Genetic variability of wild Agave angustifolia populations based on AFLP: A basic study for conservation

F. Sánchez-Teyer c, 1, S. Moreno-Salazar a, b, 1, M. Esqueda a, A. Barraza c, M.L. Robert c, * a

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ABSTRACT
Agave angustifolia is taken from wild populations in the desert of Sonora, Mexico, to make the alcoholic drink known as “bacanora”. An increase in the demand for this beverage has led to overexploitation of these agaves. We used AFLP to measure the genetic variability within and between natural populations of the species. The fixation index (FST) and population genetic distances (GD) were determined for three populations of A. angustifolia. At the clonal level, the expected heterozygosis (HE), the proportion of polymorphic loci (P), and the genetic similarity indices (GSIs) were lower in one population. Total GSI for adults and offshoots was 0.924. There were no differences in the GSI within or between the populations and dendrogram showed three groups that partially reflect the geographical distribution of the populations. Some degree of genetic variation between mother plants and their vegetatively produced rhizomes was observed. At the species level, total expected heterozygosity was high (HT = 0.514) with a P value of 78%, and intermediate gene flow (Nm = 1.18), giving rise to a moderate fixation index (FST = 0.175). Nm, FST and GD values observed, indicate that urgent measures must be implemented to preserve the genetic diversity.

1. Introduction
The genus Agave contains more than 200 species, 90% of which are present in Mexico and 17% in the Sonora desert. Species of this genus show both sexual and asexual reproduction, and genetic variation has been observed in asexual propagation by rhizomes in Agave fourcroydes (Reyes et al., unpublished data) and Agave tequilana (Gil-Vega et al., 2006).

Agave angustifolia is the most economically important Agave in the state of Sonora, Mexico, where it has been used to make “bacanora”, a traditional centuries-old drink, similar to tequila and mezcal, but with a distinctive flavor. Its industrialization, however, has not enjoyed the same development as the other spirits, because the wild populations of A. angustifolia are being overexploited, resulting in a reduction in the size of the populations and a significant genetic erosion of the species. Furthermore, immature agaves are being used: the producers cut the inflorescences as soon as they start to develop, preventing the plant from using up stored sugars but also eliminating sexual reproduction. To make things worse, the uncontrolled clearing of natural vegetation to create pastures for cattle has caused the fragmentation of the habitat where this species grows, reducing the size and increasing the spatial and reproductive isolation of its populations.

Biochemical and molecular techniques for the identification of genetic markers of single genes, mobile elements, or repetitive regions of non-coding DNA have made it possible to estimate levels of genetic diversity, within and between populations, of many species, as the “molecular profile” or “genetic fingerprint” is characteristic of each variety or group of genetically linked individuals, regardless of the environmental conditions (Lombard et al., 2000; Schulman, 2006).

Various studies on the level of genetic variability of the agaves have used different molecular markers, including: isozymes in A. fourcroydes (Colunga-Garcíamarín et al., 1999), Agave victoriae-reginae (Martínez-Palacios et al., 1999) and Agave lechuguilla (Silva-Montellano and Eguiarte, 2003); microsatellites (SSR) in Agave

* Corresponding author. Tel.: +52 999 981 3914; fax: +52 999 981 3900.
E-mail address: robert@cyix.mx (M.L. Robert).
1 These two authors contributed equally to this paper.

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cupreata and Agave potatorum (Eguiarte et al., 2003); Random Amplification of Polymorphic DNA (RAPD) in A. tequilana (Gil-Vega et al., 2001, 2006) and in the Agave deserti complex (Navarro-Quezada et al., 2003); and Amplified Fragment Length Polymorphism (AFLP) in A. fourcroydes (González et al., 2003; Demey et al., 2004) and A. angustifolia (Barraza-Morales et al., 2006).

In a previous study, we used AFLP with five primer combinations to analyze three distant wild populations of A. angustifolia from the Sonora desert in Mexico (Barraza-Morales et al., 2006). The dendrograms obtained showed a clear grouping among the individuals of each region without overlapping between regions. Since the population in Nácori Chico had a larger variability (HE = 0.295) than those of Moctezuma and Bacadéhuachi (HE = 0.253, HE = 0.249, respectively), we decided to analyze this region in greater depth in order to define the limits of variation between clones and establish the correlation between physical and genetic distances among these agaves.

The objective of this work was to analyze, by means of AFLP, the genetic variability within and between geographically close populations of A. angustifolia in the Nácori Chico region.

