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Phenotypic analysis of mezcal agaves from the Central Valleys of Oaxaca

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ABSTRACT

Objective: Mezcal agaves in the state of Oaxaca have a high economic value due to the demand for mezcal production; therefore, the purpose of this work was to assess and highlight the importance of morphological diversity within and among cultivated Agave species.

Design/Methodology/Approach: A completely randomized experimental design with 25 treatments (populations) and 11 replicates (individuals) was implemented. The plant (individual) was the experimental unit and 19 morphological descriptors proposed by the National Seed Inspection and Certification Service (SNICS) were assessed. A total of 275 individuals of the *Agave angustifolia*, *Agave karwinskii*, *Agave marmorata*, *Agave rhodacantha*, *Agave potatorum*, *Agave seemanniana*, and *Agave nussaviorum* species were assessed using a multivariate analysis to determine their phenotypic variability and existing relationships.

Results: The dendrograms for the Q-mode and R-mode were obtained by means of a cluster analysis, forming 4 groups based on the average linkage generated from the standardized BDM of the sampled species of the genus *Agave*. Four groups were formed using the k-means clustering method, in accordance with field observations and a review of the taxonomic bibliography. The first two principal components (PC) accounted for 66.4% of the total variation, according to the principal component analysis (PCA). For PC1 and PC2, the variables with the highest contribution were those related to leaf shape (Fh), size of the lateral spine (FEL), number of leaves (Nh), plant height (H), uniformity in the size of the lateral spines (UTE), and terminal spine shape (FET).

Study Limitations/Implications: A comprehensive study requires taxonomic keys to identify species, subspecies, and even varieties of *Agave*. Additionally, molecular characterization is essential to understand the variability and phylogenetic relationships of these populations, subject to a phylogenetic analysis.

Findings/Conclusions: Multivariate analysis techniques revealed that three species showed high phenotypic variability in the maturation stage under cultivation conditions. The *A. potatorum* and *A. karwinskii* species had a greater intra-population phenotypic variability, with significant differences within the same species. *Agave marmorata* showed no intra- or inter-population variability. Leaf texture (Txh) was the only variable that explained the variation within its group. This is a tall species whose diameter is larger than in the other species. The variables of the Mexican (*Agave rhodacantha*) group showed low correlation, as their behavior was highly dispersed. The variables obtained in the field from this group of populations must be meticulously assessed to identify the degree of correlation between the variables and to confirm the behavior of this group.

Key words: Morphological diversity, multivariate analysis, hierarchical clustering, clustering, k-means clustering.

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INTRODUCTION

Mexico's agave diversity is unevenly distributed, despite the presence of native and cultivated populations in most of the country. In some areas, *Agave* can be very diverse. For example, up to 20 species coexist in the Tehuacán-Cuicatlán region (García-Mendoza, 2002, 2004, 2011). Meanwhile, the state of Oaxaca is home to the largest number of *Agave* species (43) (Mora *et al.*, 2011; León *et al.*, 2013). Other states with a significant number of *Agave* species include Jalisco (38), as well as Hidalgo, Veracruz, and San Luis Potosí (32 each) (Villaseñor, 2016).

Currently, agaves within the genus *Agave* are an economically important product, mainly due to their industrial characteristics, and are consequently in high demand. Therefore, generating scientific information is of vital importance to support their use and conservation.

Data analysis and morphological and molecular characterization studies are tools that help to efficiently manage accessions within a germplasm bank (Barrera-Guzmán *et al.*, 2020). The analysis of genetic variation between and within populations is fundamental for their conservation and genetic improvement. It provides estimators of the extent of available genetic variation, contributes to germplasm monitoring, and predicts potential genetic gains (Moreno-González and Cubero, 1993). A multivariate analysis can be conducted for this purpose, enabling the comprehensive study of the attributes or characteristics of the elements of a given population. Furthermore, it provides the opportunity to quantify the intensity of the influence or association of independent variables in a specific mathematical model or to use them as a starting point to research a given phenomenon on a dependent variable (Garbanzo and Navarro, 2016). Consequently, the objective of the present study was to expand the knowledge about the genetic diversity of genus *Agave*, through the implementation of a robust multivariate analysis.

