American Journal of Agricultural and Biological Sciences 8 (1): 44-53, 2013
ISSN: 1557-4989
©2013 Science Publication
doi:10.3844/ajabssp.2013.44.53 Published Online 8 (1) 2013 (http://www.thescipub.com/ajabs.toc)

Variability and Genetic Structure in a Commercial Field of Tequila Plants, *Agave tequilana* Weber (Agavaceae)

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Received 2012-06-17, Revised 2013-01-18; Accepted 2013-02-22

ABSTRACT

Crops of the tequila plant (*Agave tequilana*) are produced mainly from offshoots of mother plants in established commercial fields. This propagation method is significant, as it is believed that it facilitates the spread of disease because of the crop's low genetic variability and is also necessary because it is regulate the use of just that variety in tequila industry. Different levels of genetic variability have been reported for *A. tequilana* and so we tested individuals from representative cultivation zones to determine the actual variability in fields and to assess the genetic structure of populations in commercial plantations. Four additional *Agave* spp. were used as a control group while *Fourcrea* spp. individuals were used as an external group. Morphological traits and molecular markers were analyzed. The differences between *A. tequilana* individuals collected from southern Jalisco state and those collected in the principal Denomination of Origin zone confirmed the existence of different genotypes, which were conserved in different regions by asexual propagation. Leaf length, plant height and number of leaves were the most significant variables that explained the variability within the *A. tequilana* group. At the molecular level, we found genetic differentiation with a minimum similarity of 0.253 (Jaccard's coefficient) and genetic structure analysis indicated five groups with significant genotypic differences. Genetic structure analysis, grouped accessions according to the dispersion of plant material from the initial sites of cultivation. These results might facilitate the correlation of different groups with crop yield or tequila quality and the establishment of elite lines for breeding programs. It is recommendable in a future, to determinate the different levels of inulines produced by each detected group.

Keywords: Molecular Markers, ISTR, Morphological Traits, Asexual Propagation

1. INTRODUCTION

Tequila is an alcoholic beverage that is known throughout the world. The Mexican tequila industry is important and complex because it supports the economy of a large region. In recent decades, there has been a revolution in the entire process used for producing tequila. First, the agave plant material must be exclusively *Agave tequilana* Weber var. azul. An official standard, the Norma Oficial Mexicana NOM-006SFI-1994, regulates the process and determines the places or regions where Denomination of Origin tequila can be produced. Second, the agronomic management of agave culture has changed greatly. Most tequila crops are cultured initially from agave offshoots produced by mother plants in commercial fields, which

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facilitates the spread of diseases (Esqueda *et al*., 2011) because asexual propagation leads to lower genetic variability (Cedeno and Alvarez-Jacobs, 2000; Colunga-Garcia and Zizumbo-Villareal, 2007). Indeed, low levels of genetic variability were reported 10 years ago (Gil-Vega *et al*., 2001). The producers and industry maintain a uniform agave phenotype via asexual reproduction using offshoots and bulbils. Agave producers also use seeds, but seeds and bulbils are used rarely in commercial plantations, although they might be used in breeding programs (Piven *et al*., 2001). Relatively low levels of genetic diversity have been found in many asexually produced populations, but it is important to note that not all asexual populations are genetically uniform. Thus, offshoots provide a certain level of agave uniformity, but different levels of genetic variability have been detected in *A. tequilana* (Gil-Vega *et al*., 2006; Torres-Moran *et al*., 2010) and several other *Agave* spp. using molecular tools (Magdub-Mendez, 2000; Demey *et al*., 2004; Infante *et al*., 2007; Sanchez-Teyer *et al*., 2009). Other studies have also reported variability in the morphological characteristics of the *Agave* genus. Colunga-Garcia and May-Pat (1997) demonstrated that there was a discontinuity in the morphological variation pattern of *A. fourcroydes* (henequen) in uniform conditions, according to Principal Components Analysis (PCA) and Analyses of Variance based on vegetative characteristics. Other morphologic characteristics and molecular markers have been used for the morphological and molecular characterization of *A. tequilana* and *A. angustifolia*, which are closely related species (Rodriguez-Garay *et al*., 2009; Vargas-Ponce *et al*., 2009). Different numbers of individuals were used to detect this variability, i.e., samples ranged from five to 42 individuals (Gil-Vega *et al*., 2001; Davila *et al*., 2007; Rodriguez-Garay *et al*., 2009; Vargas-Ponce *et al*., 2009). According to Nei (1978), an estimation of genetic distances in molecular data can be achieved with a sample of 10 individuals when the number of loci detected is ≥50. However, considerably more individuals need to be examined at each locus if the number of loci is small. In this study, we estimated the variability using 100 *A. tequilana* individuals in a crop field, which was established according to traditional production methods using offshoots acquired from different areas in the Denomination of Origin Zone (DOZ). Individuals were collected from representative cultivation zones and used to determine the variability that was actually present in the field and to assess the genetic structure of a commercial plantation population. We assessed morphological traits and Inverse Sequence Tagged Repeat (ISTR) molecular markers, which have been used previously to detect variability in the *Agave* genus (Infante *et al*., 2003; Torres-Moran *et al*., 2010). The identification of genetically different groups may facilitate the correlation of groups with yield and tequila quality, as well as the establishment of elite lines for breeding programs.

