Polymorphism determination in two natural mezquite (Prosopis laevigata) populations using RAPD

RESEARCH

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ABSTRACT

A number of different markers (proteins, isoenzymes and RAPDs) have been used to settle taxonomic disputes and to assess genotype variability in populations of the genus *Prosopis*, in order to select highly productive phenotypes. In this study the genetic variability of two wild populations of *Prosopis laevigata* located in the Mexican states of Guanajuato (population 1) and Hidalgo (population 2), as well as that of reference specimens of *P. glandulosa*, *P. juliflora* and *P. laevigata*, were determined using RAPD. The dendrograms for the analysis of genetic similarity among populations and species were generated by cluster analysis using UPGMA and Jaccard's similarity coefficient, based on 27 identified bands. The dendrograms show that the individuals from both populations of *P. laevigata* can be separated in two highly related groups A and B (similarity coefficient 0.938) . Five subgroups were identified in group A, and six subgroups in group B. A total of 11 different genotypes were identified, of which 6 are shared among both populations. The polymorphic profile of the reference specimen of P. laevigata (from the Botanical Garden) matched that of subgroup a, which included 47% of the individuals from population 1. The calculated similarity coefficient between *P. laevigata* and *P. glandulosa* was 0.79, whereas that of P. juliflora to the former was 0.63. Genotypic diversity was similar in both populations, differing in 5 genotypes and their relative abundance. These components of genotypic diversity are the result of a differential response to environmental conditions.

Keywords: Leguminosae, biodiversity, conservation, population, RAPD

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RESUMEN

Estimación del polimorfismo en dos poblaciones de mezquite (*Prosopis laevigata*) mediante RAPD. Diferentes tipos de marcadores (proteínas, isoenzimas y RAPD's) han sido utilizados en el género *Prosopis* para aclarar controversias taxonómicas y para determinar la variación de genotipos en poblaciones. Esto es importante para seleccionar fenotipos altamente productivos. La diversidad genética fue determinada mediante RAPD en dos poblaciones silvestres de *P. laevigata* localizadas en México en los estados de Guanajuato (población 1) e Hidalgo (población 2), así como ejemplares de referencia de *P. glandulosa*, *P. juliflora* y *P. laevigata*. Dendrogramas que muestran las similitudes genéticas entre poblaciones y especies fueron generados usando el análisis de agrupamiento UPGMA y el coeficiente de similitud de Jaccard. Un total de 27 bandas fueron identificadas. El dendrograma muestra que los individuos de ambas poblaciones de *P. laevigata* se distribuyeron en dos grupos (*A* y *B*) muy relacionados entre sí por su alto coeficientes de similitud (0.938). En el grupo *A* cinco subgrupos por grupo fueron identificados mientras en el *B* fueron seis. Un total de once genotipos fueron identificados, de los cuales 6 son comunes en ambas poblaciones. El patrón polimórfico de referencia de *P. laevigata* (ejemplar del Jardín Botánico) coincidió con el patrón del subgrupo *a* que incluyó el 47% de los individuos de la población 1. Un coeficiente de similitud de 0.79 fue encontrado entre las especies *P. laevigata* y *P. glandulosa* mientras que *P. juliflora* se relacionó con éstas mediante un coeficiente de similitud de 0.63. Las dos poblaciones tuvieron similar riqueza de genotipos aunque difirieron en cinco. También difirieron en su abundancia relativa. Estos componentes de la diversidad genotípica responden diferencialmente a las condiciones del ambiente.

Palabras claves: Leguminosae, biodiversidad, conservación, población, RAPD

*I*ntroduction

Nine species of *Prosopis* (Leguminosae, Mimosoideae) have been identified in Mexico based on morphological traits [1]. These species grow in both arid and semiarid lands and are important sources of wood, firewood, fodder, honey and wildlife sanctuary [2-3]. The accelerated and uncontrolled harvesting of this important natural resource has led to land degradation and desertification as well as the loss of genetic diversity within and across the *Prosopis* population. A clear understanding of the genetic structure of the wild populations is needed to establish appropriate reforestation programs for the optimal exploitation of this natural resource.