MATERIAL AND METHODS

Location of the study area

The study area is in the areas of Central Valleys of Oaxaca where *Agave* is cultivated. Sampling was conducted in the municipalities listed in Table 1, which are recognized for the sowing and production of mezcal.

Experimental design

A completely randomized experimental design was used with 25 treatments (populations) and 11 replicates (individuals). The experimental unit consisted of one mature individual from each population, defining maturity as the presence or proximity of floral scape or a minimum age of five years (SNICS, 2014). Twenty-five samples were collected at different time points over a 5-month period.

Plant species used in the study

A phenotypic analysis was conducted on populations of seven species of *Agave* that formed the experimental unit in cultivated conditions. As shown in Table 1, locations were selected based on the existence of cultivated agave populations in the area.

Table 1. Identification and description of sampled agave populations.

P	Specimen ID	Ni/P	District	Crop location	Geographic coordinate	Alt.
1	*A_angustifolia*	11	Ejutla	San Agustín Amatengo	16° 29' 59" N, 96° 46' 36" O	1380
2	*A_angustifolia*	11	Ejutla	Agua del espino	16° 35' 25" N, 96° 48' 31" O	1422
3	*A_angustifolia*	11	Ocotlán	Santa Catarina minas	16° 47' 23" N, 96° 36' 16" O	1630
4	*A_angustifolia*	11	Ocotlán	San Dionisio	16° 44' 55" N, 96° 40' 11" O	1530
5	*A_angustifolia*	11	Ocotlán	San Dionisio	16° 29' 59" N, 96° 46' 36" O	1500
6	*A_karwinskii*	11	Ejutla	San Agustín Amatengo	16° 30' 59" N 96° 46' 32" O	1440
7	*A_karwinskii*	11	Ejutla	Agua del espino	16° 35' 45" N, 96° 48' 45" O	1457
8	*A_karwinskii*	11	Ejutla	La compañía	16° 35' 40" N, 96° 48' 49" O	1380
9	*A_karwinskii*	11	Ocotlán	Santa Catarina minas	16° 47' 5" N, 96° 36' 55" O	1560
10	*A_karwinskii*	11	Ocotlán	Santa Catarina minas	16° 47' 5" N, 96° 36' 55" O	1562
11	*A_seemanniana*	11	Ocotlán	San Dionisio	16° 44' 57" N, 96° 40' 5" O	1530
12	*A_potatorum*	11	Ocotlán	Santa Catarina minas	16° 46' 41" N, 96° 36' 58" O	1630
13	*A_nussaviorum*	11	Tlacolula	San Dionisio Ocotepec	16° 48' N, 96° 24" O	1670
14	*A_seemanniana*	11	Tlacolula	San Baltazar	16° 46' N, 96° 29' O	1548
15	*A_potatorum*	11	Tlacolula	San Dionisio	16° 48' N, 96° 24' O	1670
16	*A_rhodacantha*	11	Ocotlán	Santa Catarina minas	16° 47' 5" N, 96° 36' 55" O	1630
17	*A_rhodacantha*	11	Ocotlán	Santa Catarina minas	16° 47' 5" N, 96° 36' 55" O	1630
18	*A_rhodacantha*	11	Tlacolula	San Baltazar Guelavila	16° 46' N, 96° 29' O	1548
19	*A_rhodacantha*	11	Tlacolula	San Dionisio	16° 48' N, 96° 24' O	1670
20	*A_rhodacantha*	11	Tlacolula	San Baltazar Guelavila	16° 46' N, 96° 29' O	1548
21	*A_marmorata*	11	Ocotlán	Santiago Apóstol	16° 47' 58" N, 96° 42' 56" O	1440
22	*A_marmorata*	11	Ocotlán	Santa Ana Zegache	16° 48' 42" N, 96° 43' 48" O	1480
23	*A_marmorata*	11	Tlacolula	San Baltazar Guelavila	16° 46' N, 96° 29' O	1548
24	*A_marmorata*	11	Tlacolula	Tlacolula	16° 43' N, 96° 33' O	1600
25	*A_marmorata*	11	Tlacolula	Matatlán	16° 51' 48" N, 96° 22' 58" O	1718

P: population; Alt: altitude (meters above sea level); Ni/P: number of individuals/population.

