GENETIC DIVERSITY IN POPULATIONS OF *Lupinus Elegans* KUNTH, IMPLICATIONS FOR ECOLOGICAL RESTORATION

DIVERSIDAD GENÉTICA DE POBLACIONES DE *Lupinus Elegans* KUNTH, IMPLICACIONES PARA RESTAURACIÓN ECOLÓGICA

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RESUMEN

Lupinus elegans Kunth es una especie común de la región Centro-Occidental de México. *L. elegans* se ha utilizado para la restauración de bosques de coníferas degradados porque crea un microambiente apropiado para el establecimiento de especies de coníferas al aumentar las concentraciones de nitrógeno del suelo. En este estudio se determinó la variación genética de poblaciones de *L. elegans* mediante marcadores moleculares RAPD. Se colectaron 150 individuos para cinco poblaciones de Michoacán: Llano de Pario, Charapan, Pico de Tancítaro, San Nicolás y Villa Madero. Los resultados indicaron una variación genética (h) alrededor de 0.20 con 60 % de polimorfismos. El análisis molecular mostró que la variación genética dentro de poblaciones (59 %) es mayor que entre poblaciones (41 %). Con las distancias genéticas de Nei se generó un árbol "Neighbor Joining" que agrupa a las poblaciones de Llano de Pario y de San Nicolás, seguidas de las de Tancítaro, y finalmente agruparon las de Villa Madero y Charapan. No se encontró correlación entre las distancias genéticas y geográficas. Plantas provenientes de la población local se desarrollaron mejor que las introducidas en los experimentos de jardín común. Estos resultados sugieren que a pesar del amplio rango de distribución de *L. elegans*, la colecta de semillas para proyectos de restauración se debe realizar lo más cerca (geográficamente) a la población local posible.

Palabras clave: *Lupinus elegans,* diversidad genética, población, restauración ecológica.

SUMMARY

Lupinus elegans **Kunth is a common species in the West-Central region of México. *L. elegans* has been used for the restoration of degraded conifer forests because it creates microenvironments suitable for the establishment of coniferous species by increasing nitrogen concentrations in the soil. In this study we determined the genetic variation of *L. elegans* populations using RAPD markers. One hundred and fifty individuals were collected from five populations in Michoacán: Llano de Pario, Charapan, Pico de Tancítaro, San Nicolás and Villa Madero. Results indicate genetic variation (h) about 0.20 with 60 % polymorphism. The molecular analysis showed higher genetic variation within (59 %) than between (41 %) populations. A Neighbor Joining tree was generated based on Nei's genetic distances, and the resulting tree clustered the populations of Llano de Pario and San Nicolás, followed by populations of Tancítaro, and lastly clustered Villa Madero and Charapan. No correlation was found between genetic and geographic distances. Common garden experiments indicate that plants from the local population outperform introduced plants. These results suggest that although *L. elegans* is a species with a considerable geographic range, seed collection for restoration purposes should be done from the closest (geographical) population possible.**

Index words: *Lupinus elegans*, genetic diversity, population, ecological restoration.

INTRODUCTION

Lupinus is a nitrogen fixating genus with about 200 species distributed in the Mediterranean region, Africa and America (McVaugh, 1987). Some species such as *Lupinus albus* L. and *Lupinus elegans* Kunth have been used for ecological restoration (Blanco-García and Lindig-Cisneros, 2005; Burton *et al.*, 2006). *L. elegans*, an outcrosser is a long-lived perennial shrub (Ainouche and Bayer, 1999). It is endemic to Mexico, distributed from 1800 to 3000 masl, in pine and pine-oak forests in the states of Guerrero, Jalisco, Michoacán, Morelos and Zacatecas (Dunn, 2001). *L. elegans* is a highly plastic species characteristic of early successional stages. Germination requirements are well known (Medina-Sánchez and Lindig-Cisneros, 2005), and propagation techniques have also been developed (Alvarado-Sosa *et al.*, 2007). This species inhabits seven different types of plant communities in the Meseta Purépecha region in Michoacán, having the largest distribution then any other legume species in the region (Medina *et al.*, 2000). The species has been used for

restoring areas affected by volcanism (Blanco-García and Lindig-Cisneros, 2005) and is currently used in the restoration of heavily degraded agricultural lands.