2. MATERIALS AND METHODS

2.1. Plant Material

One hundred *A. tequilana* Weber specimens were collected from 14 localities in the DOZ (**Fig. 1**) and established in commercial plantations. The samples were maintained at Centro Universitario de Ciencias Biológicas y Agropecuarias in Zapopan, Jalisco state, which is located within the DOZ. Measurements were made when individuals reached 3 years old. Ten individual specimens of *A. americana* L., *A. maximiliana* Baker and *A. salmiana* Otto ex. Salm Dyck and five individual specimens of *A. angustifolia* Haw were used as a control group (**Fig. 2**). Keys were used to describe the individual origins, as shown in **Table 1**. In the molecular marker analysis, a *Fourcrea* spp. group was also used to represent species from the Agavaceae family.

2.2. Morphological Markers

Morphological variables were measured for 100 *A. tequilana* specimens and the comparison species. The variables quantified were: Number of leaves (NH), plant height (AP), Diameter of cone (DT), Leaf length (LH), leaf width (AH) and Number of teeth per 10 cm (NE).

2.3. DNA Isolation and Molecular Markers Application

Leaf tissues were collected from the *A. tequilana* specimens and comparison groups. Total DNA was isolated from each individual using the CTAB procedure of Keb-Llanes *et al*. (2002). Retrotransposon fragments were amplified using the following primer pairs: FI/B6 (d-5'[GCA CTC CAC CAA GAA TAC C]3'/d-3' [GGT TTC ACT TTG TCC TTA G]5') and F91/31 (d-5'[ATA TGG ACT TAA GCA AGC CA]3'/d-3' [ATT CCC ATC TGC ACC AAT]5'). PCR amplifications were performed according to the method of Torres-Moran *et al*. (2010), with 25 ng of DNA in 20 μL reaction volumes containing 1 unit of Taq DNA polymerase (Promega®), 3 mM $MgCl_2$, 0.3 μM of each primer, 0.25 mM of each dNTP and the buffer supplied with the enzyme.

