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Morphological Characterization and Variation in the Total Content of Reducing Sugars in Wild Populations of *Agave angustifolia* Haw

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Abstract: Problem statement: Bacanora, a spirit, is distilled from wild populations of *Agave angustifolia* Haw. Loss of biodiversity must be taken into account when proposing sustainable management actions for this resource. In this study we identified the morphological variants of this species, as well as the weight and Total Content of Reducing Sugars (TRS) in the stem of the agave. **Approach:** Twenty-three morphometric variables were measured in three wild populations of *A. angustifolia* distributed along the western slopes of the Sierra Madre Mountain Range in Sonora, Mexico. The relationship of plant weight to stem TRS was evaluated using multivariate analyses. **Results:** Canonical discriminate analysis explained 100% of the morphological variation with just two canonical variables ($p<0.01$) and the Mahalanobis distances support the finding of significant differences among the three populations. TRS content did not vary significantly among populations, but did differ between individuals ($p<0.01$). Cluster analysis then revealed eight groups of morphologically related plants. **Conclusion:** Based on this analysis and previous studies of genetic variability and cytogenetic on the same individuals, morphologically and genetically related groups of agave were detected and also had heavier stems and a higher TRS content. These plants can be considered the basis for the selection of germplasm.

Key words: *Agave angustifolia*, morphological variability, total reducing sugars, wild populations, chroma values, cluster analysis, Denomination of Origin (DO), Genetic Similarity Index (GSI)

INTRODUCTION

Agave angustifolia Haw. stands out from the diversity of the *Agave* genus in northeastern Mexico owing to its potential as both a crop and for industrialization in the production of the spirit known locally as *Bacanora*. Its use represents both economically and socially promising activities for the development of marginal communities in the Sierra Madre Mountain Range in Sonora, Mexico (Solano and Nunez-Noriega, 2003). This species is found along the Mexican Pacific Coast down to Central America and is also one of the main species used to make mezcal in Oaxaca, Mexico (Garcia-Mendoza, 2002).

The economic aspect of Bacanora is backed by its Denomination of Origin (DO) and a *Norma Oficial Mexicana* (NOM, Official Mexican Standard). Approved by the Mexican government in November 2000, the DO establishes a set of rules and geographic delimitations, conferring the exclusive right to make Bacanora on growers in 35 municipalities located in the mountains of the state of Sonora. The NOM-168-SCFI-2004, i.e., the set of rules and criteria designed to standardized quality parameters for Bacanora, was approved on October 2005. Ironically, these laws have favored the overexploitation of wild populations of *A. angustifolia*, making it immediately necessary to implement actions for the sustainable management of this species.

The morphology of this agave differs depending on where it grows. Near the coast of Sonora, the leaves are shorter and broader in the middle. In the mountains, under the shade of the trees, the leaves are narrower, long and flexible. In the desert, its xerophytic

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characteristics are more marked: leaves are shorter, narrow and have more teeth. The inflorescence's peduncle grows an average of 3 m in height, though at times it reaches 6 m. The panicle is open, narrow and up to 1 m long. The flowers are greenish-yellow *ca*. 5 cm long. Fruit are dry capsules, dehiscent, trilocular, 2-2.5 cm in diameter by 4-4.5 cm long. Seeds are black or dark brown and triangular in shape (Gentry, 2004).

Agave angustifolia reproduces sexually and asexually; the latter via rhizomes and bulbils. The rhizomes are modified stems that grow underground from the main stem, generating offshoots that develop at the base and around the mother plants (Fig. 1) which, once they reach the age of three years, can produce up to six offshoots. The bulbils originate in the vegetative buds that are present on the peduncle and the lateral flowering branches, forming as a survival response of the plant when, for any reason, it loses its flowers. Sexual reproduction occurs via seeds. Flowering generally begins at six years of age and the flowers produce a copious amount of seeds. However, owing to the fact that the majority of the seeds fall into an adverse environment and are subject to a high degree of predation, germination indices are low (Gentry, 2004).