The field of restoration genetics is concerned with the genetic performance of introduced species (Hufford and Mazer, 2003) and, among other things, focuses on delineating seed collecting zones. Restoration ecology projects introduce native species into degraded sites. Hufford and Mazer (2003) and Galloway and Fenster (2000) call for studies on genetic diversity of the source populations used for reintroduction, along with evidence from common garden experiments, to ensure appropriate plant performance in restoration projects, thus avoiding under or outperformance of the new phenotypes in local populations (Gustafson *et al.,* 2005; McKay *et al.*, 2005). Studies on the genetic diversity of restored populations are also needed (Liu *et al.*, 2008).

RAPD markers have been used for many years to assess genetic diversity in populations because this technique provides a quick and reliable tool (Williams *et al.*, 1990). RAPD have been widely used for genetic diversity studies within the legumes, for example: *Crotalaria longipes* Raf. (Jayanthi and Mandal, 2001), *Dalea purpurea* Vent. (Gustafson *et al.*, 2002), *Desmodium sumichrastii* (Schinder) Standley (Bedolla-García and Lara-Cabrera, 2006) and *Vicia pisiformis* L.(Black- Samuelsson *et al.*, 1997; Black-Samuelsson and Lascoux, 1999). Talhinhas *et al.* (2003) tested the reliability of molecular markers to assess inter-species genetic diversity among lupin species and obtained similar results when using AFLPs, ISSRs and RAPDs. No genetic diversity studies using RAPD markers have been reported for lupin; there are no reports either of studies undertaken to assess genetic diversity of lupin species for ecological restoration purposes.

The present study was undertaken to assess the genetic diversity of natural populations of *Lupinus elegans*, to determine how genetic variation is distributed among and within populations from different regions in order to aid in the decision-making process for determining seed collection ranges, and finally to perform common garden experiments under restoration conditions with seeds collected from the same natural populations.

MATERIALS AND METHODS

Plant material and RAPD amplification

Thirty individuals from five populations of *Lupinus elegans* were collected in Michoacán (Table 1), and all samples were made at random avoiding close neighbors when possible. The collection sites were: San Nicolás (where the restoration site is located), Llano de Pario (the population nearest San Nicolás), and Tancítaro and Charapan within 450 to 500 kms from San Nicolás, and the farthest Villa Madero at 670 kms. From the five populations, those from Tancítaro and Villa Madero were present in well preserved pine-oak forests. San Nicolás plants were found in abandoned agricultural fields near forest edges. Llano de Pario and Charapan plants were found in the most degraded areas. The Llano de Pario population was in an area affected by volcanic ash deposition from the Paricutín volcano which erupted more than 50 years ago and recently by cattle grazing. Charapan is a secondary pine-oak forest severely overgrazed and next to a state highway.

Young and healthy leaves were randomly collected in the five populations for a total of 150 individuals (Table 1). Voucher specimens were deposited at MEXU and IEB. DNA extraction was performed following Lefort and Douglas (1999). DNA concentration was determined using a Spectrophotometer (Jenway Designed model 6305) at 260/280 wavelength. Each polymerase chain reaction (PCR) included 0.05 μg μL^{-1} of DNA.

Thirty decamer primers (Series OPA, OPB, OPC, OPD, OPF, OPH and OPR from Operon Technologies, Alameda, California, U.S.A.) were screened and of these, ten primers in five of the series (Table 2) exhibited high polymorphism and repeatability. In most instances, DNA from each plant was amplified with the same primer more than once and the banding patterns compared. Final volume for PCR reaction was 25 μL: : 15.5 μL of water, 1 μL MgCl$_2$ (50mM), 1.5 μL of oligonucleotides (10nm), 1 μL BSA (10mg μL^{-1}), 2.5 μL of 10X PCR Buffer, 2 μL (10mM) dNTP's (dATP, dCTP, dGTP, dTTP), 0.5 μL of recombinant *Taq* DNA polymerase (Invitrogen, San Diego, California, U.S.A.) and 1 μL (0.05 μg μL^{-1}) of DNA. The thermal cycle program was run on a Techne TC-412 thermal cycler. The program consisted of 44 cycles, each at 94 °C for 1 min, 36 °C for 1 min, 72 °C for 2 min and final extension at 72 °C for 7 min.