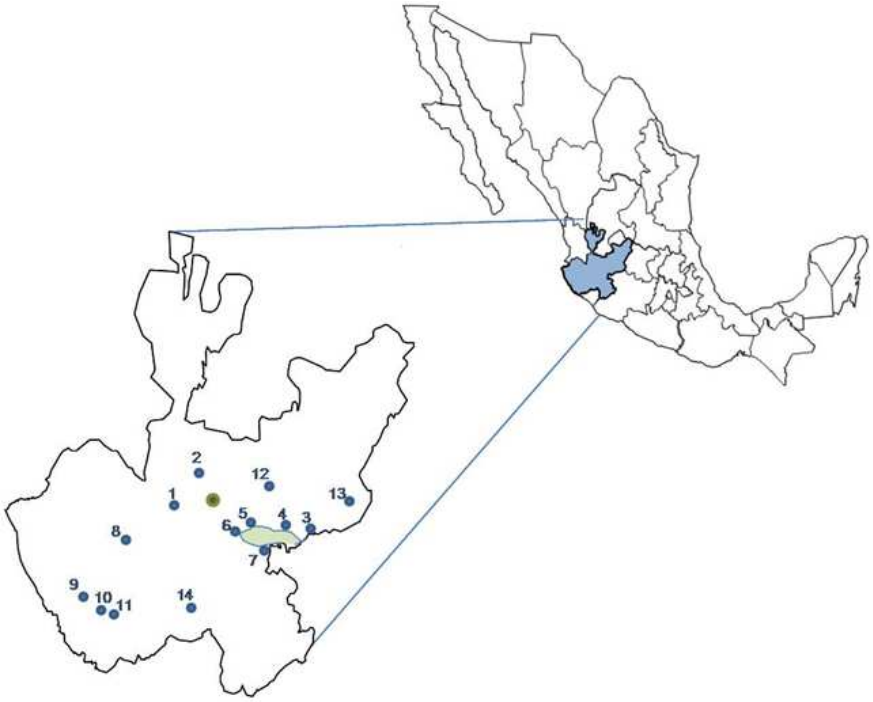

Fig. 1. *Agave tequilana* collection sites. 1: Ameca; 2: Arenal; 3: La Barca; 4: Ocotlán; 5: Chapala; 6: Jocotepec; 7: Tizapán el Alto; 8: Juchitlán; 9: Unión de Tula; 10: El Grullo; 11: El Limón; 12: Acatic; 13: Jesús María; 14: San Gabriel

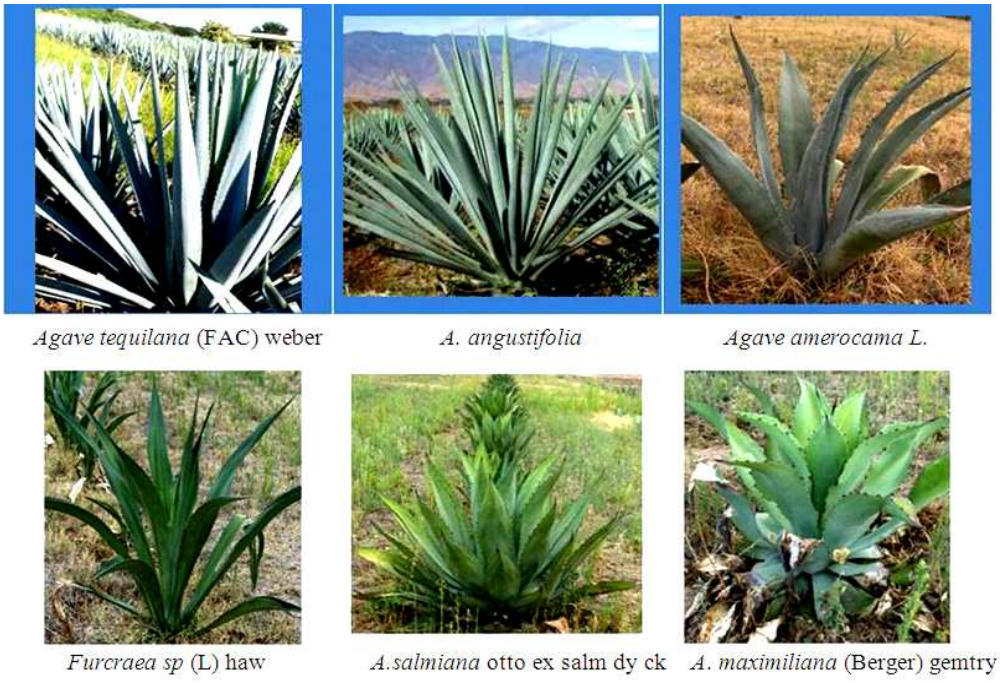

Fig. 2. Phenotypic differences among species from the Agavaceae family

Table 1. Origin and identification keys for agave individuals from the control and reference groups used in the present study

Species	Origin	Individual keys
Agave tequilana	Ameca, Jal.	A_azul AM 1 to 12
Agave tequilana	Acatic, Jal.	A_azul AC 1 to 10
Agave tequilana	San Gabriel, Jal.	A_azul SG 1 to 3
Agave tequilana	Unión de Tula, Jal.	A_azul UT 1 to 5
Agave tequilana	Jesús María, Jal.	A_azul JM 1 to 3
Agave tequilana	Juchitlán, Jal.	A_azul JU 1 to 7
Agave tequilana	El Limón, Jal.	A_azul EL 1 to 10
Agave tequilana	Tizapán, Jal	A_azul TI 1 to 8
Agave tequilana	La Barca, Jal.	A_azul LB 1 to 7
Agave tequilana	El Grullo, Jal.	A_azul EG 1 to 10
Agave tequilana	Jocotepec, Jal.	A_azul JO 1 to 3
Agave tequilana	Arenal, Jal.	A_azul AR 1 to 6*
Agave tequilana	Chapala, Jal.	A_azul CH 1 to 6
Agave tequilana	Ocotlán, Jal.	A_azul OC 1 to 10
Agave maximiliana	Mascota, Jal.	A_maxi MA 1 to 10
Agave angustifolia	Nextipac, Jal.	A_angu NE 1 to 5
Agave salmiana	Ahualulco, Jal.	A_salm AH 1 to 10
Agave americana	San José de Gracia, Mich.	A_amer SJ 1 to 10
Fourcrea spp.		F_AH1 to 4

