

Molecular analysis of sexual and asexual genetic variation of two sympatric *Agave angustifolia* varieties

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Abstract

The genetic diversity using RAPD markers, between plants obtained through sexual and asexual reproduction of *Agave angustifolia* var. Lineño and var. Cimarrón collected in the southern region of the State of Jalisco, Mexico was evaluated. The two varieties studied are particularly important in the making of mezcal, and have been selected by producers in the region based on morphological characters, attempting to obtain an increasingly homogenous production with an improved quality. Despite this selection process, results obtained through RAPDs showed that there is a large genetic variation in these varieties, which is very important to preserve the genetic heritage in this species. In this study, RAPDs allowed a clear separation between the Lineño and Cimarrón varieties, being Lineño more genetically homogeneous than Cimarrón, putatively due to ancient domestication, and all analyzed individuals tended to group according to their mode of propagation. In terms of the clear separation of the two varieties in the case of seed–generated individuals, it is clear that although these varieties develop in the same habitat, they grow in a sympatric manner in their environment. Otherwise, there would be a closer relationship among formed groups, and it would be hard to separate seed–originated individuals of both varieties.

Key words: Molecular markers, Mezcal, Cimarrón, Lineño, Genetic diversity.

Introduction

The *Agave* genus is endemic to America and includes 166 species and is the largest genus in the family *Agavaceae* (Good–Avila *et al.*, 2006), 75% is distributed in Mexico, and 51% are endemic species (García–Mendoza, 2002). Species of this genus show both sexual and asexual reproduction, and genetic variation has been observed in asexual propagation by bulbils in *Agave fourcroydes* (Infante *et al.*, 2003), *A. tequilana* (Gil–Vega *et al.*, 2006) and *A. angustifolia* (Sanchez–Teyer *et al.*, 2009).

Twenty two mezcal–producing species of the *Agave* genus are known in Mexico. From these, *A. angustifolia* is used more often for this purpose and is the most widely distributed in the country. Most of these species are wild, and constitute a very important genetic resource and some are cultivated in different regions of Mexico for the production of mezcal.

Agave angustifolia reproduces itself in three manners: (1) early in development it produces rhizomes, in which apical meristems emerge close to the mother plant and form new plantlets; (2) late in development a branched flower stalk (10–12 m high) appears and forms bulbils, which reproduce asexually; and (3) fruits with seeds are produced in the flower stalk at the top of the plant (Gentry, 1982).

Molecular markers have been employed for the solution of relevant problems in the identification, conservation and use of plant genetic resources, especially in those cases where identification by morphological characters is complex (Hodgkin, 1995). The RAPD (Random Amplified Polymorphic DNA) technique (Williams *et al.*, 1990) is one of the most used since it is not necessary to have previous knowledge of the individual's DNA. Short primers are used which are distributed randomly in the genome, and these provide a series of segments characteristic to each species and variety (McPherson and Moller, 2000).

RAPD molecular markers have been previously used to determine genetic diversity and phylogenetic relationships in a wide range of species, including garlic (*Allium sativum*) (Al-Zahim *et al.*, 1997), grape (*Vitis vinifera*) (This *et al.*, 1997), strawberry (*Fragaria ananassa*) (Degani *et al.*, 1998), sugar cane (*Saccharum* spp.) (Nair *et al.*, 1999), and soybean (Xu, 2003). In the *Agavaceae* family it has been proven that this technique is useful to evaluate the diversity in the *Agave tequilana* var. Azul, the raw material for the production of tequila (Gil-Vega *et al.*, 2001) and in the *Agave deserti* complex (Navarro-Quezada *et al.*, 2003). In previous studies, we used RAPDs to evaluate the genetic diversity in *Agave tequilana* var. Azul, and *Agave angustifolia* var. Lineño (e.g. Rodríguez-Garay *et al.*, 2009).