The taxonomical classification of *Prosopis* has been based on morphological traits [4]. Most of the approaches used to determine genetic diversity in this genus have involved isozyme analysis. However, this method has methodological limitations because of restrictions in the number of loci examined and the possible tissue, developmental stage, or environmental specificity of gene expression [5-12].

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During the last decade, molecular markers such as Random Amplified Polymorphic DNA (RAPD) have been widely used to detect polymorphism [13], analyze phylogenetic relations [14-15], identify cultivars [16-17], discriminate between wild and cultivated species [17-18], and detect agronomic traits [19-20].

The RAPD markers in particular, have been successfully used to determine intraspecies genetic diversity in tropical and semitropical forest plants. These include *Cedrus* [21] and *Pinus* [22, 23]. In contrast, fewer reports are available on the distribution of forest trees in arid and semiarid regions. In the *Prosopis* genus, recent RAPD studies have shown differences in allele frequencies between species of section Algarobia and it has identified species from South America, Africa and Asia [11, 12, 24]. These markers have been used to differentiate accession levels of the *Prosopis* genus [25]. Few reports are available on the genetic diversity of *Prosopis* species in Mexico [15] using molecular markers. The objective of the present study was to apply RAPD and cluster analysis to determine the interspecific similarity among *P. laevigata*, *P. glandulosa* and *P. juliflora*, and the genotype diversity in two wild populations of *P. laevigata* located in two separated geographical locations of Mexico considering the richness of genotypes (number of genotypes identified in the populations) and the relative amount or proportion of individuals per genotype.

Materials and methods

Plant material

Sixty *P. laevigata* trees per population were randomly sampled within an area of 5 ha in each of two Mexican semi-arid locations: Dolores Hidalgo, Guanajuato (population 1) and Santiago de Anaya, Hidalgo (population 2). Additionally, 14 herbarium samples of *P. laevigata* from the Herbarium (MEXU) of the National Autonomous University of Mexico, a sample from one tree of *P. laevigata* located at the Botanical Garden of the National Autonomous University of Mexico, Mexico city, 15 samples of *P. glandulosa* from the countryside of Ciudad Juarez, Chihuahua, and 15 samples of *P. juliflora*, from Culiacan city, Sinaloa, were collected for the analysis. The locations of the two wild *P. laevigata* populations and reference samples are indicated in Figure 1.

DNA isolation

Two grams of healthy leaves from each of the 60 individual samples of populations 1 and 2 and from the tree of *P. laevigata* located at the Botanical Garden of the National Autonomous University of Mexico were frozen and ground, and the individual high molecular weight genomic plant DNA was isolated according to Dellaporta [26] with the addition of 1% of polyvinylpyrrolidone to the extraction buffer.

In order to determine the interspecific relationships among *P. laevigata*, *P. glandulosa* and *P. juliflora*, the genomic DNA from the leaf tissue of 15 individuals of each species was extracted following the same protocol; however, in this case 200 mg of tissue was used and the solutions involved in the extraction were

Figure 1. Geographical distribution of locations where Prosopis sp. samples were collected. 1) Wild population of P. laevigata. Dolores Hidalgo, Guanajuato. 2) Wild population of P. laevigata. Santiago de Anaga, Hidalgo. 3) P. laevigata. Botanical Garden UNAM, Mexico City 4) P. glandulosa. Ciudad Juárez, Chihuahua. 5) P. juliflora. Culiacán, Sinaloa.

appropriately adjusted. All DNA samples were diluted to a working concentration of 20 ng/µL with TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0). Three bulk samples were prepared by pooling equal amounts of purified genomic DNA from *P. laevigata*, *P. glandulosa* and *P. juliflora* and aliquots from these combined samples were used for PCR amplifications.