The genetic variability of the three wild populations of *A. angustifolia* sampled in this study has been analyzed using Amplified Fragment Length Polymorphism (AFLP), as were genome and karyotype. The Index of Genetic Similarity (IGS) did not vary significantly at the intra- or interpopulation level and the dendrogram revealed three groups that partially reflect the geographic distribution of the populations. Some degree of genetic variation was observed in the vegetative reproduction of this agave, with an IGS of 0.924 between the mother plants and their offshoots. Total expected heterozygosity was high at 0.314, with 78% of the loci polymorphic and an intermediate level of gene flow at 1.18 (Sanchez-Teyer *et al*., 2009). Cytogenetic studies reveal that, although the level of ploidy and the mean 2C-DNA were equal, the karyotype formula, total genome length and the asymmetry index varied significantly among the wild populations of this agave.

The morphological, genetic and cytogenic variability observed in *A. angustifolia* has potential for the selection of promising biotypes with characteristics that are desirable for future genetic improvement programs. Based on the above, the relationship between the morphological variability, the weight and total reducing sugars content in the stem of *A. angustifolia* were analyzed for three wild populations located in the Sierra Madre Mountain Range in Sonora, Mexico.

Fig. 1: *Agave angustifolia* Haw. mother plant and her offshoots in the Sonora Sierra Mountain Range, Mexico

MATERIALS AND METHODS

Sampling area: The plants studied belong to three wild populations of *Agave angustifolia*: Site 1 (S1) *El Bajio*: 29°46'88'' LN, 109°00'28'' LO, Site 2 (S2): *Los Mochomos*: 29°55'58'' LN, 108°60'04'' LO and Site 3 (S3) *El Chorro*: 29º42'45'' LN, 108º68'21'' LO, located in the municipality of Nacori Chico, Sonora, Mexico.

Sample size: The random quadrat method was used. Three 50×100 m quadrats were set up per population, for a total of 15000 m^2 and the number of *A. angustifolia* in each sampling site was counted to estimate the size of the entire population. Plants were also classified according to their height (h). For the morphological analysis a number of adult plants were selected ($h \geq 1$ m), so that sample size was approximately 10% of the total number of adults observed. Thus, 10, 10 and 14 plants were collected from S1, S2 and S3 respectively, with each assigned a number from 1-34. Eight months after sampling, the adult plants were harvested and their fresh weight and total content of reducing sugars determined in order to analyze them in the context of the morphological variables.

Morphological parameters: For each plant selected, height and total diameter were measured. Three leaves were cut from the center of the plant to determine the dry/fresh weight of the leaves, mid-leaf width/total leaf length, total length/width at leaf base, number of teeth/leaf length, distance between teeth, tooth length, width at tooth base, distance from the spine to the first tooth, spine length and width at the base of the spine. Color parameters were also measured using a Minolta CE-300 portable colorimeter: luminosity (L*), ºHue and purity (chroma). This colorimeter records L* values, as well as a* and b* factors, which are used to estimate ºHue [arcTan (b*/a*)] and chroma $(a^{*2} + b^{*2})^{-1/2}$.

According to the CIElab system, L* has values from 0 (black) to 100 (white), a* -60 (green) to +60 (red) and b* -60 (blue) to +60 (yellow). Chroma defines the degree of purity of a color; higher chroma values indicate greater color saturation. °Hue defines the tone and a value of 0° indicates red, 90° indicates yellow, 180° green and 270° blue.

Content of total reducing sugars: The adult plants sampled for the morphological analysis were harvested in order to determine their fresh weight and TRS. Each stem was cut in half longitudinally and a 50-100 g sample was taken from the center of each half. The sample was dehydrated at 60°C to a constant weight over 72 h. TRS was determined on a dry basis, following the enzyme colorimetric method used to measure total fructans (AOAC, 1995), modified for TRS by eliminating the step for reducing the monosaccharide's obtained from the hydrolysis of starch and sucrose and their respective derived alcohols. These carbohydrates are quantified together with the glucose and fructose obtained from the enzymatic hydrolysis of inulin, the main polysaccharide in agaves.

Statistical analysis: To determine the cluster patterns among populations canonical discriminate analysis was used with the Pair wise squared distance between Groups method and differences between populations were measured as the Mahalanobis statistical distance using the (SAS, 2008) version 9.2 statistical software. Also, to define the groups of plants that were morphologically similar with noteworthy characteristics, cluster analysis was done using the UPGMA method and fixing the dissimilarity index at a limit of 1.0. Variation in TRS content was evaluated using a nested ANOVA and to detect differences among the means, Tukey's test was used with $\alpha = 0.05$.