Amplification products were electrophoresed in 1.5 % agarose gels with 0.5X TBE buffer (0.045M Trizma base, 0.045M boric acid and 0.001M EDTA) at 120V for 3 h and stained with ethidium bromide (0.1 μg mL^{-1}). Gels with amplification fragments were visualized and photographed under UV light in a Transilluminator UVP-BioDoc-It^{TM} System (Bioimaging system). Molecular mass of the RAPD bands was estimated by comparison to a 123-bp ladder (Invitrogen, San Diego, California, U.S.A.).

Table 1. Sampled populations of *Lupinus elegans* with geographical location, voucher specimen (collectors name and number), number of individuals per population, altitude and vegetation type.

Locality	Voucher specimen	GPS coordinates	Population size	Altitude (m)	Vegetation
San Nicolás	*Alejandre-Melena 260*	19° 24' 60" N 102° 14' 46" W	>500	2850	Agricultural land with POF
Llano de Pario	*Alejandre-Melena 261*	19° 29' 6" N 102° 11' 46" W	237	2470	PF, Ash
Tancítaro	*Alejandre-Melena 259*	19° 22' 13"N 102° 20' 24" W	>500	2343	POF, PF
Charapan	*Alejandre-Melena 265*	19° 34' 4" N 102° 14' 51" W	124	2399	Secondary POF
Villa Madero	*Alejandre-Melena 264*	19° 18' 59" N 101° 21' 52" W	48	2183	POF

POF=Pine-Oak forest; PF= Pine forest.

Table 2. Oligonucleotides employed and RAPD markers obtained for the 150 individuals examined.

Oligonucleotide	Sequence (5'-3')	No. of monomorphic bands	No. of polymorphic bands	Total no. of bands
OPA-01	CAGGCCCTTC	0	9	9
OPA-02	TGCCGAGCTG	0	14	14
OPB-11	GTAGACCCGT	0	20	20
OPB-17	AGGGAACGAG	0	18	18
OPC-02	GTGAGGCGTC	0	16	16
OPD-20	ACCCGGTCAC	1	10	11
OPF-01	ACGGATCCTG	0	13	13
OPH-03	AGACGTCCAC	0	17	17
OPH-13	GACGCCACAC	0	16	16
OPH-15	AATGGCGCAG	2	6	8
Total		3	139	142

RAPD data analysis

Amplified products were scored as the presence (1) or absence (0) of homologous bands; because RAPDs are dominant markers (Williams *et al.*, 1990) we assumed that each band represented a single band locus. Only those fragments amplifying strongly in each instance were included in the analyses. Based on these data, we calculated the percentage of polymorphism (%P) and Shannon´s diversity index (Ho, Observed heterozygosity)

(Nei, 1973) assuming that populations are not under Hardy-Weinberg equilibrium, using the program POPGEN ver. 1.31 (Yeh and Yang, 1999).

Analysis of molecular variance (AMOVA) was used to calculate the partitioning of genetic variation between and within populations. The significance of the different variance components was estimated from distributions generated from 10 000 random permutations in ARLEQUIN ver. 3.01 (Excoffier *et al.*, 2006).

A genetic distance and identity matrix was calculated using Nei's genetic distance. A Neighbor Joining (Saitou and Nei, 1987) dendrogram based on pairwise measures of genetic distance was generated in PAST v. 1.55 (Hammer *et al.,* 2001). The reliability of the Neighbor Joining dendrogram was tested by bootstrap analysis with 1000 replications.

The normalized Mantel statistic (r_M) (Mantel, 1967) was calculated between the genetic and the geographic distance matrices in the program Tools for Population Genetic Analyses (TFPGA) v. 1.3 (Millar, 1997). The significance of r_M was tested by a permutation test (10 000 iterations) to determine if there is a relationship between the genetic composition and the geographic distribution of the populations.