Genomic DNA concentration was quantified spectrophotometrically and the purity condition of samples was calculated from the 260/280 absorbance ratio. DNA stock samples were diluted into a final concentration of 20 ng/µL with the TE buffer. The quality of the DNA samples was evaluated by agarose gel (0.8% w/v) electrophoresis prior to the RAPD analysis.

DNA amplification conditions and gel electrophoresis

PCR amplifications were performed in reaction mixtures of 25 µL containing 2.5 µL of 10x Taq DNA polymerase reaction buffer (10 mM Tris pH 8, 50 mM KCl), 2 to 3 mM $MgCl_2$, 200 µM of each dNTP, 1.5 units Taq DNA polymerase (Gibco BRL), 20 pmol primer (Table 1), and 20 to 60 ng genomic DNA. The reaction mixtures were covered with 20 µL of mineral oil to prevent evaporation. Amplification was performed using a MJ Research thermocycler, Model PTC 100 using an initial denaturation cycle (94°C, 1 min.) followed by 38 cycles of a 94°C denaturing step (30 s each for cycles 1 to 3; 15 s each for cycles 4-38), a 35°C annealing step (30 s) and a 72°C elongation step (90 s). Amplification reactions were performed at least twice each and products were separated by electrophoresis in 1.2% w/v agarose gels with 1x TAE buffer and were then stained with ethidium bromide and UV photographed using a Gel Doc 1000 video gel

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Table 1. Nucleotide sequences of primers used here

Number	Primer name	Sequence (5'- 3')
1	GIBCO 1	GGT GCG GGA A
2	GIBCO 2	GTT TCG CTC C
3	GIBCO 3	GTA GAC CCG T
4	GIBCO 4	AAG AGC CCG T
5	GIBCO 5	AAC GCG CAA C
6	GIBCO 6	CCC GTC AGC A
7	ROTH-H 01	GGT C GG AGA A
8	ROTH-H 02	TCG GAC GTG A
9	ROTH-H 03	AGA CGT CCA C
10	ROTH-H 04	GGA AGT CGC C
11	ROTH-H 05	AGT CGT CCC C
12	ROTH-H 06	ACG CAT CGC A
13	ROTH-H 07	CTG CAT CGT G
14	ROTH-H 12	ACG CGC ATG T
15	ROTH-H 13	GAC GCC ACA C
16	ROTH-G 02	GGC ACT GAG G
17	ROTH-G 04	AGC GTG TCT G
18	ROTH-G 06	GTG CCT AAC C
19	ROTH-G 08	TCA CGT CCA C
20	ROTH-G 09	CTG ACG TCA C
21	ROTH-G 15	ACT GGG ACT C
22	ROTH-G 18	GAA TCG GCC A

documentation System (Bio-Rad). The molecular weights of the resulting amplification products were determined by comparing to a 100 bp ladder standard (Gibco BRL).

Cluster analysis

Fragment sizes of the RAPD markers were calculated from a standard curve based on the known size of DNA fragments of the 100 bp ladder (Gibco BRL). Each fragment was scored as an independent locus with two alleles. The presence (1) or absence (0) of data from the RAPD samples were used to calculate genetic similarities using Jaccard's similarity coefficient [27] calculated according to the expression:

$$C_{jk} = \frac{a}{a + b + c} \qquad 0.0 \leq C_{jk} \leq 1.0$$

Where C_{jk} is a similarity coefficient that measures the resemblance between individual *j* and *k*, *a* represents the number of times a band is present in individuals *j* and *k* (1.1), b represents the number of times a band is present in individual *j* and absent in individual *k* (1.0), and c represents the number of times a band is absent in individual *j* and present in individual *k* (0.1). The corresponding similarity matrix was submitled to a cluster analysis using the unweighted pair-group method with arithmetic averages (UPGMA), and dendrograms were generated using NTSYS-PC version 1.8 software [28].