The objective of this work was to evaluate the genetic diversity, using RAPD markers, between plants obtained through sexual and asexual reproduction of *Agave angustifolia* var. Lineño and var. Cimarrón collected at different locations in the southern region of the state of Jalisco, Mexico.

Material and methods

Plant material

Shoots, bulbils, and plantlets from seed of two *Agave angustifolia* varieties were collected: Lineño y Cimarrón, in the towns of Canoas and Toliman, located in the south of the state of Jalisco (Figure 1). In total, 18 shoots (LH), 20 bulbils (LB) from the same plant, and 40 plants (LS) from the Lineño variety seed were collected; while 13 shoots (CH) and 40 plantlets from seed (CS) of the Cimarrón variety were collected (no bulbils were available for this variety). This yielded a total of 131 individuals analyzed. In addition to the above materials, an *A. tequilana* individual was included, as well as three from the *A. angustifolia* var. Soka as external controls.

Healthy leaves from each accession were collected, labeled, packed in ice and transported to the Plant Biotechnology Laboratory at Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco (CIATEJ), Guadalajara, Jalisco, Mexico, and stored at -80°C until used for DNA extraction.

DNA Extraction

DNA was extracted using the Zhang *et al.* (2001) protocol. Sample concentration was determined by means of agarose gels, stained with ethidium bromide and diluted in TE (10 mM Tris-HCL, pH 8.0, 1 mM EDTA) to achieve a final concentration of 10 ng/ μl .

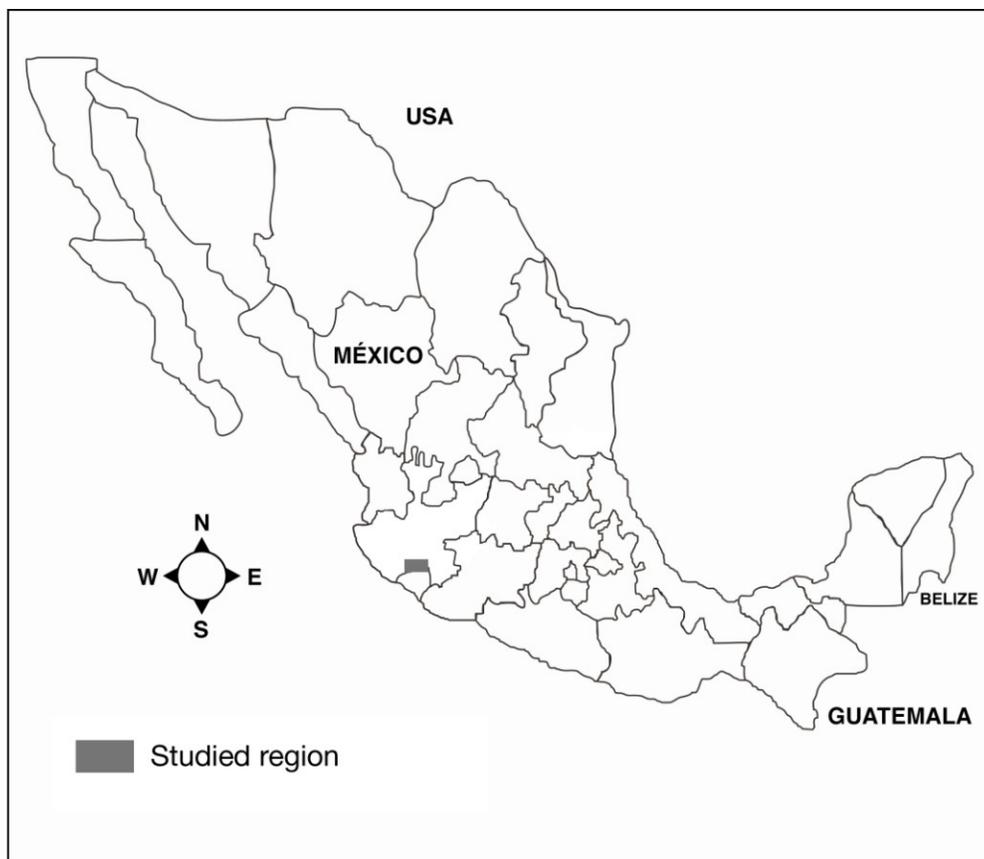


Figure 1. Map showing the region where *Agave angustifolia* vars. Lineño and Cimarrón were collected.