2. Material and methods

2.1. Plant material

Specimens were collected from three wild populations of A. angustifolia: El Bajo (site 1) [29° 47′ 28″ N, 109° 00′ 28″ W], Los Mochomos (site 2) [29° 55′ 58″ N, 108° 60′ 04″ W] and El Chorro (site 3) [29° 42′ 45″ N, 109° 08′ 21″ W], all located within the previously studied Nácori Chico population in the state of Sonora. The random quadrant method was used with three 50 × 100 m (5000 m²) quadrants set up at each site. Within these areas, the plants were counted to estimate the size of the population. A total of 40, 40 and 56 individuals were collected from El Bajo, Los Mochomos and El Chorro, respectively. Of these, 10, 10 and 14 individuals, respectively, were mother plants (genets) and three were offshoots from each mother plant (ramets), thus satisfying the requirement of 30 individuals per population for the reliable estimation of allele frequency established by Nei (1987) and representing more than 10% of the total number of individuals per population.

2.2. AFLP analysis

Genomic DNA was extracted according to Keb-Llanes et al. (2002). The AFLP analysis was carried out following the original protocol described by Vos et al. (1995) with slight modifications to reduce the volume of the reaction to 10 μl. Briefly, DNA (250 ng) was digested with MseI (frequent cut) and EcoRI (rare cut) restriction enzymes and specific adapters were ligated using T4 DNA Ligase (Invitrogen®). The ligated fragments were used as templates for preamplification with primers having a single selective nucleotide MseI-C and EcoRI-A. For selective amplification, three combinations of primers were used based on the level of polymorphism observed in A. angustifolia (Barraza-Morales et al., 2006): EcoRI-ACA/MseI-CAT, EcoRI-AAC/MseI-CAC and EcoRI-ACG/MseI-CTT. The EcoRI primers were radioactively end-labeled at 5′ with [32P]-γ-ATP, using T4 polynucleotide kinase (Invitrogen®). The PCRs were carried out as described by Vos et al. (1995). At the end of the reaction, the fragments were denatured with a volume of stop solution containing 95% formamide.

The amplified fragments were separated by electrophoresis on denaturing polyacrylamide gels (6%), at 1870 V, 40 mA and 70 W over 2 h. The gels were transferred to paper and vacuum dried at 80 °C for 2 h before exposing them to X-ray film (Kodak®) for at least 48 h. The fragments were visualized by autoradiography. The analyzed fragments ranged in size from 100 to 600 bp, an estimate based on the 100 bp Ladder DNA marker (GIBCO BRL).

Reproducibility of AFLP was evaluated by comparing AFLP profiles of a duplicated sample (treated as a new sample) in the same reaction using two primer combinations. The patterns of bands were 98% identical among duplicated samples in both primer combinations, suggesting high reproducibility. The duplicated sample was not included in the analysis.

2.3. Data analysis

A binary matrix (1 = band present, 0 = band absent) was prepared based on the pattern of AFLP bands. A polymorphic band was defined as one that was absent from at least one individual. Genetic similarity index (GSI) was determined according to Nei and Li (1979). The resulting dendrogram was obtained with the Unweighted Pair Group Method with Arithmetic Mean (UPGMA), using the SAHN (Sequential Agglomerative Hierarchical Cluster Analysis) statistical module of NTSYS (Numerical Taxonomy and Multivariate Analysis System) software version 2.02.

Population parameters such as expected heterozygosity (HE), proportion of polymorphic loci (P) and fixation index (FST) were calculated using Tools for Population Genetics Analyses (TPPGA) reported by Miller (1997). The genetic variation within and between populations was evaluated by applying the correction reported by Lynch and Milligan (1994) for dominant markers (such as AFLP), with the assumption that the populations are in Hardy–Weinberg equilibrium. Clones were considered subpopulations in the analysis.

The proportion of polymorphic loci was obtained as follows: \[ P = x/m \] (where \( x \) is the number of polymorphic loci in a sample of \( m \) loci). The hierarchical structure of the populations was estimated using Wright’s fixation or endogamy index (Wright, 1965). Gene flow was measured with the parameter \( N_m \) (number of migrants), using the formula: \( N_m = N_m [1 - (1 - FST)]/FST \), and genetic distance (GD) was based on the Nei’s (1972) identity parameter. The phenogram of genetic distances was obtained with UPGMA and bootstrapping of 1000 repetitions.

3. Results

3.1. Plant material

In total, 136 individuals were collected from three sites of Nácori Chico, out of which 34 individuals were mother plants and the rest were offshoots, three from each mother plant, that were all collected less than 1 m away from the mother. The collecting sites are shown in Fig. 1. It is important to stress that, although the distance between El Chorro and Los Mochomos is only 0.8 km, the latter is located in a gorge surrounded by hills that act as a geographical barrier.