Results

Intraspecific relations in two natural populations of *P. laevigata*

Twenty-two randomly selected primers were initially tested using four DNA samples randomly selected from population 1 in the search for primers generating highly reproducible RAPD polymorphic fragments that could be unmistakably scored. The sequences of these primers are specified in Table 1. Primers 7, 8, 9, 17 and 18 met these requirements and were used in the analysis. The RAPD analysis of 120 DNA samples (60 from each population) amplified with the five primers permitted the evaluation of genetic associations among individuals within each population, as well as the number and proportion of each genotype within and between the two populations.

Amplified products from each primer were found to contain 6 to 12 fragments. With the five primers, a total of 27 fragments ranging from 450 to 2250 bp were detected and used for cluster analysis. Four fragments (15%) were polymorphic.

Based on the calculated Jaccard similarity coefficients, a dendrogram of both populations was drawn using UPGMA cluster analysis. The dendrogram differentiated two groups (*A* and *B*), by similarity coefficients of 0.938. The two populations were similarly rich in genotypes. Five subgroups (*a* to *e*) were identified in group *A*, whereas six (*f* to *k*) were identified in group *B* (Figure 2), and they differed in five of the 11 identified genotypes (*b*, *e*, *g*, *i* and *k*). They also differed in their relative abundance (number of individuals per subgroup). Group A was composed of 39 individuals (65%) from population 1 and 24 individuals (39%) from population 2. Group *B* was composed of 21 individuals (35%) from population 1 and 36 individuals (60%) from population 2. Subgroups *a*, *c*, *d*, *f*, *h* and *j* were common in both populations (individuals of each subgroup shared the same polymorphic pattern with the five primers tested) (Table 2). Subgroups *a* and *f* had the largest number of individuals (Figure 2). Subgroup *a* had 28 (47%) and 14 (23%) individuals from population 1 and 2, respectively (Table 2), while subgroup *f* had 16 (27%) and 21 individuals (35%) from population 1 and 2, respectively.

Most of the genotypes found in population 1 were also found in population 2. Hence, genotypes *e*,*g* and *i* of population 2 were not present in population 1, whereas genotypes *b* and *k* from population 1 were absent in population 2 (Table 2). Six out of 11 RAPD genotypes were common to both populations, which accounts for 92.5% of the individuals sampled in the two populations (Figure 2).

The RAPD patterns obtained using primers 7, 8, 9, 17, and 18 on the sample of *P. laevigata* from the Botanical Garden matched the genotype of subgroup *a*, the largest subgroup in population 1 and the second most abundant phenotype (23%) in population 2 (Figure 3).

Table 2. Comparison of subgroups established from the two populations of *P. laevigata* and the number of individuals in each one of the subgroups

Group	Subgroup	Number of individuals (%)	
		Population 1	Population 2
A	a	28 (47)	14(23)
	b	2 (3)	-
	c	3 (5)	3 (5)
	d	6 (10)	5 (8)
	e	-	2 (3)
B	f	16 (27)	21 (35)
	g	-	2 (3)
	h	2 (3)	10 (17)
	i	-	2 (3)
	j	2 (3)	1(2)
	k	1 (2)	-