Common garden experiments

A reciprocal transplant study was attempted at two restoration sites adjacent to the populations of San Nicolas and Llano de Pario. Nevertheless, all plants died at Llano de Pario before the experiment started; only the experiment at San Nicolás is described. One hundred and twenty seeds, in groups of two, were sown from each population following the design of a Latin square lattice with a distance of 40 cm between planting points. When both seeds within the same planting point germinated, the second seedling was eliminated in order to have 60 plants from each population. The experiment was set up during the first week of June, 2005 and followed every month for a complete growing season, until December of the same year. The number of plants present at the end of the growing season and the height for each plant were recorded. Plants from the Llano de Pario population were eliminated from the San Nicolás site at the end of the growing season to prevent cross pollination the following year.

A common garden experiment was set up at the University grounds located in the City of Morelia (19º41'W, 101º12'N, 1921 masl) using five tubs of 210 Leach. Seeds were collected from four populations: San Nicolás, Llano de Pario, Charapan and Tancítaro. These produced enough seeds the same year that leaf samples for genetic analyses were collected. In each tub, eight plants from each population were grown for a total of 40 plants per population. The experiment was set up during the first week of June 2005 and followed for a complete growing season, until December of the same year. The number of plants present at the end of the growing season and the height for each plant were recorded.

Establishment was analyzed using Chi squared tests or analyses of deviance on generalized linear models (GLM) for binomial distributed data considering each plant as an experimental unit (Dobson, 2002). ANOVA was applied to test differences in height among populations. Residuals were checked for compliance with ANOVA assumptions. For the common garden experiment, missing height data resulting from nine dead plants were replaced with the mean for the treatment; each tub was considered a block for the statistical analysis. Data shown are means and standard errors of the mean. All analyses were carried out using the statistical software R (R Development Core Team 2008).

RESULTS AND DISCUSSION

Genetic Diversity

Of 142 amplified bands (a sample is shown in Figure 1) 96 % were polymorphic (Table 3). The percentage of polymorphic bands ranged from 49 to 56 %, with the highest percentages in San Nicolás and Tancítaro (56 and 54 % respectively), and the lowest in Charapan (49 %). Sampled populations differed in genetic diversity as shown by Shannon's diversity index (Table 3), the highest genetic diversity (I) of 0.2144 for Tancítaro and Villa Madero, followed by San Nicolás, Llano de Pario and Charapan. These values are similar to that of *Dalea purpurea* (0.21), another widely distributed legume (Gustafson *et al.,* 2002).
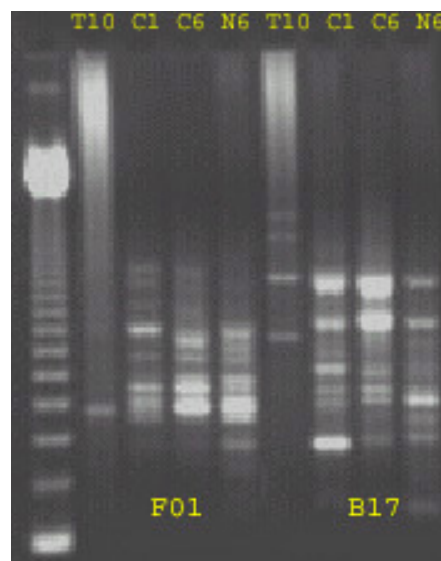

Figure 1. An example of polymorphisms found with amplification of two oligonucleotides F01 and B17 for individuals of *Lupinus elegans*. The first lane shows the 123bp DNA ladder. The same four individuals were included for each oligonucleotide, the codes indicate the population (T= Tancítaro, C= Charapan and N= San Nicolás), and the number refers to the particular individual.

Table 3. Genetic variation within populations of *Lupinus elegans* based on RAPD data.

Population	Number of alleles per locus (No)	Effective number of alleles per locus (Ne)	Nei´s genetic distance (h)	Shannon Index (I)	Number of polymorphic loci	Percentage of polymorphic loci (%)
Llano de Pario	1.5070	1.1961	0.1210	0.1922	72	50.70
San Nicolás	1.5634	1.2115	0.1335	0.2131	80	56.34
Tancítaro	1.5423	1.2245	0.1368	0.2144	77	54.23
Villa Madero	1.5211	1.2147	0.1360	0.2144	74	52.11
Charapan	1.4859	1.1907	0.1203	0.1905	69	48.59
Among populations	1.9577	1.2928	0.1884	0.3078	136	95.77