Agave from representative DOZ

The PCR cycling conditions were 3 min at 95°C, followed by 40 cycles of 30 s at 95°C and annealing at 45°C for 1 min, with extension at 72°C for 2 min and a final extension at 72°C for 10 min. DNA amplification was performed using a Techne Flexigene® thermocycler. Amplification fragments were separated by electrophoresis using 6% polyacrylamide gels and stained with silver salts, according to the methods of Sanguinetti *et al*. (1994) and Sambrook and Russell (2001).

2.4. Data Analysis

The statistical analysis of variability within species used the classical BART test written in SAS code for the SAS program (SASI, 1994) and homogeneity of variances using the procedure DISCRIM, according to the methods of Morrison (1976). A cluster analysis was also performed based on the correlation matrix: $r_{ij} = (\Sigma_k x_{kj} x_{ki})/(\Sigma_k x_{ki}^2 \Sigma_k x_{kj}^2)^{½}$ where ij corresponds to the plants and k indicates the variables, which were standardized to a mean of zero and a variance of one in this study. Clustering was performed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) in NTSYS 2.11 (Rohlf, 1994). The results were represented using a dendrogram. The PCA was represented using a biplot graph, as described by Rawlings (1988) and Sanchez (1995). To examine genetic structure a Bayesian cluster analysis using STRUCTURE software was performed (Pritchard *et al*., 2000). This analysis estimates membership of each individual to a population K we selected, the admixture model and correlated allele frequencies with a burn-in period and MCMC of 10^5 iterations. To verify the most probable number of cluster (K), the method of Evanno *et al*. (2005) was following using a range of K from 2 to 8.

3. RESULTS

3.1. Morphological Markers

The PCA indicated that the first two components explained 70% of the total variation observed. The first Component (C1) explained 45% and it was defined by the variables AP, LH and NH. The second Component (C2) explained 25% of the variability among groups and was defined by the variables AH and DT. The results are shown in **Table 2**.

Figure 3 shows that *A. tequilana* and *A. angustifolia* are located on the positive side of the biplot graph and both species belong to the Rigidae group with elongated stems and sword-shaped leaves. The variability and spatial location of this group were defined by LH, AP and NH and these species shared similar phenotypes. Dots represent individuals in **Fig. 3** and they are dispersed according to the variables that group their components. It can be seen that the dispersion of the comparison groups was greater than that of *A. tequilana*.

Table 2. Principal components analysis eigenvector values for the morphological characters of *A. tequilana* and five species of Agavaceae

Variable	Key	C1	C2	C3	C4	C5	C6
Number of leaves	NH	0.3600	0.1400	0.6400	-0.6000	-0.1500	0.170
Plant height	AP	0.5800	0.0065	0.0350	0.0920	0.3600	-0.710
Diameter of cone	DT	0.2300	0.6200	0.1600	0.5900	-0.4000	0.070
Leaf length	LH	0.5600	-0.1700	-0.1800	0.2000	0.3700	0.670
Leaf width	AH	-0.3000	0.6400	0.0560	-0.1100	0.6800	0.100
Number of spines	NE	-0.2500	-0.3700	0.7100	0.4600	0.2500	0.020
Variation (%)		0.4533	0.2530	0.1577	0.0887	0.0393	0.008

Table 3. Estimation of variance for the morphological traits among five species of Agavaceae using Bartlett's test