Morphological variables

A descriptive analysis of 25 populations under cultivation conditions was conducted and the basic data matrix (BDM) was integrated. The study data consisted of 19 morphological variables, 10 of which were quantitative (QN) and 9 were qualitative (QL) (Table 2). These variables were recorded for the 275 SU (Study Units) and were obtained from the *Guía técnica para la descripción varietal de Agave* (Technical Guide for the Varietal Description of Agave) handbook developed by the National Seed Inspection and Certification Service (SNICS). The whole multivariate analysis was based on the differences between the SUs. Only 19 out of the 32 variables assessed in the technical guide provided an acceptable explanation, according to Barrientos (2019).

Data were collected using a flexometer, a tape measure, a ruler, and a vernier scale. A GPS was used to determine the coordinates and altitude of the sampled sites.

Table 2. Quantitative (QN) and qualitative (QL) variables assessed for the phenotypic analysis of agaves from the Central Valleys of Oaxaca.

Quantitative (QN) characteristics	Variable ID
Plant: height	H
Plant: rosette diameter	DIAM
Plant: number of leaves	Nh
Leaf: wide	Ah
Leaf: length	Lh
Leaf: size of the lateral spine in 10 cm	Tel
Leaf: number of lateral spines in 10 cm	Nel
Leaf: distance between the lateral spines	Del
Leaf: length of the terminal spine	Let
Plant: flowering initiation cycle	CIF
Qualitative (QL) characteristics	
Leaf: terminal spine shape	FET
Leaf: color intensity	Ich
Steam: visibility	VT
Leaf: shape	Fh
Leaf: texture	Txh
Leaf: shape of lateral spines	FEL
Leaf: profile of the lateral spine	PEL
Leaf: uniformity in the size of the lateral spines	UTE
Plant: growth habit	HC

Statistical analysis

Cluster analysis includes a set of techniques that are available during the installation of the R Project software (RStudio, 2021.09.0.0).

Q-mode and R-mode hierarchical clustering

Following Palacio *et al.* (2020) and based on a BDM (basic data matrix), the hclust () function and the method argument were used to determine the optimal clustering method to be applied. Additionally, the Pearson correlation coefficient was estimated, and a correlation matrix was generated using the RStudio statistical package.

The cophenetic matrix was calculated using the cophenetic function (), based on the new similarity matrix (SM). Based on these data, the Pearson correlation between the cophenetic matrix and the original SM was calculated, using the cor () function and the Pearson argument. This procedure was repeated with Ward's method (simple, average, and complete linkages). Table 3 shows the cophenetic correlation values obtained with the application of the different methods. The average linkage method was applied and the groups that included the study units (SUs) were formed using the Factoextra graphics package.

Table 3. Values of the cophenetic correlation coefficients (CCC) for Ward's methods (simple, complete, and average linkages), based on the BDM of the species of the genus *Agave*.

Linkage technique	CCC
Ward's Method	0.755528
Simple Linkage	0.870786
Average Linkage	0.887935
Complete Linkage	0.835104

The distortion of a linkage technique calculated by means of the Pearson correlation is deemed suitable when it has a ≥0.8 value. Since the highest value was obtained for the average linkage (0.887), this method was employed for the development of dendrograms.