RESULTS

Morphological variability among wild populations: There were 503, 202 and 199 agave plants h^{-1} in S3, S2 and S1 and the percentage of adult plants was 15.6, 14.4 and 22.5% respectively. Population density and the proportion of adult *A. angustifolia* plants were significantly greater in S3. The diameter and height of the plants ranged from 1.4-2.1 and from 1.1-1.6 respectively. Leaf length was 90-120 cm and leaf width was 5-7 cm (Table 1). The color parameters varied from 44-61 for L, 119-27 for ºHue and 27-43 for chroma, with means of 52.6, 123.2 and 34.7 respectively; dark to yellowish green colors were recorded.

The canonical discriminant analysis is a powerful tool for determining genetic or morphological distances between groups of organisms. This multivariate analysis extracts the components that maximize interpopulation Vs. intrapopulation variability (Vaylay and Santen, 2002). It was found that two canonical functions were significant ($p<0.01$) and account for 100% of the morphological variation between populations, with 70.8 and 29.2% explained by canonical functions 1 and 2 respectively. Centroid values for the three populations are shown in Fig. 2 and were significantly different ($p<0.01$) with Mahalanobis distances (D^2) of 23.58 between 1 and S2; 12.74 between 2 and S3 and 11.02 between 1 and S3.

When the limit of dissimilarity was set at less than or equal to 1 based on the morphological parameters, eight groups of plants were identified (Fig. 3). The mean values of the morphological parameters of the agave clusters present in this dendrogram are shown in Table 2. Not all the plants that fall into a given cluster belong to the same population (Fig. 3).

Based on these eight clusters and from less to greater dissimilarity, Cluster I has the lowest dissimilarity, with plants 19 and 20. These two agaves had low diameter/height ratio, low dry/fresh weight, narrow mid-leaf, wide leaf base, medium teeth and fewer of them, quite separate from each other, large (long, wide) spines that were quite far from the first tooth.

Table 1: Mean value, standard deviation and coefficient of variation for the morphological variables analyzed in *Agave angustifolia*

Morphological characteristic	Mean	Standard deviation	Coefficient of variation
Plant diameter (m)	1.75	0.37	0.21
Plant height (m)	1.33	0.26	0.19
Diameter/height ratio	1.33	0.21	0.15
Plant volume (m^3)	1.16	0.73	0.63
Leaf length (cm)	104.00	17.89	0.17
Mean leaf width (cm)	5.69	1.03	0.18
Mean width of leaf base (cm)	4.44	0.74	0.17
Leaf fresh weight (g)	269.70	128.00	0.47
Leaf dry weight (g)	45.96	18.16	0.39
Leaf dry to fresh weight ratio	0.17	0.02	0.09
Length/Width of leaf base	23.60	3.24	0.20
Length/Width at mid-leaf	18.64	3.54	0.19
Number of teeth	85.17	24.89	0.29
Number of teeth/leaf length	0.83	0.22	0.26
Width at the base of the spine (cm)	0.42	0.08	0.20
Length from the first tooth to the spine (cm)	7.81	2.56	0.33
Distance between central teeth (cm)	2.49	0.72	0.29
Tooth width (mm)	7.70	3.52	0.46
Tooth length (mm)	3.70	0.98	0.26
Length of spines (mm)	21.27	6.09	0.29
Luminosity	52.63	8.13	0.15
Hue angle (ºHue)	123.21	3.69	0.03
Purity (chroma)	34.69	8.04	0.23

Table 2: Mean values for the morphometric parameter, weight and total content of reducing sugars in the stem of *Agave angustifolia* grouped into eight clusters, based on a dissimilarity less than or equal to 1