RAPD reactions were performed in a volume of 12.5 μ l containing: 15 ng of genomic DNA, 2.5 μ M of an arbitrary primer, 0.20 mM dNTPs, 1.33 mM MgCl₂, 0.75 unit of Taq polymerase (Invitrogen™) and 1X reaction buffer 10X (200 mM Tris–HCl pH 8, 500 mM KCl) provided by the enzyme supplier. (Primers 2, 5 y 6 from Amersham Biosciences) previously used in the characterization of *Agave tequilana* Weber var. Azul Mayahuelt and *Agave angustifolia* var. Lineño (Rodríguez–Garay *et al.*, 2009). Amplifications were done using a thermocycler (Techne model TC412) using an initial cycle of 5 min for denaturation at 95°C, followed by 45 cycles of 1 min denaturation at 95°C, 1 min of annealing temperature 36°C and 2 min extension at 72°C. RAPD–PCR products were size–fractionated in a 2% agarose gel with the addition of ethidium bromide and visualized using an UV transilluminator. The results of electrophoresis were documented with the use of a digital camera.

RAPD Analysis

RAPD bands were scored based on the presence (coded as 1) or absence (coded as 0) of polymorphic fragments for each primer, and used for calculating a genetic similarity matrix using the Jaccard (J) coefficient. Cluster analysis was performed on a similarity matrix using the unweighted pair group method using the arithmetic means (UPGMA) algorithm, from which dendrograms depicting similarity among varieties were drawn and plotted with the NTSYS–pc software, version 2.0 (Exeter software, East Setauket, NY). The cophenetic correlation was calculated in order to find the degree of association between the original distance matrix and the

tree matrix. Also, the Nei index of diversity (H) was calculated using POPGENE, ver. 1.31 (Yeh *et al.*, 1999; available free on: <http://www.ualberta.ca/~fyeh/>).

Results and discussion

Polymorphism level

With the three tested primers in this study, 91 total bands (33.3 fragments per primer in average) were obtained, of which 48 (52.7%) were polymorphic. Primer 5 generated the highest number of fragments (34), of which 20 were polymorphic (58.8%); however, primer 2 presented a higher polymorphism in the 32 fragments generated (23 polymorphic fragments, 71.8%), while primer 6 presented the lowest values of generated bands and polymorphism percentage (25 and 20%, respectively) (Table 1).

Table 1. Polymorphism levels, genetic similarity values, and genetic diversity index found in two varieties of *Agave angustifolia* in southern Jalisco, México.

Group	Polymorphic bands	Polymorphism (%)	Average similarity*	H
SL	39	42.86	0.80	0.122
SC	44	48.35	0.79	0.127
LH	11	12.09	0.92	0.044
CH	20	21.98	0.86	0.053
LB	16	17.58	0.93	0.057
Total	48	52.70	0.71	

*Jaccard's index; H= Nei's index.

In this work, the use of only three primers was convenient due to the number of analyzed individuals and to the fact that the level of polymorphism detected with these was high. On the other hand, only a few RAPD primers have been used in earlier works to evaluate the genetic diversity among individuals of *Agave angustifolia* y *A. tequilana*, and these results, although not correlated with the evaluated morphological characters, they do present a grouping pattern similar to the one obtained with morphological characters, and both species could be separated in both cases (Rodríguez-Garay *et al.*, 2009).