The number of groups was determined using Figure 1 as a reference, which was generated using arguments from the standardized BDM and the fviz_nbclust() function of the Factoextra package, under the hierarchical clustering parameters. This image identifies four groups as the optimal minimum. From group 4 onwards, an abrupt decrease in intra-group variation was observed. Finally, the corresponding dendrogram was developed using the fviz_dend() function. To obtain a dendrogram in which the variables are clustered instead of the SU, the matrix is simply transposed (R-mode hierarchical clustering).

k-means clustering

This analysis was conducted using a classification provided by a hierarchical algorithm, in which the k-means clustering method was used without specifying the centers of the clusters. In the case of unknown centers, the k-means clustering method begins with a division of the data set into (x) randomly configured groups. Subsequently, the method seeks to improve this initial classification by reassigning the elements to the centroid of the nearest cluster or group, in order to reduce the average distance between each element of a group and its centroid (de la Fuente, 2011).

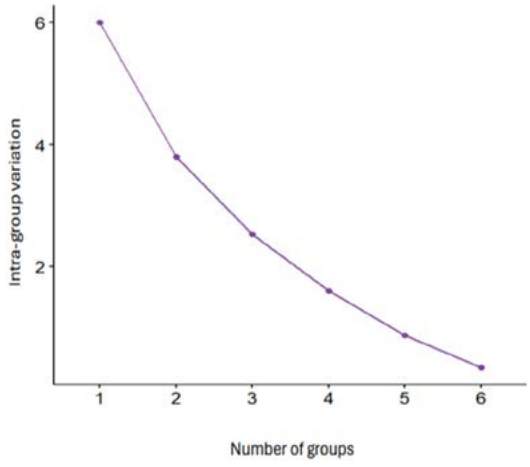

Figure 1. Number of groups *vs.* intra-group variation obtained with the function fvz_nbclust() on the hierarchical clustering of the *Agave* BDM.

Figure 2 shows the result of the *a posteriori* determination of the optimal number of groups applying the function fviz_nbclust() to the k-means clustering method. This figure illustrates the relationship between the number of clusters versus the sum of squares (intra-cluster variation), concluding that the optimal number of clusters is four.

The software selects the configuration with the lowest intra-cluster variation. Once the BDM has been standardized, the output results in a matrix (cluster means) whose values are the average of each standardized variable per group and the percentage of variation explained by the cluster (within-cluster sum of squares by cluster) and calculated as the variation between groups (between-SS) over the total variation (total-SS). In this case, this variation was calculated using the following formula:

$$\frac{between\_SS}{total\_SS} = 73.3\%$$

A principal component analysis was applied to visualize the k-means clustering result and to reduce the number of dimensions. The values of the first components were then used to create the graph (Kassambara and Mundt, 2017).

RESULTS AND DISCUSSION

Dendrogram of the hierarchical clustering (Q-mode)

The phased structure shown in Figure 3A suggests that the individuals under analysis exhibit significant differences between each SU.

The dendrogram resulting from the hierarchical clustering (Q-mode) —based on the average linkage generated from the standardized BDM of the species of the genus *Agave*— comprised four groups (Figure 3A). The first group in the dendrogram (from the bottom to the top) was formed at a distance of 1.2 with populations of the *Agave potatorum* (Figures 3A and 4E), *Agave seemanniana* (Figures 3A and 4F), and *Agave nussaviorum* species (Figures 3A and 4D). The second group was established at a distance of 1.4 with Tepeztate (*Agave marmorata*) populations (Figures 3A and 4A), with a low level of similarity to the rest of the

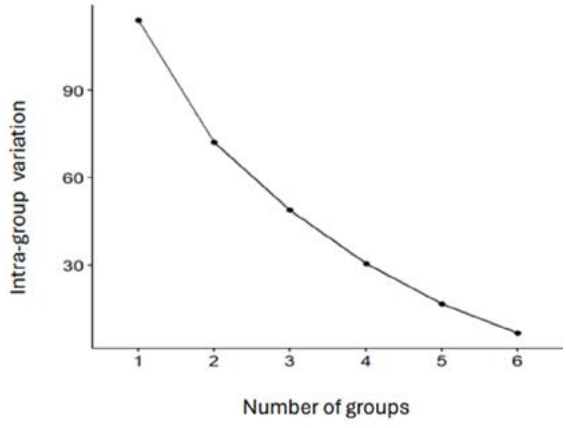

Figure 2. Number of groups *vs.* intra-group variation on the k-means clustering method applied to the *Agave* BDM.