Species	VNH	VAP	VDT	VLH	VAH	VNE	logD
Agave tequilana	87.490	0.020	20.8600	93.410	0.48000	1.21000	5.59000
Agave salmiana	83.600	0.010	83.1200	56.710	8.67000	1.38000	7.89000
Agave maximiliana	22.220	0.010	7.3900	46.490	3.11000	9.43000	3.96000
Agave americana	18.890	0.030	59.7900	131.880	5.38000	0.18000	3.21000
Agave angustifolia	26.800	0.040	8.7000	89.300	0.20000	1.20000	-31.01000
χ^2	12.500	7.030	21.2800**	3.090	87.13000**	41.39000**	267.19000
$p>\chi^2_c$	0.013	0.134	0.0002	0.543	0.00001	0.00001	0.00001

Bold = higher variance; underlined = lower variance; VNH = variance of the number of leaves; VAP = variance of plant height; VDT = variance of diameter of cone; VLH = variance of leaf length; VAH = variance of leaf width; VNE = variance of number of spines; logD = log of the determinant

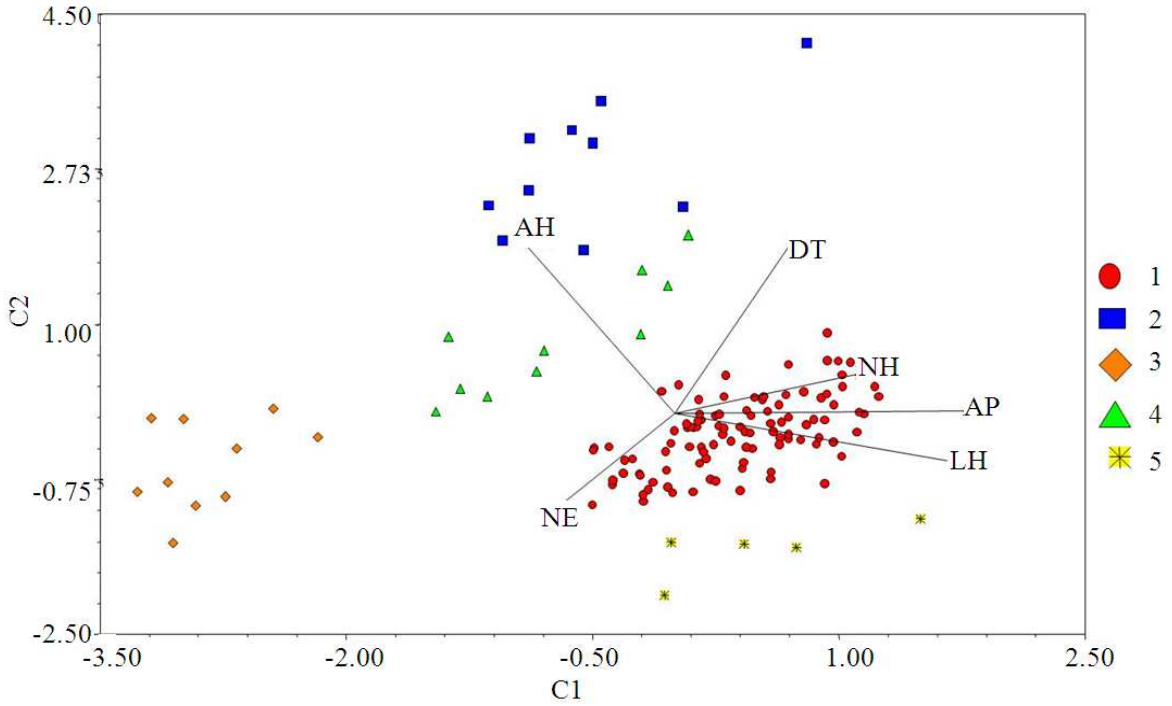

Fig. 3. Biplot of the principal components analysis for *Agave* spp. showing the vectors of the variables measured. 1: *A. tequilana*, 2: *A. salmiana*, 3: *A. maximiliana*, 4: *A. americana*, 5:*A. angustifolia*. C1: first component, C2: second component. NH: number of leaves, AP: plant height, DT: diameter of cone, LH: leaf length, AH: leaf width, NE: number of spines