Also, Navarro-Quezada *et al.* (2003) used RAPDs to evaluate the genetic relationships in populations of the complex *A. deserti*; they finally selected only two primers to analyze all their populations, and found 41 fragments 100% polymorphic. Dávila *et al.* (2007), using only three ISSR primers to analyze interspecific variability in the *Agave* genus, reported 85.3% polymorphism in the case of wild *A. angustifolia*. While Vargas-Ponce *et al.* (2009) only used two ISSR primers in *A. angustifolia* from the south of Jalisco, México.

In terms of polymorphism levels found for different types of material used in this work, as it was expected, individuals stemming from seeds, in this case SL and SC, presented a higher percentage of polymorphism (42.86 and 48.35%, respectively) (Table 1). This level of polymorphism is logical due to the allogamous nature of *A. angustifolia*. Therefore, any *A. angustifolia* individual generated from a seed presented a high heterozygosity in a majority of its alleles and, hence, a high polymorphism. Navarro-Quezada *et al.* (2003) showed 100% polymorphism in the *A. deserti* complex. Barraza-Morales *et al.* (2006), using AFLP markers, found 82% polymorphism in wild *A. angustifolia* from the State of Sonora, Mexico, and more recently, Sánchez-Teyer *et al.* (2009) reported a 72% polymorphism. While in the case of wild *A. angustifolia* from the south of Jalisco, it was found 100% polymorphism (Vargas-Ponce *et al.*, 2009).

In the case of individuals from vegetative structures (shoots and bulbils), the levels of polymorphism were lower: HL 12.09%, and HC 21.98%. However, these values could be considered high if we consider that the same genetic information is preserved by vegetative propagation in relation to the tissue donor plant (Table 1). A first answer to this might be that these structures (shoots) were not taken from the same plant, and given that the Cimarrón and Lineño varieties are practically under a domestication process, the levels of diversity in these varieties must be high. Likewise, polymorphism was present in the case of bulbils, BL 17.5% even when all individuals came from the same donor plant. This same condition has been reported for *A. fourcroydes* by González *et al.* (2003), who using AFLP markers reported 25.2% of polymorphism in commercial plantations (normally of asexual propagation), attributing this polymorphism to the natural variations in the species. In addition, González *et al.* (2003) reported 19.9% of polymorphism in plants generated from somatic embryogenesis, similar to what has been reported by Infante *et al.* (2003). Although in previous works Gil-Vega *et al.* (2001) reported little variation in *A. tequilana* Weber var. Azul, which reproduces in an asexual manner, in later works a higher variation is reported for this variety (Gil-Vega *et al.*, 2006). Likewise, there have been reports of variation in *A. angustifolia* and *A. tequilana* plants among individuals from the same plantation (Rodríguez-Garay *et al.*, 2009).

Variation in plants propagated in vegetative mode in *A. angustifolia* has been reported by Sánchez-Teyer *et al.* (2009), who using AFLP markers found a great variation between shoots and their respective mother plants in wild *A. angustifolia* from Sonora, México.

Li *et al.* (2006), using RAPD markers, analyzed 74 individuals of *Taxus cuspidata* var. Nana vegetatively propagated from one single mother plant, and found high levels of polymorphism (31.1%). This variation found can be attributed to the fact that the mother plant hosted a large amount of somatic variations, and that this *Taxus* variety presented a high genetic instability. It is recognized that it might be one of the reasons why *T. cuspidata* adapted itself to a wide variety of environments and climate conditions. Likewise, studied varieties of *A. angustifolia* may be subject to this variation phenomenon so even if commercial propagation is achieved asexually, it is very feasible that due to a high natural genetic instability, these varieties present a considerable genetic variation.