Total genetic diversity for all populations (Hsp) was 0.1884. From these values the proportion of total genetic variation among populations (Hpop/Hsp) was 0.6873. The proportion of genetic diversity shared between populations as calculated by G_{ST} =0.3128 (Nei, 1973) indicated that ca. 30% of genetic diversity occurred among populations. G_{ST} values are similar to those found in other legume species: *Desmodium sumichrastii* (G_{ST} =0.3271) (Bedolla-García and Lara-Cabrera, 2006) and *Medicago truncatula* (F_{ST} = 0.32) (Bonnin *et al.*, 2001). Higher population structure scores have been found for outcrossing crop species (Hamrick and Godt, 1997). The estimate of gene flow between the populations in one generation (Nm) was 1.1 (one individual per generation) which would suffice to overcome the homogenizing effects of genetic drift (Ellstrand and Ellam, 1993).

Genetic distance and genetic identity (Table 4) indicated that the populations showing the greatest similarity were Villa Madero and Tancítaro and those differing the most were Charapan and Llano de Pario. The partitioning of genetic diversity as revealed by AMOVA (Table 5) showed the larger fraction of variation (%Φ-st, percentage of the overall differentiation among populations) was within populations (59.2 %, P < 0.00001), compared to a 40.7 % (P < 0.00001) variation among populations; in accordance with large cross pollinated species (Agrimonti *et al.*, 2007; Huff *et al.*, 1993; Nebauer *et al.,* 1999; Yu and Pauls, 1993). Similar results have been found in *Desmodium sumichrastii* with 61% within population variation reported (Bedolla-García and Lara-Cabrera, 2006), and higher within population variation for *Trifolium pratense* with 76 % variation (Campos de Quiroz and Ortega-Klose, 2001).

RAPD evidence for *L. elegans* showed no correlation between genetic and geographic distances following the Mantel correlation test (P = 0.619) for these populations. The Neighbor Joining tree (Figure 2) for all populations based on Nei's genetic distances, showed two clusters with 100 % of bootstrap confidence splitting Llano de Pario and San Nicolás (69 % of bootstrap support), and Tancítaro (>50 % of bootstrap support) from Villa Madero and Charapan (70 % of bootstrap support).

Common garden experiments

The reciprocal transplant study at the restoration site near the population of San Nicolás showed that plants from local stock had a significantly higher survival rate, and were nearly 20 % taller than plants from Llano de Pario, despite the fact that these populations are more closely related genetically. Survival differed between populations: 30 % of the plants from the Llano de Pario population survived and 60 % of the plants from San Nicolás, the local population, survived. Differences were statistically significant (X^2= 9.73; P = 0.002). Plant eight at the end of the growing season also varied between populations, with plants from San Nicolás (110 ± 7 cm) being taller than plants from Llano de Pario (90 ± 7 cm). The difference in height between populations was 18 %, but only marginally significant ($F_{(1,52)}$ = 2.9; *P* = 0.09).

The common garden experiment showed that if conditions are less demanding no differences in survival occur (only nine plants from different populations died), but populations still differ in growth (Figure 3). The tallest plants were those from the Llano de Pario population (66 ± 2 cm) and Tancítaro (66 ± 2 cm), followed by plants from Charapan (62 ± 2 cm) and finally plants from San Nicolás (55 ± 2 cm), the differences being statistically significant (Table 6).