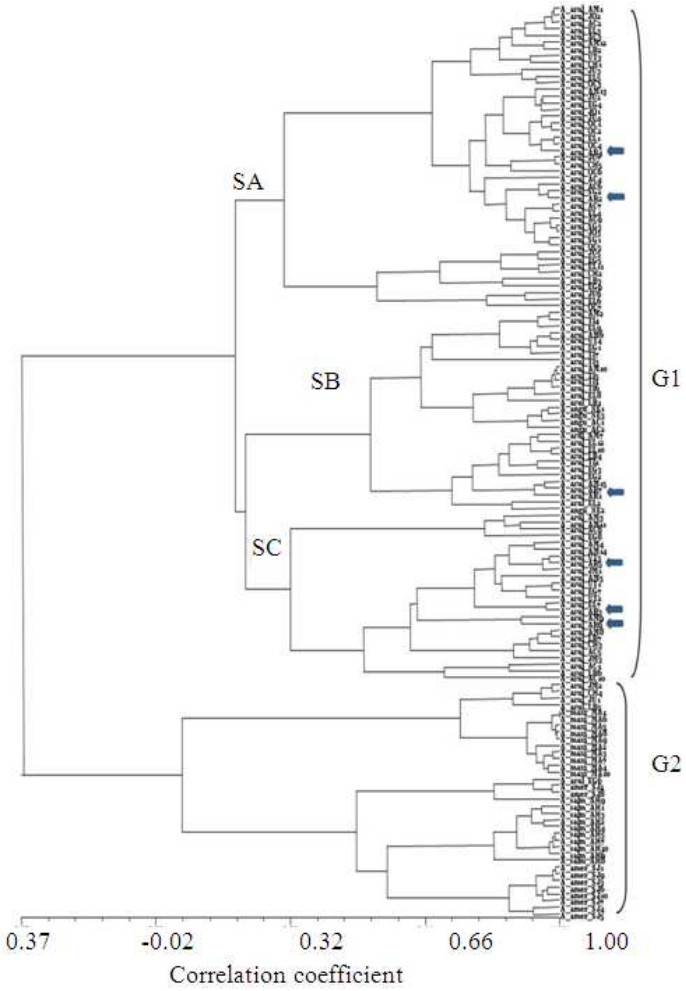

Fig. 4. Dendrogram generated by clustering analysis for five Agavaceae species based on morphological traits. G1: Group 1, G2: Group 2, SA: Subgroup A, SB: Subgroup B, SC: Subgroup C. Agave individuals collected from representative DOZ were marked with an arrow

In terms of the second Component (C2), *A. salmiana* differed with respect to AP and so its position in the biplot is opposite that of *A. tequilana* and *A. angustifolia* (see morphological differences in **Fig. 2**).

The cluster analysis divided the samples into two main groups (**Fig. 4**).The main division was that of *A. tequilana* and *A. angustifolia* from the other Agavaceae species. Subgroup SA was distinguished in the collections of *A. tequilana* and was characterized in individuals collected from the four regions of Jalisco, particularly in the southern zone (points 8, 9, 10, 11 and 14; **Fig. 1**). The dendrogram shows that specimens in subgroups SB and SC were grouped from different collection sites. The results indicate a high correlation between the morphological characters of *A. tequilana* from Tizapán el Alto, San Gabriel and Juchitlán, which do not belong to the main traditional culture areas (Tequila region and "Los Altos de Jalisco" region). Group 2 (G2, **Fig. 4**) contained the remaining groups of Agavaceae that were used to compare variability. In this group, *A. maximiliana* had lower average values for the variables AP, DT, LH and AH compared with *A. salmiana* and *A. americana*.

3.2. Variability within Species

Table 3 shows the variance estimates for the six variables studied. The highest variances were for NH in *A. tequilana* and *A. salmiana*.