Cluster	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
19,20	1.12	0.16	23.99	16.31	0.47	0.47	10.77	3.51	8.30	4.11	33.72	47.55	123.73	37.14	8.80	50.39
7,18	1.32	0.17	25.59	21.47	0.86	0.47	7.18	2.25	5.01	2.82	26.88	69.78	117.83	37.77	2.75	37.92
16,26	1.34	0.17	19.10	14.63	0.72	0.49	5.72	2.83	15.06	5.97	27.74	41.94	124.88	25.75	6.79	59.64
3,12	1.53	0.15	23.99	18.86	0.67	0.44	10.12	2.97	11.57	5.25	20.62	50.54	123.07	35.64	13.65	48.14
6,11,21,29	1.28	0.17	24.88	20.86	1.17	0.35	8.38	1.66	4.96	3.24	15.81	45.40	128.22	23.72	3.38	36.02
4,5,31,32,33,34	1.23	0.18	24.84	19.34	0.96	0.34	7.05	1.99	5.61	3.45	17.27	55.44	121.05	39.83	3.42	48.95
9,10,13,23	1.34	0.19	24.99	20.37	0.65	0.44	6.98	3.30	7.94	3.75	18.54	52.87	122.51	36.95	4.01	47.66
2,17,24,25,27	1.43	0.18	20.11	15.23	0.94	0.45	5.60	2.07	6.64	3.52	22.41	55.95	123.56	36.35	2.96	37.09

where, (A) plant diameter/height ratio, (B) dry to fresh leaf weight ratio, (C) length/width of leaf base, (D) length/width at mid-leaf, (E) number of teeth/leaf length, (F) width at the base of the spine in cm, (G) distance from the first tooth to the tip of the spine in cm, (H) distance between central teeth in cm, (I) tooth width in mm, (J) tooth length in mm, (K) spine length in mm, (L) luminosity, (M) °hue, (N) chroma, (O) fresh weight in kg of stems harvested and (P) total percentage of reducing sugars in the dry base

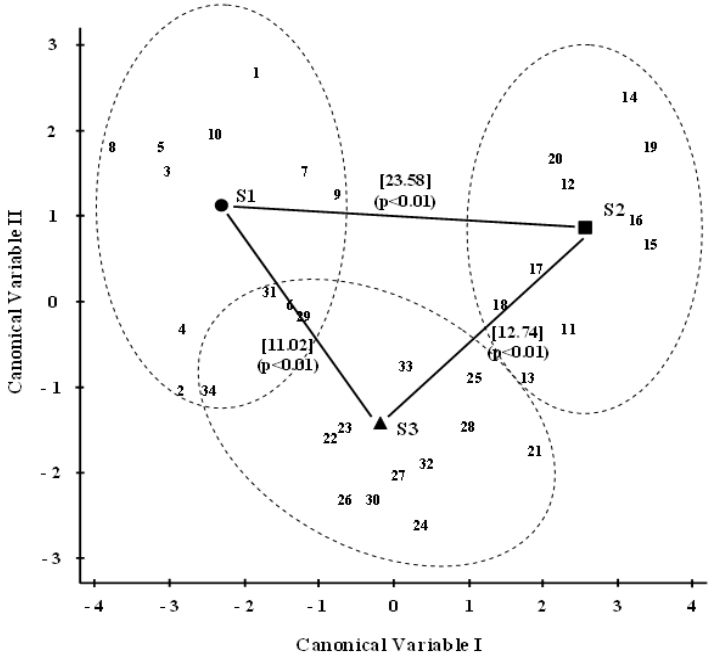

Fig. 2: Scatterplot of centroid values for three wild populations of *Agave angustifolia* (● S1 = *El Bajio*, ■ S2 = *Los Mochomos* and ▲ S3 = *El Chorro*) on two canonical discriminant variables. Mahalanobis distances in square parenthesis and their probability values in parenthesis measure the extent of morphological diversity between the populations. Dashed lines group the plants (numbered) of each population

Cluster II is made up of plants 7 and 18 which had long, narrow leaves with the corresponding high values in the length/width ratio for the leaf base and mid-leaf, very small teeth, moderately separated, large spines far the first tooth, with very high L* and very low °Hue (Table 2).

Plants 16 and 25 make up Cluster III and were characterized by short, broad leaves and the corresponding low values for the length/width ratio for the leaf base and mid-leaf, very large teeth, large spines, with little space between the spine and the first tooth, with very low L* and chroma values.

Cluster IV includes plants 3 and 12, which had a high diameter/height ratio, a low proportion of dry material, leaves narrow at mid-leaf and wide at their base, large, scarce teeth that are spaced out and a large distance from the first tooth to the spine. Cluster V has four plants (6, 11, 21 and 29) with leaves that were narrow from the center to their base; numerous teeth that were very narrow and close together, with small spines and very close to the first tooth. L* was low and chroma and °Hue were high (Table 2).

Plants 4, 5, 31, 32, 33 and 34 make up Cluster VI and were characterized by leaves narrow at the center, numerous teeth that were very narrow, very close to each other and with very small spines and close to the first tooth. L* was high and chroma and °Hue were low.