Genetic diversity

Levels of similarity between pairs of individuals were between 1 and 0.36 (similarity matrix not shown). As expected, all individuals that presented similarity values of 1 (due to an identical band pattern) belonged to groups of plants obtained from asexual reproduction, mainly with shoots of the Lineño variety: LH1/LH2, LH3/LH4 and LH11 with LH12; bulbils of the Lineño variety LB16/LB18, LB19/LB20; and only one pair of individuals generated from shoots of the Cimarrón variety: CH2/CH3. This suggests that Lineño variety is more homogenous, while that Cimarrón variety presents a higher genetic variation among individuals. The lowest similarity values were obtained between pairs of individuals CH7/CS20 (0.36), and pairs CH8/CS20, CS9/CS20, CH9/CS20 and LB8/CS18, whose similarity values did not exceed 0.39. Median similarity in all analyzed individuals was 0.71. However, the lowest average similarity values were found in populations generated from seeds in both varieties, while the highest average values (above 0.9) occurred in the case of the Lineño variety propagated by shoots and bulbils, thus confirming its higher homogeneity versus the Cimarrón variety (Table 1).

Barraza-Morales *et al.* (2006) reported a similarity average of 0.81 in wild *A. angustifolia* from the state of Sonora, and more recently Sánchez-Teyer *et al.* (2009) found similarities of 0.80–0.82 within populations and 0.749–0.786 among populations.

The Nei genetic diversity index, another indicator of diversity levels in populations, behaved in the same manner as the similarity data. The highest genetic diversity values were in the case of individuals generated from seed in both varieties (LS and CS, with 0.122 and 0.127, respectively). Also, a lower diversity was found in populations of an asexual nature (Table 1).

The two varieties of *A. angustifolia* (Lineño and Cimarrón) studied in this work are particularly important in the making of mezcal in the south of the state of Jalisco, and have been selected through the years by producers in the region based on morphological characters, attempting to obtain an increasingly homogenous production with an improved quality. Despite this selection process, results obtained through this work show that there is a large genetic variation in these varieties, which is very important to preserve the genetic heritage in this species. Other works also report that the diversity of these varieties is comparable to populations in-the-wild in the same region (Vargas-Ponce *et al.*, 2009).

Cluster analysis

The cophenetic analyses from the UPGMA cluster analysis, using the similarity matrix, showed a correlation of $r=0.82$, indicating that the data in the matrix was well represented by the dendrogram. Both *Agave angustifolia* varieties were separated into four main groups; and they diverged at a genetic similarity coefficient of 0.67, based on the dendrogram. Also, the materials of *A. tequilana*, and three individuals of *A. angustifolia* var. Soka were separated from the groups formed with both varieties under study.

The four groups formed matched the varieties and types of material used for propagation (seed, bulbils, and shoots). Group I was formed exclusively by individuals coming from seeds of the Lineño LS variety; Group II, by seed-generated individuals of the Cimarrón CS variety; Group III, by shoot-generated individuals of the Cimarrón variety; while Group IV was formed by shoots and bulbils of the Lineño variety, and although less related, two individuals from the Cimarrón variety (CH5 and CH6) were located in this clade, these plants not corresponding to the arrangement expected in the dendrogram, being them probably originated by a natural cross between both lineages (Figure 2).

The individuals LS36 and CS1 generated by seed did not fit into any of the groups formed by the cluster analysis. This could be due to a putative natural hybridization between both varieties. On the other hand, the individual CS18 could be the result of a cross of the Cimarrón variety with pollen of any other unknown agave variety, since it positioned itself quite externally from the generated groups, even less related than individuals of the Soka variety that were used as external controls (Figure 2).

Results observed in the principal coordinates analysis (ACoP) were similar to the ones shown in the dendrogram, clearly defining groups of individuals by variety and type of reproduction; however, the individuals that were not correctly grouped in the dendrogram (CH5 y CH6), as well as those that were not part of a group (LS36, CS1, y CS18), were seen in this case closer to their corresponding group, which suggests that these can be part of the natural variation of the variety (Figure 3).



Figure 2. UPGMA dendrogram (based on Jaccard's similarity coefficients) of two *Agave angustifolia* varieties (Cimarrón and Lineño), $r=0.82$. LH=Lineño shoots, LB=Lineño bulbils, LS=Lineño seed, CH=Cimarrón shoots, and CS=Cimarrón seed.