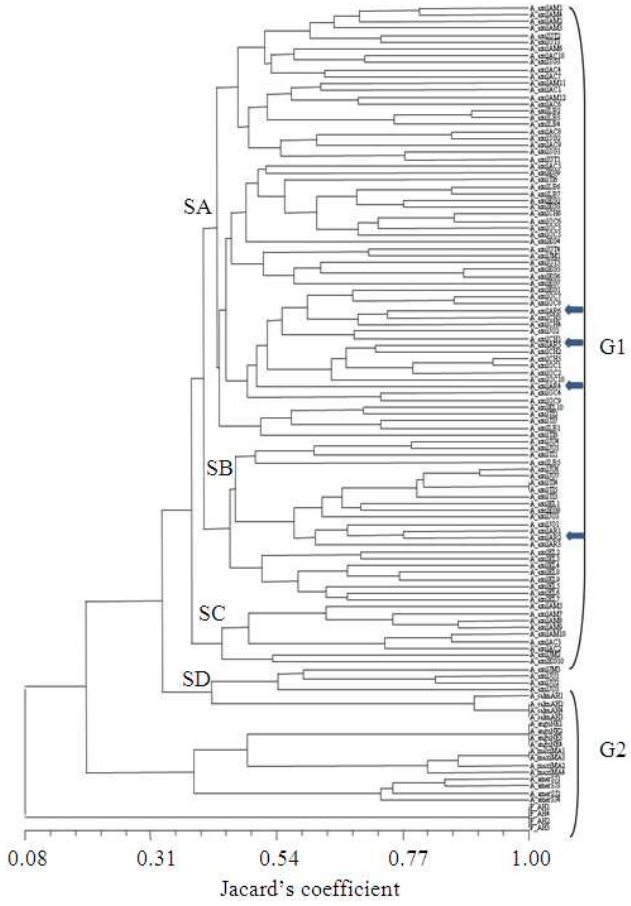

Fig. 5. Dendrogram generated by clustering analysis for six species of Agavaceae based on molecular data. G1: Group 1, G2: Group 2, SA: Subgroup A, SB: Subgroup B, SC: Subgroup C. Agave individuals collected from representative DOZ were marked with an arrow

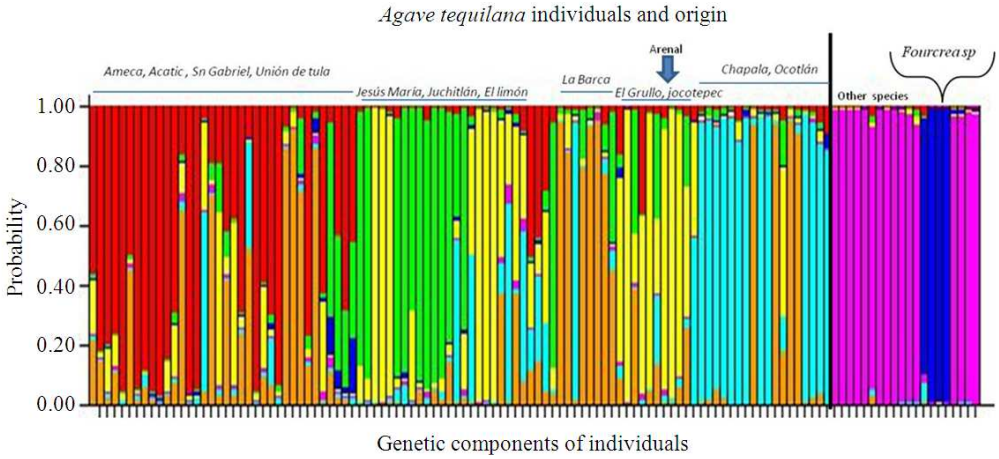

Fig. 6. STRUCTURE analysis using a simulation with $K = 7$ for six species of Agavaceae

There were major differences between species for DT, AH and NE. Values in **Table 3** where χ^2>20 were highly significant. The test for homogeneity of variance and the covariance matrix showed that the highest value for the logarithm of the determinant was in the *A. salmiana* group, which suggests high variability among individuals in this species. This can also be seen in the dispersion of dots representing these individuals in **Fig. 3**.

3.3. Molecular Analysis

The amplification patterns produced by the ISTR molecular marker were species-specific. A total of 53 bands were obtained and 94% were polymorphic. The similarity and clustering analysis based on molecular data detected a significant separation between *Fourcrea* spp. and the *Agave* group (**Fig. 5**) with a Jaccard's coefficient of 0.18. The *A. tequilana* specimens were placed in a single Group (G1) on the dendrogram. The division of the subgroups within the principal group and the cluster analysis based on morphological data clearly demonstrate the genetic variability. Agaves are asexually propagated, but the differences in variability of the DNA were remarkable, with Jaccard's coefficient being as high as 0.40. It is important to note the separation of four subgroups (SA, SB, SC and SD), which probably indicates the origins of the specimens.