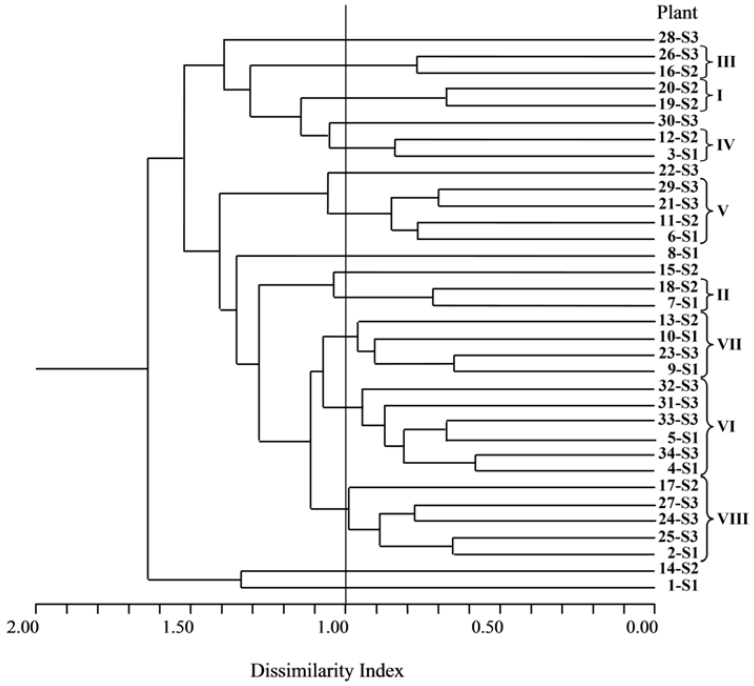

Fig. 3: Dendrogram generated by the cluster analysis showing the eight groups of morphological variants based on a dissimilarity less than or equal to 1. Site 1 (S1: 1-10), Site 2 (S2: 11-20) and Site 3 (S3: 21-34)

Cluster VII has plants 9, 10, 13 and 23, which in general had a high dry/fresh weight ratio, long, narrow leaves, scarce medium-sized teeth that were spread out and had average sized spines. L*, chroma and ºHue fell within the overall mean. Cluster VIII includes plants 2, 17, 24, 25 and 27, which were characterized by a high diameter/height ratio, short, broad leaves, corresponding to the low values for the ratios of length/width for the leaf base and mid-leaf, numerous teeth, relatively close together and average in size and a short distance from the first tooth to the spine. L*, chroma and ºHue fell within the overall mean (Table 2). For the weight of the harvested stems, the clusters with the highest mean biomass were those that grouped plants (3, 12) and (19, 20), had a fresh weight of 13.6 and 8.8 kg, respectively.

Variation in the content of total reducing sugars: Although no significant differences were observed in TRS between populations (mean TRS: 44.7, 42.8 and 39.6% at 2, 3 and S1 respectively) with an interpopulation mean of 42.4%, TRS did vary significantly between individuals ($P < 0.01$). Plants 17, 11, 13, 19 and 16 from S2; 22, 34, 23 and 33 from S3 and only plant 3 from S1 all had % TRS significantly above the mean.

DISCUSSION

Although several factors-the subtropical scrub vegetation mainly associated with sarcocrassicaul scrub, the sandy-loam texture of the soil and the soil nutrients-were similar in the three wild populations of *A. angustifolia*, the higher density and greater development of the agaves in S3 can be attributed to the microclimate of that site which receives natural protection from the gentle surrounding hills resulting in greater plant cover which, in turn, also offers some protection. The agaves were associated with trees and shrubs, particularly *Acacia cochliacantha* Humb. and Bonpl. ex Willd., *Fouquieria macdougalii* Nash, *Opuntia* spp., *Prosopis* spp. and *Sapium biloculare* (S. Watson) Pax.