3.2. Genetic polymorphism

In a previous work (Barraza-Morales et al., 2006), we evaluated three wild populations of A. angustifolia from the Mountains of Sonora using five primer combinations of AFLP, out of which only the three that produced most polymorphism were selected for this work. The selective amplification using three primer combinations produced a total of 155 bands, out of which 114 (73.5%) were polymorphic. The combination that produced the largest number of bands (71) was EcoRI-AAC/MseI-CAC, of which 72.1% were polymorphic. The combination that detected the most polymorphic bands (44) was EcoRI-ACG/MseI-CTT, out of a total of 53. The
combination EcoRI-ACA/MseI-CAT produced only 41 bands, 26 of which were polymorphic.

The genetic similarity index (GSI), including all adult plants and their rhizome offshoots, was 0.828. Narrow GSI average values were observed within (intra) populations (0.853), or between (inter) populations (0.834). The analysis between mother plants and their clonal offshoots from El Bajio, Los Mochomos and El Chorro gave GSI average values of 0.905, 0.938 and 0.930, respectively.

The dendrogram generated from GSI (Fig. 2) showed three major groups. Group A includes all plants from El Bajio plus the mother plants and their rhizomes of individuals 11 (except 11C) and 12. Group B includes the rest of the individuals from Los Mochomos plus 22 and 23 (and their rhizomes) collected at El Chorro. Group C contains the rest of the individuals from El Chorro. Group C diverges from Groups A and B at 0.80 and the latter two separate from each other at 0.81.

It is important to stress that, although most plants and their offshoots are grouped very closely together, only in a few cases individuals appeared widely separated from their group. This is the case of plant 11 and its rhizomes (11a and 11b) which were located at Los Mochomos within group A, while 11c is located within group C.

3.3. Genetic structure and variability

The value of expected heterozygosis (\(H_e\)) and the proportion of polymorphic loci were slightly lower in the population from El Bajio (0.250 and 64.5%) than in Los Mochomos (0.264 and 67.7%) and El Chorro (0.263 and 66.2%). At the species level, the index of heterozygosity was 0.314 with 78% of polymorphic loci. A low value for the genetic structure among populations (0.175) and a relatively moderate genetic flux of 1.18 were observed (Table 1).

3.4. Genetic distances and phenograms

The grouping observed from the genetic distances calculated between pairs of populations, considering all samples of each site as a single population (Fig. 3), showed that El Bajio and Los Mochomos are more closely related to each other than to El Chorro.

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**Fig. 1.** Distribution and distances from sample sites. A and C show distances between different individuals in El Bajio (individuals 1–10), Los Mochomos (individuals 11–20) and El Chorro (individuals 21–34), respectively. B shows distances between El Bajio and El Chorro and Los Mochomos regions. Distance between plus symbols represents 100 m.
4. Discussion and conclusions

4.1. Similarity index

In a previous work (Barraza-Morales et al., 2006), we used AFLP to evaluate the level of genetic variation among three separated wild populations of *A. angustifolia* in the mountains of Sonora, Mexico. We found that one of them (Nácori Chico) had a much larger variability (HE = 0.295, P = 68.53) than the other two populations (Moctezuma HE = 0.25, P = 65.4 and Bacadéhuachi HE = 0.24, P = 61.57). The similarity indices varied from 0.80 to 0.82 within the populations and 0.749 to 0.786 between them. We
therefore decided to analyze the Nácori Chico region in more detail in order to define the limits of variation between clones and to establish the correlation between physical and genetic distances among these agaves.

Using AFLP markers we analyzed the level of genetic variation in three locations: El Bajío, El Chorro and Los Mochomos, within the Nácori Chico region. The results show that the total intra- and interpopulation GSI values for *A. angustifolia* analyzed in this study were higher than those reported for Moctezuma, Bacadéhuachi and Nácori Chico populations, whose similarity ranged from 0.749 to 0.786 among populations and from 0.800 to 0.827 within populations (Barraza-Morales et al., 2006). This can be explained by the greater geographic separation between the populations evaluated by Barraza-Morales et al. (2006): 70 km between Moctezuma and Bacadéhuachi, 30 km from Bacadéhuachi to Nácori Chico and 90 km from Moctezuma to Nácori Chico; whereas the distances between the populations of the present study, all within the Nácori Chico region, fluctuated between 2 and 9.5 km with some geographical barriers (Los Mochomos is located in a gorge surrounded by hills). It is important to point out that the sampling of the wild populations of Nácori Chico used in this study includes a representative number of all the individuals currently in the region.

The groups formed from the GSI values (Fig. 2) do not match completely with the geographic distances that separate each site (Fig. 1). Group A comprises all individuals of El Bajío but includes two mother plants and their rhizomes from Los Mochomos (the distance from *El Bajio* to *Los Mochomos* and to *El Chorro* was 9.06 and 9.62 km, respectively). Group B includes all other individuals from Los Mochomos and two mother plants and their rhizomes from *El Chorro*, which is only 2 km away. Group C incorporates the remaining individuals from *El Chorro* and a single rhizome from El Bajío.