3.4. Genetic Structure

The different species used in this study were separated by the analyses of molecular and morphological traits. In the genetic structure analysis using admixture-based models, each individual was assumed to have inherited some proportion of its genetic material from each of *K* distinct populations. These proportions were determined as the admixture proportions for each individual and a key goal of these methods is to estimate the allele proportions and frequencies in each population (Engelhardt and Stephens, 2010). The genetic structure of 130 individuals of Agave based on the Bayesian-based model is shown in **Fig. 6** for K = 7. Evanno's method determined K = 7 as the most likely number of genetic clusters for the entire set of agave plants. The genetic structure indicated in **Fig. 6** is in close agreement to geographic origin. *Fourcrea* spp were clearly separated from other Agavaceae members (**Fig. 6**). *Agave tequilana* individuals were subdivided into five groups by STRUCTURE, partly along geographic lines. We also calculated the proportion of variation within subpopulations (FST) relative to the total variation. The values were >0.25 in the external and comparison groups, indicating a very high differentiation in terms of allelic frequencies. The FST values ranged from 0.15 to 0.25 in *A. tequilana*, which also suggested high differentiation among individuals in this species.

Figure 6 shows the different *K*-simulated groups (in different colors). Among the *A. tequilana* specimens, it was possible to identify genetic differences that could explain the spread of genotypes among crop fields throughout the state and the DOZ. Accessions representing the sites of origin of Agave Azul, are indicated by arrows in **Fig. 4-6**.

4. DISCUSSION

This study suggests that genetic variability is present in asexually propagated plants, i.e., *A. tequilana*, as previously reported by other authors (Infante *et al*., 2003; Rodriguez-Garay *et al*., 2009; Torres-Moran *et al*., 2010; Esqueda *et al*., 2011). The differences between A. *tequilana* individuals collected from southern Jalisco state and those collected in the principal DOZ (Arenal, Acatic and Jesús María) confirmed the existence of different genotypes, which were conserved in specific regions by asexual propagation, as reported by Gil-Vega *et al*. (2001). The similarities between *A. tequilana* and *A. angustifolia* have been reported previously, which indicates that *A. angustifolia* is an ancestor of *A. tequilana* (Gentry, 1982; Davila *et al*., 2007; Vargas-Ponce *et al*., 2007). The current work clearly demonstrates the morphological and molecular differences between *A. tequilana* specimens and demonstrates the genetic variability within individuals of all the species examined. We detected differences within *A. tequilana* individuals and our study was conducted using a large sample (100 individuals), which contrasts with the small samples used in previous studies (Gil-Vega *et al*., 2001; 2006). The FST values indicate genetic differentiation within A. *tequilana* and *A. angustifolia,* as reported by Sanchez-Teyer *et al*. (2009). The model-based clustering method using multilocus genotype data based on ISTR molecular markers provided information on the population structure of *A. tequilana* and other Agavaceae species. The simulation program used K = 7 to produce a set of allele frequencies that probabilistically assigned each individual to a specific population, as reported by Pritchard *et al*. (2000). Morphological and molecular tools can detect genetic differences in plants that have previously been considered clonal individuals. These tools allowed us to estimate the relationship between *Agave* spp. and cluster in a group *A. tequilana* together with *A. angustifolia,* whereas a separate group was formed by *A. maximiliana*,

A. americana, *A. salmiana and Fourcrea* spp. Torres-Moran *et al*. (2010) have reported the separation of different agaves using ISTR. It is important to note that grouping based on genetic structure analysis shows relationships according to the dispersion of plant material from the initial sites of cultivation. It was indicated that the area of origin of Agave Azul is on the foothills of Colima volcanoes, near the Jalisco coast; from there, plants migrated along a geographical corridor up to the Tequila area, where Tequila beverage takes its name (Vargas-Ponce *et al*., 2009). Recently, following the commercial expansion, promoted interchange of plant materials into very distinct environments, enabling them to genotypic differentiation as seen in structure analysis.

5. CONCLUSION

In this study, we detected genetic variability within and among individuals and different species in the genus *Agave*. We used a large sample and this allowed us to detect different groups among field crops of the plant *A. tequilana*, which is propagated asexually. We also tested a mixture of samples from all localities in the DOZ. The differences we detected in *A. tequilana* could allow producers and industry to establish breeding programs by selecting elite phenotypes, while also preserving genetic resources by sustainable management in the immediate future.

6. ACKNOWLEDGMENT

The researchers wish to acknowledge Dr Ana Lília Guzmán Vigueras in the Departamento de Botánica y Zoología, Universidad de Guadalajara, for supporting the taxonomic identification of agaves in this work. To Dr. JJ Sanchez-Gonzalez for invaluable contribution.

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