groups. The third group was integrated at distance of 1.3 with populations of the *Agave karwinskii* species, commonly known in the region of Central Valleys of Oaxaca, including as Tobasiche, Kuish, and Marteño (Figures 3A, 4G, 4H, and 4I). The fourth group was structured at a distance of 0.80, with populations of the *Agave angustifolia* (Figures 3A and 4B) and *Agave rhodacantha* (Figures 3A and 4C) species. The dendrogram in Figure 3A indicates that the closest species (in phenotypic terms) are *A. angustifolia* and *A. rhodacantha*, while the most remote are *A. marmorata* and *A. karwinski*.

Dendrogram of relationships between variables (R-mode)

Figure 3B illustrates the relationships between the variables assessed in the dendrogram. Four groups were subsequently identified. The first group was formed at a distance close to 1.0 by the Nel, PEL, FET, UTE, Let, Tel, and Fh variables. In this group, the related variables were Nel (number of lateral spines) and PEL (profile of the lateral spine), in addition to FET and UTE, which belong to the lateral spine shape pattern and have a uniform lateral spine size. Furthermore, the Let, Tel and Fh variables demonstrated a high correlation between lateral spine size and leaf shape. The second group was formed at a distance of 0.70 by HC (growth habit), Nh (number of leaves), and VT (stem visibility). These variables were found to be highly related, since stem visibility implies that agave plants grow upwards and have a greater number of leaves. The third group was constituted at a distance of 0.95 by the Del (distance between the lateral spines), H (plant height), DIAM (rosette diameter), and Lh (leaf length) variables, based on which, leaf diameter and leaf length were observed to be highly related. Finally, a fourth group was formed at distance of 1.2, based on FEL (shape of lateral spines), CIF (flowering initiation cycle), Txh (leaf texture), Ah (leaf width), and lch (color intensity). The most closely related variables were DIAM (rosette diameter) and Lh (leaf length), suggesting that the greater the leaf length, the greater the rosette diameter.

The variable that distinguishes *Agave marmorata* from the other species is its rough leaf texture. For *Agave potatorum*, *Agave nussaviorum*, and *Agave semanniana*, the variable with the

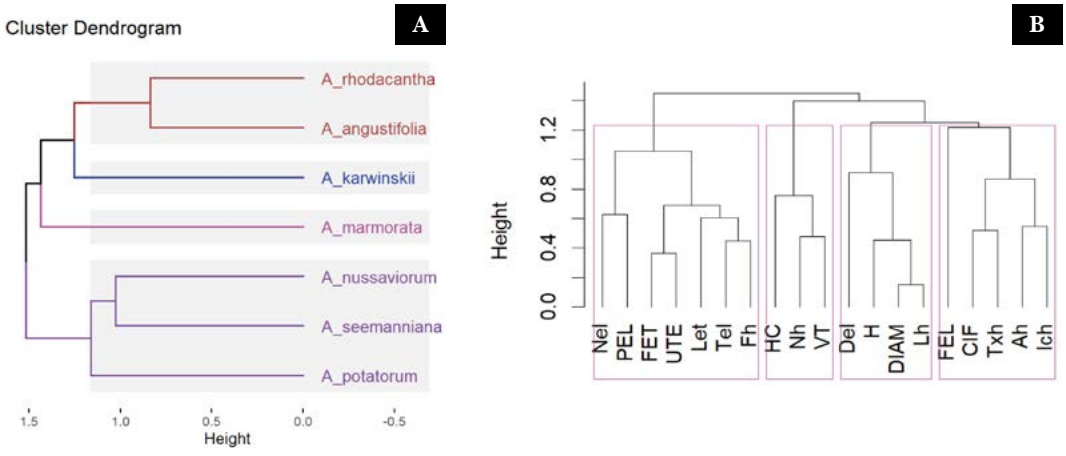

Figure 3. Dendrogram A: hierarchical clustering (Q-mode). Dendrogram B: (R-mode) based on the average linkage. Generated from the standardized BDM of the species of the genus *Agave*.