The grouping observed in group B, as well as the values of the fixation index and the genetic flux among the populations analyzed in this work, are probably due to some mother plants having originated by cross-pollination (by pollinators like bats or bees), or seed dispersion between the closer populations as reported for other *Agave* species (Eguiarte et al., 2003; Silva-Montellano and Eguiarte, 2003). Our observations, however, only revealed floral structures in less than 4% of the plants observed. Despite this assumption, we have observed a similar range of genetic variation among rhizomes and plants derived from seed in species like *A. tequilana* and *A. fourcroydes* (unpublished data).

Our results also show that there is some degree of genetic variation between the mother plant and their rhizomes in wild populations, in spite of the fact that they are clones produced by asexual propagation.

Vegetative propagation through rhizomes, that maintain the new individuals joined to the mother plant, guarantees the nourishment and survival of the offspring under the very stressful conditions of the desert environment. Although some agaves can set seed no young seedlings are normally observed in the wild. Plants with predominantly asexual reproduction (like *Agave* species), where recombination does not operate, must have some ways to generate variation. Genetic variation in clonal plants has been reported previously for *Saxifraga* (Gabrielsen and Brochmann, 1998), *Polygonum viviparum* (Diggle et al., 1998), *A. fourcroydes* (González et al., 2003) and *A. tequilana* (Gil-Vega et al., 2006). This variation could be explained, at least partially, by the presence of several repetitive elements including LTR-retrotransposons, as reported recently by Bousios et al. (2007), who isolated three families of LTR-retrotransposons for *A. tequilana*, showing variation in copy numbers across the genome. The authors reported active and putative ancestral elements present in *A. tequilana*. Such active elements could be responsible for part of the observed genetic variation, by means of segmental duplication that contributes to rapid genomic reshuffling (Ma and Jackson, 2006).

Endophytic organisms have been observed in several plant species (Zinniel et al., 2002; Arnold et al., 2003; Lata et al., 2006), therefore we cannot rule out the possibility that the presence of endophytic organisms, or asymptomatic diseases (at the time of sampling) caused by fungi and bacteria, in wild *A. angustifolia* is responsible for part of the variation observed in some individuals (i.e. mother plant 11 and its rhizomes) that are completely out of place in the dendrogram.

It has been suggested that clonal propagation as a reproductive strategy permits ramet populations to induce changes in the genet, by increasing the proportion of ramets with more advantageous specific traits (Pan and Price, 2001).

The GSI values for *A. angustifolia* plants studied were lower than those recorded for *A. fourcroydes* (González et al., 2003) and *A. tequilana* var. Azúl (Gil-Vega et al., 2006), where decades of vegetative propagation, forced by the growers as a result of the exclusive propagation through rhizomes have likely contributed to a decrease in the genetic variation of cultivated agaves.

### 4.2 Genetic variability and fixation

The lowest values of expected heterozygosity and polymorphic loci were observed at El Bajío and these values were similar to the ones reported by Barraza-Morales et al. (2006). Such values, however, were lower than those reported for wild populations of *A. victoriae-reginae* and *A. lechuguilla* (Martínez-Palacios et al., 1999;...
cytogenetics, morphological and genetic variability in Morales et al., 2006).

The fixation index in the Nácori Chico populations was greater than those reported for most other wild Agave populations (Martínez-Palacios et al., 1999; Silva-Montellano and Eguiarte, 2003; Eguiarte et al., 2003; Navarro-Quezada et al., 2003), suggesting that wild populations of A. angustifolia from Sonora Mountain Range have an intermediate range of genetic variation.

The results of this study support our previous observations on cytogenetics, morphological and genetic variability in A. angustifolia, all of which indicate that speciation is under way. Although genetic variation occurs via sexual and asexual processes, our results suggest that some of this variation is generated during asexual reproduction (formation of the offshoots). This strategy to induce new variability per generation is important for both genetic and adaptive plasticity of this species under extreme environmental conditions. A detailed analysis on reproductive ecology and pollinators is necessary for A. angustifolia. These studies are essential for clarifying the taxonomy and genetic development of Agave, for selecting biotypes for recovery and conservation programs in its natural distribution areas, and also for setting up zones for the sustainable use of this species.

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Silva-Montellano and Eguiarte, 2003), but greater than those reported for A. potatorum, A. cupreata, Agave cerulata, A. deserti and Agave subsimplex (Eguiarte et al., 2003; Navarro-Quezada et al., 2003), suggesting that wild populations of A. angustifolia from Sonora Mountain Range have an intermediate range of genetic variation.

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