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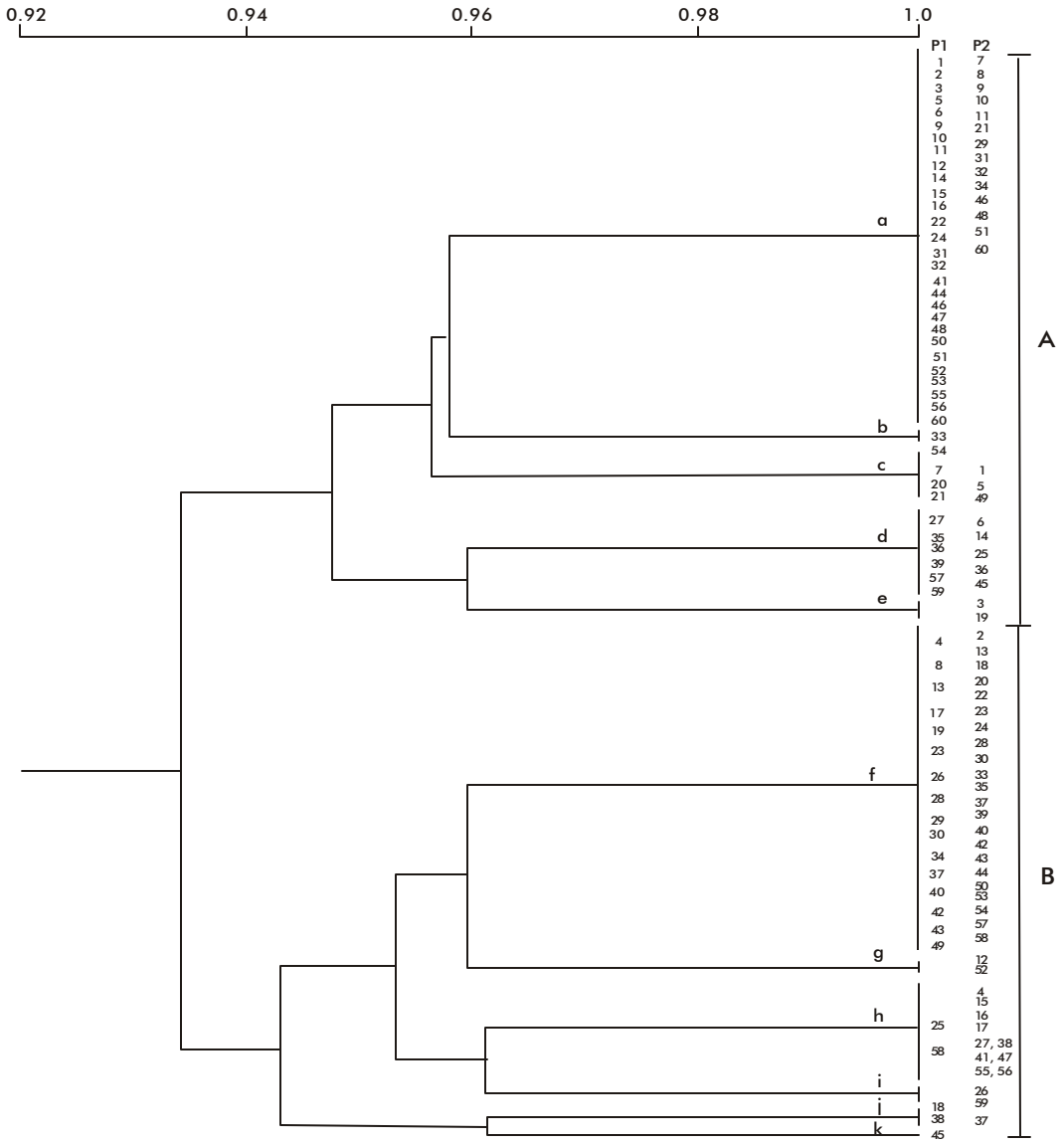

Figure 2. Dendrogram based on polymorphisms found in 60 *P. laevigata* trees (numbers) of the population from Dolores Hidalgo Guanajuato (Population 1) and 60 *P. laevigata* trees of the population Santiago de Anaga Hidalgo (Population 2), generate by Jaccard's similarity coefficient and UPGMA cluster analysis. Capital letters indicate groups, and small letters, subgroups. Column P1 indicates the individuals of population 1 and P2 the individuals of population 2.