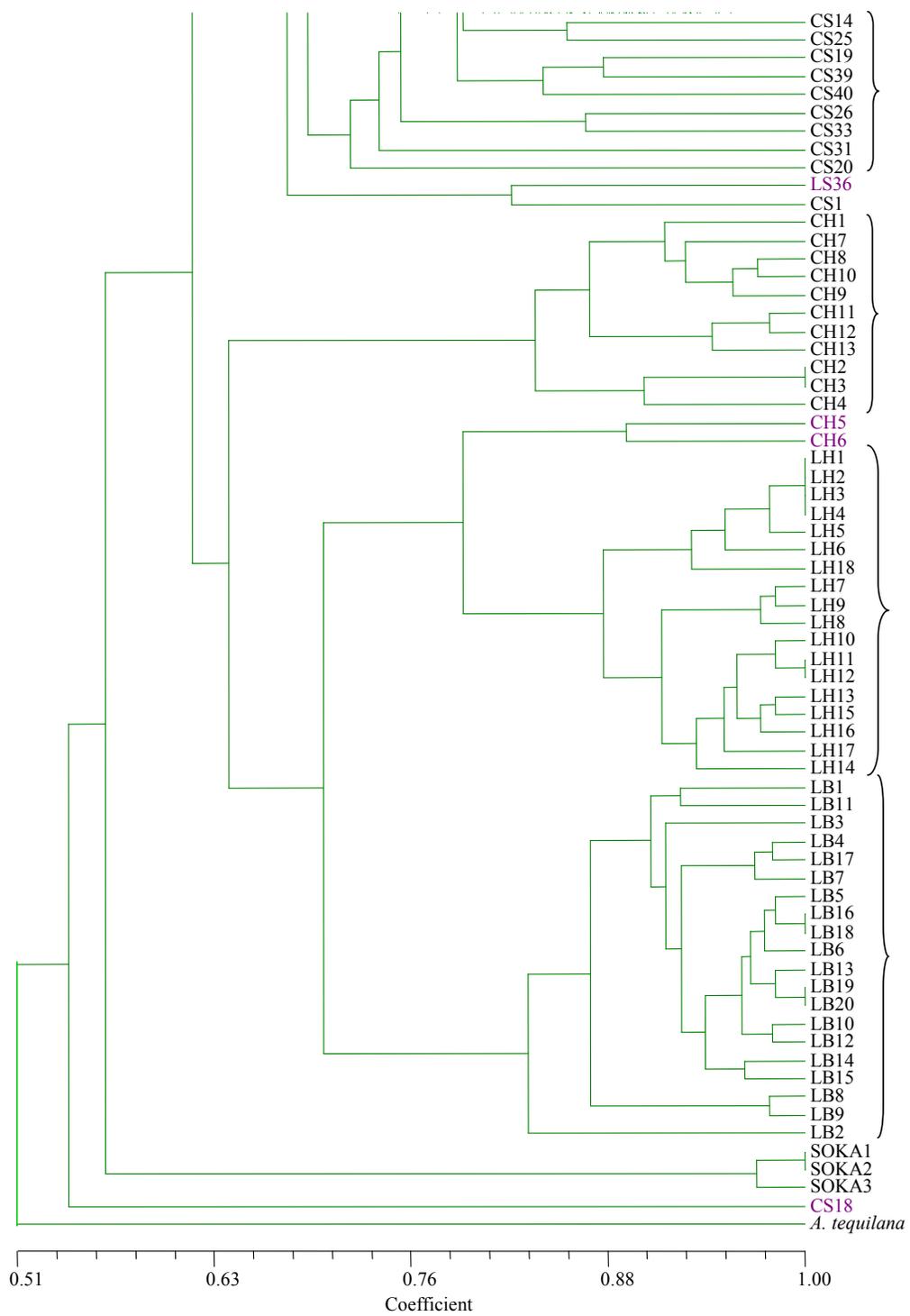


Figure 2. Continuation

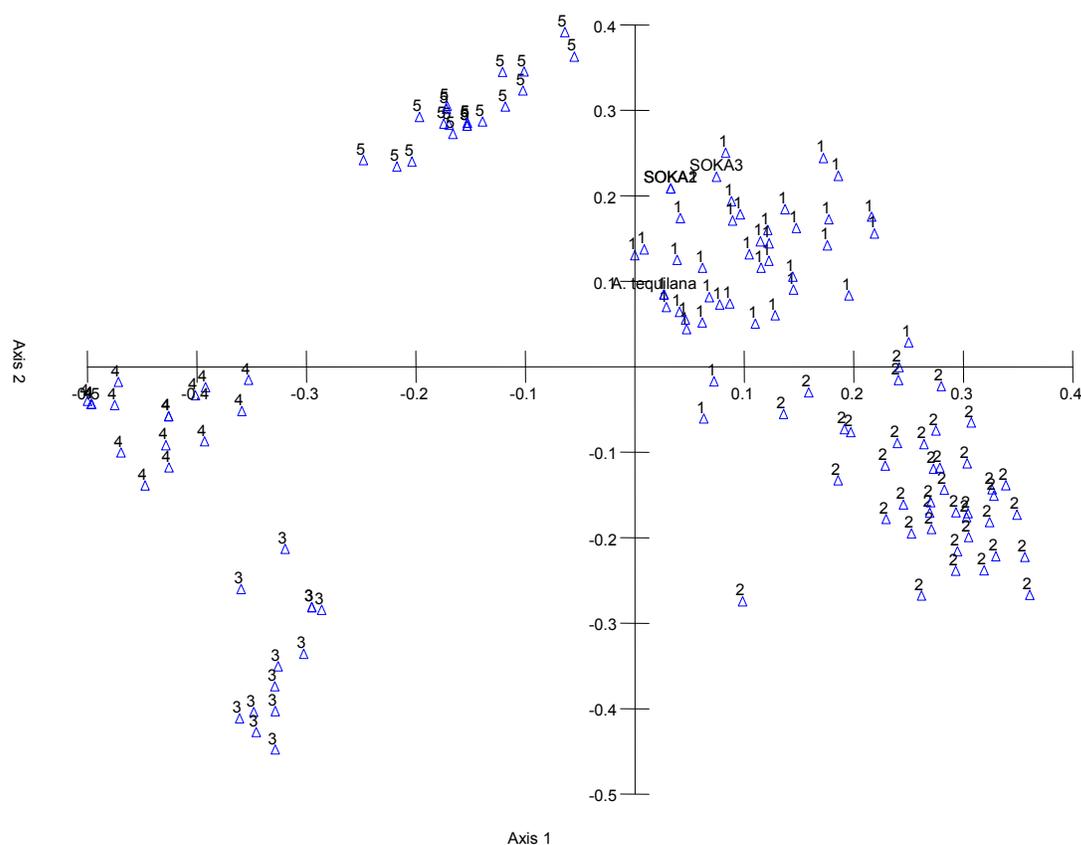


Figure 3. Principal Coordinates plot of two *Agave angustifolia* varieties (Lineño and Cimarrón) using RAPD data. 1=Lineño seed, and 2=Cimarrón seed, 3=Cimarrón shoots, 4=Lineño shoots, 5=Lineño bulbils.

Conclusions

Since all analyzed individuals tended to group according to the origin of the material and the variety, it can be concluded that the variations found are due to natural genetic variations in these populations.

In terms of the clear separation of the two varieties in the case of seed-generated individuals, it is clear that although these varieties develop in the same habitat, they grow in a sympatric manner in their environment. Otherwise, there would be a closer relationship among formed groups, and it would be hard to separate seed originated individuals of both varieties.

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