous descent have values close to 1.85. This result suggests that Mexican oregano plants analyzed are fertilized in this manner. Also the author reported that if the allele average values are close to 1.55, it suggests that plants are pollinated by animals, which confirms the mechanism of fertilization conducted by Mexican oregano plants. Hamrick (1983) attributed the high genetic variability to wide distribution of the specie, high fertility, allogamy and long generation time; oregano meets all of these requirements according to the results of this study and the biology concepts reported for *Lippia*.

Analysis of molecular variance

Variance between populations is five-fold times higher than the variance within populations. These results are in agreement with the expected values, since plants were collected from different locations of the state of Chihuahua. It is interesting to find variability within a single collection, since no differences would be expected, because samples were from the same population. According to the reports of Katsiotis *et al.* (2009) whom worked with *Origanum* spp. plants, the results confirms the hypothesis that plants from semi-desert areas have high genetic variability, allowing them to survive in extreme environments.

Wright statistics

When the populations are not in Hardy-Weinberg (HW) equilibrium, there is an excess or deficiency of heterozygotes and a correlation between alleles is generated. The correlation can be among alleles, within individual genotypes or among separate populations and are described by Wright´s F statistics (Berg &

Hamrick 1997). The D3, D2, S3 and N2 groups may be explained by gene flow among these populations; results of D8, D7 and D5 can be attributed to geographical circumstances such as hills, rivers or valleys that prevent the exchange of genetic information within oregano populations (Sánchez-Teyer *et al.* 2009). F_{IS} values helps on the understanding of self-pollination and inbreeding degree among the populations studied, all the collections included in this study were not self-pollinated. Because values were close to 0; this is in disagreement with Ocampo-Velázquez *et al.* (2009) that suggest the self-pollination of *Lippia berlandieri* Schauer plants. A F_{IT} value between 0 and -1 indicates an excess of heterozygotes, values between 0 and 1 indicates a deficit of heterozygotes, and for this reason we had two groups.

Conglomerates analysis

The results showed that collects from the same region were not grouped together; instead share characteristics with other collects; this is consistent with the results of *A*yanog *et al.* (2006) where no genetic similarity was found in plants of *Origanum onites* L. from the same region. The differences among the collects cannot be only attributed to the biochemical essential oil characteristics which make a population from one region distinct from other populations (Jacinto-Soto *et al.* 2007). This is because oregano genetic variability of *L. berlandieri* Schauer is significantly high for all collections and cluster analysis grouped all the collects together. Chemical composition basically depends on the oregano growth conditions, plant phenology and collecting season.

Principal coordinates analysis

The low variation (39%) explained for the two principal coordinates is a reason that does not allow to analyze the distances with the real line measurements (Arroyo *et al.* 2005), but it is possible to identify the formed groups according to the obtained distances. These groups are the same that were identified in the dendogram. One includes D7, D5 and D2 collections; another group consists of the D3, S2 and N2 collections, and finally D8 and S3 collections are grouped together.

CONCLUSIONS

The analyzed data of wild oregano populations from the Central and South region of the state of Chihuahua suggest that there is high genetic diversity and gene flow, probably due to pollinators. Also it is suggested that oregano cross-pollination is done mainly by insects. Analysis of molecular variance showed that significant differences among Mexican oregano populations are higher than

the differences within populations. Analysis of F_{IS} of Wright´s statistics showed that *Lippia berlandieri* Schauer plants from the Chihuahua regions are not self-pollinating. The dendogram derived from genetic distances showed that all collections are different from each, besides all population from the same geographical region were not located in the same group. Regarding the number of samples to work for subsequent genetic analysis of wild plants of *Lippia berlandieri* Schauer, the optimum number of samples is more than 13, in order to reduce sampling errors.

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