Interspecific relationships

In order to determine interspecific similarity coefficients, pooled DNA from 15 individuals each (14 samples from the Herbarium (MEXU) and a sample taken at the Botanical Garden of the National Autonomous University of Mexico), *P. laevigata P. glandulosa* and *P. juliflora* were prepared. Although the previous experiment had indicated that some of these 22 primers were not informative in *P. laevigata*, it was expected that additional polymorphisms might be seen in the two other species. In this analysis five primers (10, 11, 12, 13 and 21) were excluded since they did not generate highly reproducible and unmistakable fragments. With the 17 primers selected, 57 fragments were clearly identified and used in the production of a dendrogram (Figure 4). Similarity coefficients were found to be far lower than those determined in the previous experiments, which is expected when comparing different species. *P. laevigata* and *P. glandulosa* were found to be related, with a similarity coefficient of 0.79 and they were both related to *P. juliflora* with a similarity coefficient of 0.63 (Figure 5). Polymorphic fragments were obtained with primers 1, 2, 3, 4, 5, 7, 8, 14, 17, 18, and 20. Of these, polymorphic fragments 2, 3, and 7 were specific for *P. laevigata*, *P. glandulosa* and P. *juliflora* respectively (Table 3).

Discussion

Genotype diversity and the relative amount or proportion of individuals per genotype present in the two natural populations of *P. laevigata* were analyzed

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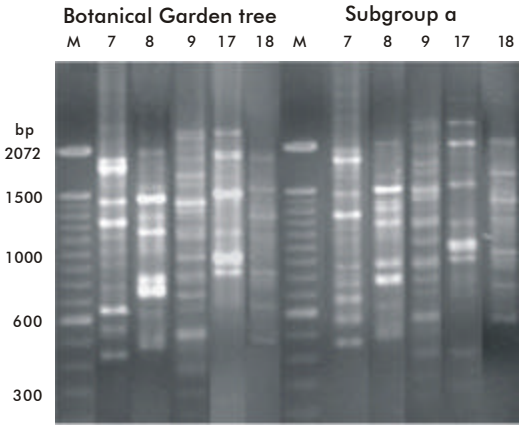

Figure 3. RAPD polymorphic patterns obtained with the primers indicated (numbers) which shared the reference tree *P. laevigata* and the subgroup *a*.

by RAPD markers. All RAPD markers from both populations were used to calculate Jaccard's similarity coefficient and to build a dendrogram using the UPGMA cluster analysis. A high intraspecific similarity coefficient was found between groups *A* and *B* (0.938) which is consistent with genotype similarities within the populations.

When populations 1 and 2 were compared, it was found that both populations were similarly rich in genotypes. Genotype diversity is determined not only by the number of different genotypes within a specific population (richness of genotypes), but also by the relative amount or proportion of individuals per genotype. Of the 11 genotypes identified in both populations, 6 were shared. The genotype predominantly found in population 1 (47%) was the second most frequent one in population 2 (23%). In population 2 the most frequent genotype (35%) occurred in 27% of population 1, making it the second most common genotype in population 1. This may suggest that both populations had a common origin or had once formed a continuous population, an idea that is supported by the fact that the two *Prosopis* populations have many common alleles. There is a well-accepted hypothesis of Rzedowski [1] who examined the distribution of *P. laevigata* in three geographically separate areas (plateau, Balsas depression and coastal plain) and proposed that the parent plants of this species probably grew for ages in areas in which mountainous systems were not a barrier for dispersion. These geographic regions are capable of harboring genotypes with similar life strategies and adaptations; however, these two populations that became isolated by geographic barriers tended to diverge in their genotypes as well. Our results agree and offer additional details to support this hypothesis; new efforts are made to determine the genetic diversity of other populations of *P. laevigata* more accurately. The identification of the real extent of genetic diversity is important to ensure the preservation of *P. laevigata* since it is a valuable multipurpose biotic resource and may be potentially used for the reforestation of eroded land. Genotypic diversity and the proportions of individuals per genotype respond differently to different environmental conditions. Small populations, as may be the case of our populations, or regions which do not have a wide variety of habitats are usually phenotypically-poor. Thus, gene frequencies are altered and even, non-adaptive traits may become established, which may be eventually harmful for the few phenotypes that are able to grow in the region which are consequently reduced. Genetic drift is often observed among reproductively isolated populations. A situation that overcomes these drawbacks is that in the last 200 years these species have become widely spread and in some countries the introduced *Prosopis* plants have escaped cultivation becoming invasive weeds [29].