The morphometric parameters were similar to those recorded by Gentry (2004), who mentioned that the wild plants of *A. angustifolia* in Sonora, Mexico have acaulescent, rosette growth, measure 1.0-1.5 m in height by 1.5-2.0 m in diameter. Leaves are linear from 50-120 cm long, by 4-8 cm wide, ending in a strong apical spine and with margins armed with small hard teeth. Compared to the characteristics observed for wild populations of *A. angustifolia* in the Yucatan, Mexico by Colunga-Garciamarin and May-Pat (1997), the

plants in the mountains of Sonora are generally taller, have narrower leaves, longer, broader spines and larger, more separate teeth. Also, *A. durangensis* shows a high morphological variability within and among populations, what makes its taxonomic delimitation a hard task (Almaraz-Abarca *et al*., 2009).

It is not possible to conclude that the high variation observed in the TRS content and in stem size is exclusively the result of genetic variability among populations. Since these are wild plants, the growth and development of the agave, combined with environmental factors play an important role in the variability (Nobel, 2009; Nobel *et al*., 2002). However, if we compare the dendrogram for morphological variability (Fig. 2) with the genetic information obtained for the same plants by Sanchez-Teyer *et al*. (2009), we can see that plants (3,12) and (19,20), which stand out for their weight and stem TRS content (Table 2), are morphologically and genetically related.

The first study of the genetic variability of *A. angustifolia* in Sonora was done by Barraza-Morales *et al*. (2006), with three wild populations located in different municipalities. The greatest variation was detected for the municipality of Nacori Chico, with an expected mean heterozygosity at the population and total levels of 0.26 and 0.31, respectively. This is why this municipality was selected for later studies on morphology, cytogenetics and genetic variability using AFLP. Torres-Moran *et al*. (2008) observed that ISTR molecular markers are worthy to typify species of Agavaceae and detect intrapopulation variability too. For S1 and S3, identified cytotype 42m+4sm+10st+4t and for S2: 48m+2sm+6st+4t. Total genome length varied significantly among the three populations with means of 137.4, 113.7 and 129.6 µm for S1, S2 and S3 respectively. The asymmetry index was similar for 1 and S3, which were different from S2.

Both cytotypes varied in proportion to the small metacentric, submetacentric and subtelocentric chromosomes, but were similar in the morphology of their long chromosomes. Nuclear DNA content and genome composition were equal in the plants collected from the three sites (2C-DNA = 8.499 pg and 1Cx = 4165), all diploid. Together these differences provide evidence for a process of differentiation among the populations owing to structural rearrangements in the group of small chromosomes.

The study of genetic variability of these three populations done by Sanchez-Teyer *et al*. (2009) indicated that the Genetic Similarity Index (GSI), including all adult plants and their rhizome offshoots, was 0.828. Narrow GSI average values were observed within (0.853) or between populations (0.834), with no significant differences. Average GSI values between mother plants and their clonal offshoots from S1, S2 and S3 were 0.905, 0.938 and 0.930 respectively. The value of expected heterozygosis and the proportion of polymorphic loci were slightly lower in S1 (0.250 and 64.5%) than in S2 (0.264 and 67.7%) and S3 (0.263 and 66.2%). A low value for the genetic structure among populations (0.175) and relatively moderate genetic flux of 1.18 were observed, giving rise to a moderate fixation index of 0.175 at the species level. The dendrogram generated from the GSI had three major groups that partially reflect the geographical distribution of the populations.

This genetic differentiation is in line with the geographic distances observed by (Barraza-Morales *et al*., 2006; Sanchez-Teyer *et al*., 2009) and could indicate that the wild populations of *A. angustifolia* grow in an allopatric manner with the isolating mechanism likely being physical barriers that prevent gene flow, with the result that the populations are differentiating.

The morphological variants could provide the basis for the selection of promising biotypes, taking into account the best characteristics and similarity between plants regardless of where they come from. The detection of plants that are genetically distinct from the others, with a different morphology, greater weight and higher stem TRS content, offers indicators that strengthen the process of selecting sources of germplasm, material for propagation, crops and processing.

CONCLUSION

Leaf dimensions and structure and the arrangement of teeth and apical spines (i.e., morphological variables not related to the age of the species), allowed for the differentiation of groups of plants. Weight and the total content of reducing sugars in the agave stem, added to the analysis of morphological variability, allowed us to identify at least two clusters of plants which could be selected as promising biotypes and a source of propagation material. This is the first study on the morphology and sugar content of *A. angustifolia* from the Sierra Madre Mountains in Sonora, Mexico. Taxonomic, genetic and biotechnology studies would be very useful for selecting specimens with desirable characteristics for the sustainable use of this biotic resource.

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