These differences in performance in the common garden experiment can be explained by the geographic origin of the plants. San Nicolás, growing at the highest altitude, is located at 2850 masl and the common garden experiment was set-up at 1950 masl, a difference of 900 m. The other populations differed in altitude from 393 to 520 m from the altitude of the site of the common garden experiment. Differences in performance across altitudinal gradients have been found for other forest species in the region, puch as *Pinus oocarpa* and *P. pseudostrobus* (Viveros-Viveros *et al.*, 2005; Sáenz-Romero *et al.*, 2006).
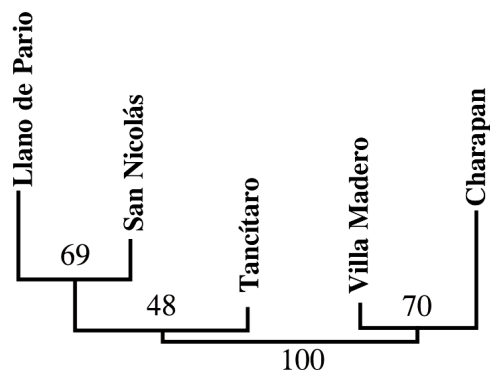
Figure 2. Neighbor joining dendrogram based on pairwise measures of Nei's genetic distances. Numbers on branches represent bootstrap confidence values for 1000 replicates.
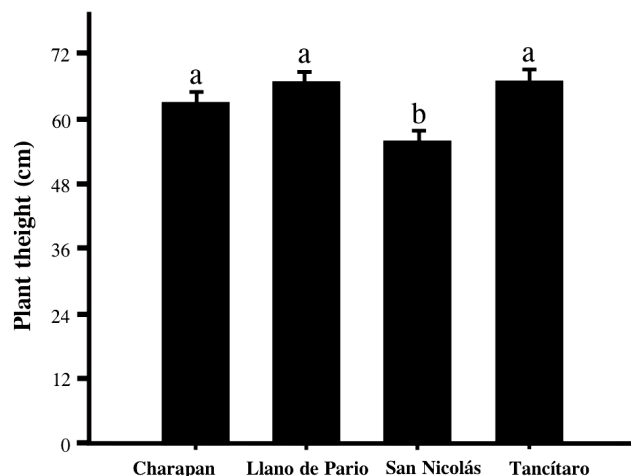

Figure 3. Heights of *Lupinus elegans* plants at the end of the common garden experiment. Different letters represent significant differences among populations according to Tukey test (data are means and standard errors of the means).

Table 4. Genetic distance (GD) among populations: Nei's genetic identity (GI) above diagonal and genetic distance below diagonal.

Population	Llano de Pario	San Nicolás	Tancítaro	Villa Madero	Charapan
Llano de Pario	-	0.9128	0.9155	0.9189	**0.8880**
San Nicolás	0.0912	-	0.9226	0.9114	0.9061
Tancítaro	0.0883	0.0806	-	**0.9481**	0.9119
Villa Madero	0.0846	0.0927	**0.0533**	-	0.9191
Charapan	**0.1187**	0.0986	0.0922	0.0844	-

Table 5. Analysis of molecular variance (AMOVA).

Source of variation	df	Sum of squares (SSD)	Variance component estimates (CV)	Percentage of variation (PV)	Probability of significance (PS)
Among populations	4	944.4	7.5	40.7	<0.00001
Within populations	144	1581.1	10.9	59.2	<0.00001
Total	148	2525.5	18.5		

df = degrees of freedom.

Table 6. ANOVA table for heights of *Lupinus elegans* plants at the end of the common garden experiment.

	df	Sum of squares	Mean squares	F	*P*
Block	4	2917	729	5.25	< 0.0001
Population	3	3322	1107	9.97	< 0.00001
Block:Population	12	2982	248	1.79	0.06
Residuals	140	19457	139		

These results have direct consequences for restoration practice because although *L. elegans* is a species with a considerable geographic range, seed collecting for restoration purposes should be done from the closest population possible. Genetic evidence reported here and our trial at San Nicolás indicates that populations as close as 10 km perform differentially. This range is considerably lower than the range recommended for seed collection for Mexican temperate tree species (Vargas *et al.*, 2004) although it is similar to the range recommended for some native prairie species in North America (Gustafson *et al.*, 2005).

CONCLUSIONS

Populations of *Lupinus elegans* for the West-Central part of Mexico are highly variable both within and among populations when using RAPD's. The Neighbor Joining tree clustered together the populations of Llano de Pario and San Nicolás, followed by populations of Tancítaro and lastly clustered Villa Madero and Charapan. Nevertheless

no correlation was found between genetic and geographic distances. Common garden experiments indicate that plants from the local population outperform introduced plants. The reciprocal transplant study showed that plants from local stock (San Nicolás) performed better than plants from the other population tested (Llano de Pario), a similar trend of significant differences in growth was observed in the common garden experiment, suggesting that populations might be highly adapted to local conditions.

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