The population structure we see today may have been influenced by the selective use by both men and

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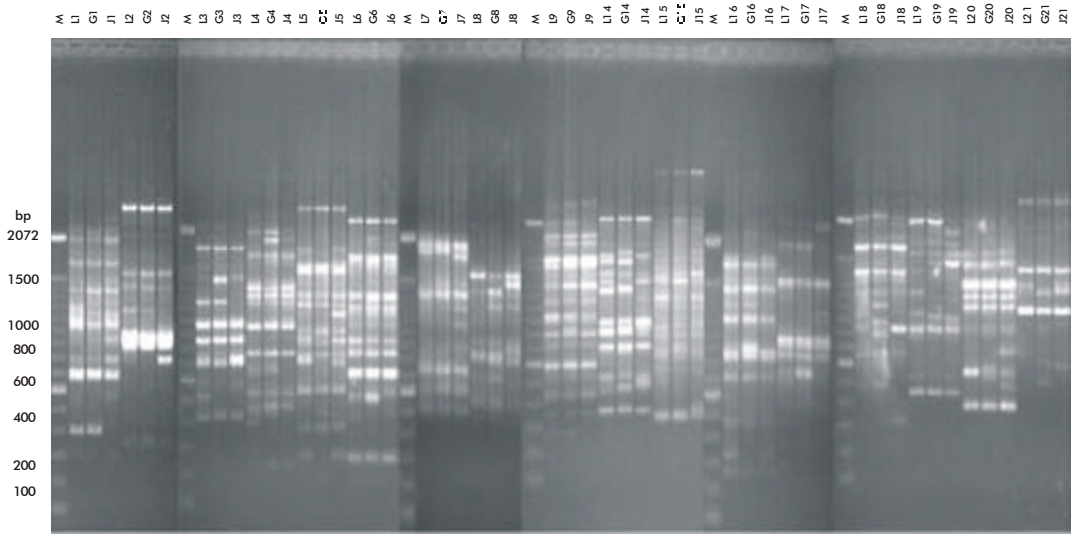

Figure 4. Polymorphism detected with 6 primers Gibco and 11 primers Roth in *P. laevigata* (L), *P. glandulosa* (G) and *P. juliflora* (J). Numbers indicate the primer used and M the molecular weight marker.

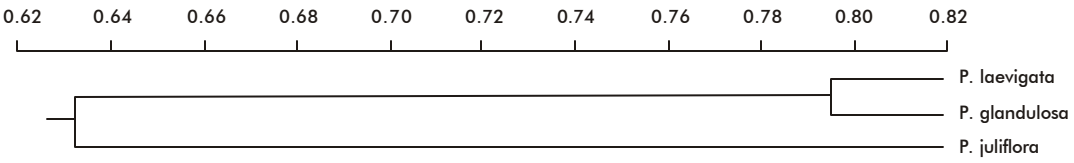

Figure 5. Dendrogram mode with data of the species of *Prosopis* indicated, based on the UPGMA cluster analysis and Jaccard's similarity coefficient.

animals of trees that express certain desirable phenotypes. The fast degradation of local environments may have favored the propagation of selected hardy phenotypes, and the reproductive biology of these species might have played a role in this, since in protogynous plant flowers, stigmas become receptive before male anthesis occurs. This may help reduce selfing and promote outcrossing [4]. Moreover, a genetic flow (n_{em} = 3.2) has been detected between these two populations, which are currently 200 km apart [15]. Considerations on a phylogenetic analysis performed elsewhere [15] to estimate the genetic diversity among populations of *P. glandulosa*, *P. laevigata* (two populations), and *P. juliflora* showed that *P. glandulosa* was grouped with a highly heterogeneous distribution, the populations of *P. laevigata* were found to be one monophyletic clade and *P. juliflora* was separated from the others. According to this distribution, the clade of *P. juliflora* may have given rise to the other two species.