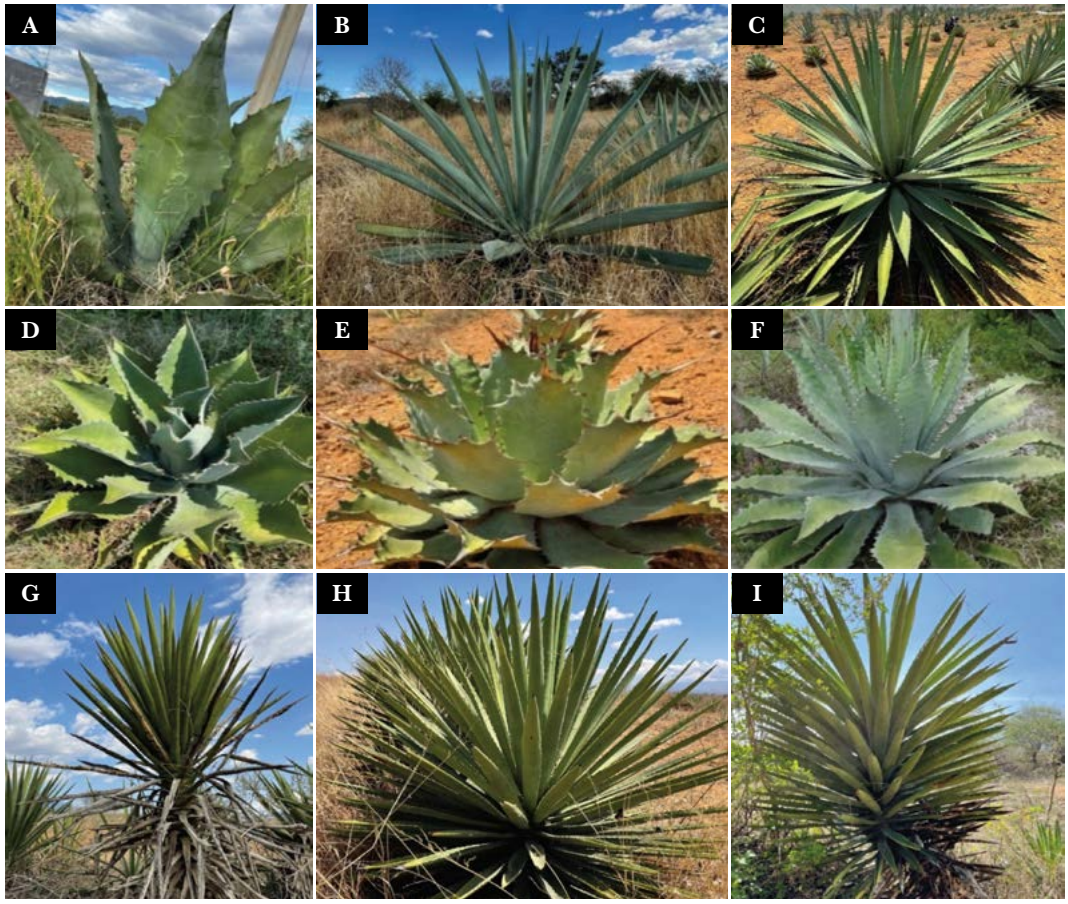

Figure 4. Phenotypes of the different species. A: Tepeztate (*Agave marmorata*, B: Espadín (*Agave angustifolia*), C: Mexican (*Agave rhodacantha*), potatorum group, D: *Agave nussaviorum*, E: *Agave potatorum*, F: *Agave seemanniana*, Karwinskii group, *Agave karwinskii*, G: Kuish, H: Barril, and I: Marteño.

highest correlation value was FET (terminal spine shape). These three species have curved terminal spines. The variables that distinguished *Agave karwinskii* were its growth habit and the number of leaves. In the case of the Mexican (*Agave rhodacantha*) group, the constituting variables were not correlated. However, these populations share a certain morphological similarity with the Espadín (*Agave angustifolia*) complex.