Here, and based on similarity coefficients, a reduced range between the species *P. laevigata, P. glandulosa* and *P. juliflora* was found (0.63-0.79). These results can perhaps be attributed to the high hybridization capacity shown to occur in the species of the section Algarobia [30] due to the absence of reproductive isolation, phenology, habitat, geographic barriers [4], and shared distribution areas. In these cases, individuals display intermediate phenotypes, and a tendency toward the homogenization of allele frequency and the consequent loss of a clear species identity [31, 32].

Saidman and Vilardi [8] used isozyme analysis to obtain a similarity coefficient of 0.96 within populations of *P. ruscifolia*, while the comparison of *P. ferox*, *P. torquata*, *P. pubescens*, *P. reptans* and *P. strombulifera*, showed interspecific similarity coefficients of about 0.49, clearly demonstrating the difference of the between- and within species similarity coefficients.

Intraspecific similarity coefficients using four arbitrary primers and populations of *P. glandulosa* were determined [33]. The number of RAPD bands varied from 2 to 10 and genetic variability within populations was high (0.06 to 0.23) while the genetic distance between the species *P. glandulosa* and *P. velutina* were very low and within a narrow range (0.19 to 0.25). These results clearly demonstrated the difference between inter and intraspecific similarity coefficients.

Recently RAPD analysis has permitted the differentiation of species and varieties in the *Prosopis* genus [24, 34]. Ramírez *et al.* [12] using RAPD analyzed the genetic variability of 15 species of *Prosopis* from the New World (Argentina and Chile) and the Old World (Asia and Africa). They found clear differences within the genus, permitting their grouping within a range of similarity coefficients of between 0.05 and 0.8. The interspecific similarities of the Old World species had the lowest values (least similarity) compared to the species of the New World, which in the section Algarobia were similar to 0.68 (*P. alba* and *P. chilensis*) and to 0.59 (*P. nigra* and *P. flexuosa*). These values are in the range of those calculated in this paper for the interspecific relations among the species of *P. laevigata*, *P. glandulosa* and *P. juliflora*, which are members of the section Algarobia [4], indicating that RAPD is a quick, viable technique for defining the relatedness among these plants. Therefore, we present evidence on inter- and intraspecies relatedness within the *Prosopis* genus for a better understanding of the genetic health of populations that form a necessary Mexican natural resource.

For the sustainable and economically viable exploitation of *Prosopis*, it is necessary to: 1. identify and classify the naturally occurring species, phenotypes and genotypes; 2. estimate their proportions, and 3. identify the factors that help maintain genetic diversity. We report here the use of a relatively simple methodology and means to fulfill several of the above requirements. Hopefully, it will be possible to progress into developing innovative sustainable approaches for reforesting vast areas of degraded, desertified arid lands with selected indigenous and exotic phenotypes.

In the future, it may be possible to respect our forest heritage while still meeting the needs of native populations that depend on these forests. In several parts of the world, species of *Prosopis* have been introduced to reforest vast areas [35, 36], with doubtful success. It is possible that a better understanding of the genetic diversity within and between species of

Table 3. Specific random amplified polymorphic DNA (RAPD) marker identified in each *Prosopis* species

Species	Number of bands	Size (bp)	Primer
P. laevigata	2	510	3
		390	4
P. glandulosa	3	1020	18
		910	18
		790	18
P. juliflora	7	1850	7
		1430	8
		780	2
		690	20
		590	1
		490	20
		310	18

30. Galindo-Almanza S, García-Moya E, Wendd TL, González-Cossio FV. Potencial de hibridación natural en mesquite (*Prosopis laevigata* y *P. glandulosa* var. Torreyana, Leguminosae) de la altiplanicie de San Luís Potosí. Acta Bot Mex 1992;20:101-17.

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Prosopis will allow for a better choice of varieties for reforestry. We present here a method for the analysis of this genetic diversity, and its application to inter- and intraspecific examination.

This is a preliminary paper and as a conclusion we consider that more efforts are required to identify the genetic diversity in these populations using a considerably larger number of primers to detect more RAPD markers and consider a larger number of populations and geographical areas, to fully determine, the extent of the biodiversity of these populations.

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