Hierarchical clustering analyses are useful tools for grouping agave specimens according to the similarities or dissimilarities of their morphological variables (Castro, 2010). In this study, quantitative and qualitative characteristics were assessed and morphological relationships among *Agave* species were identified. According to Rodríguez-Garay *et al.* (2009), additional genetic analysis would provide further information about the variation among populations and species.

K-means clustering

The K-means clustering analysis showed the integration of four distinct groups (Figure 5). The most distantly related group was constituted exclusively of the Tepeztate (*Agave marmorata*) species. A second group was formed with the Espadín (*Agave angustifolia*)

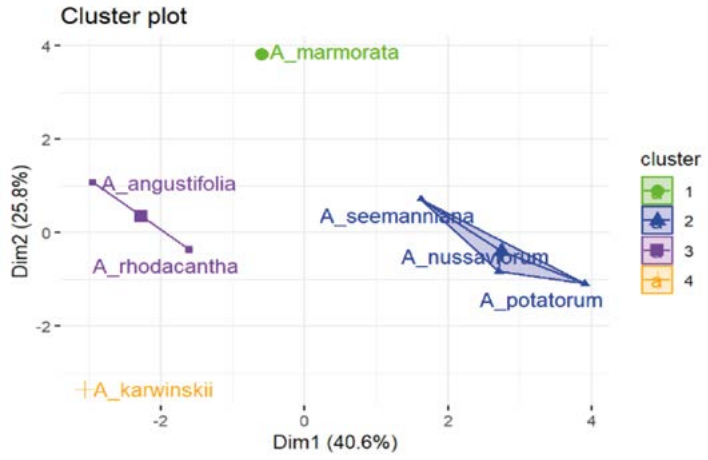

Figure 5. Groups of related *Agave* species, according to a k-means analysis using a principal component analysis. Percentages on the axes indicate the variation per component.

and Mexican (*Agave rhodacantha*) species. The third group was comprised of the *Agave seemanniana*, Agave potatorum, and *Agave nussaviorum* species, from the Tóbala group. Finally, the fourth group was integrated with *Agave karwinskii*.

The analysis of the assessed variables facilitated the determination of some morphological characteristics by means of which the populations and species of *Agave* will be differentiated. As stated by Narez-Jiménez *et al.* (2014), the demand for raw material for mezcal production has increased in recent years, making *Agave* spp. cultivars a valuable genetic resource that should be studied to improve their use and conservation.

CONCLUSIONS

The Q-mode hierarchical clustering analysis indicated that the closest *Agave* species studied are *Agave angustifolia* and *Agave rhodacantha*. In contrast, the R-mode hierarchical clustering analysis showed the relationship, selective value, and variation patterns of the characteristics assessed among the species. According to the K-means analysis, *Agave marmorata* was the least related species and had the lowest morphological variability; its rough leaf texture was defined as the differential variable of this species. The *Agave potatorum* and *Agave karwinskii* species recorded the greatest phenotypic variability. The populations of the Espadín (*Agave angustifolia*) group did not show morphological variability. Regarding *Agave potatorum*, *Agave nussaviorum*, and *Agave semanniana*, the FET (terminal spine shape) variable showed a high correlation value. The three species in question have curved terminal spines. The characteristic variables of *Agave karwinskii* are its growth habit and number of leaves. The variables of the Mexican (*Agave rhodacantha*) group showed no correlation, even though these populations share certain morphological similarities with the Espadín (*Agave angustifolia*) complex.

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