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Original Article

Phenotyping the Genetic Diversity of Wild *Agave* Species that Coexist in the Same Spatial Region

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Abstract

Phenotypic characteristics are important to identify species and provide valuable information for the uses in plant breeding. The aim of this study was to characterize through morphological traits the genetic diversity of the *Agave* genus under wild and semi-wild culture conditions in Maguey Largo region in Oaxaca, Mexico. Through field trips, eleven morphological characteristics of the *Agave* species were recorded. Principal component analysis (PCA), phylogenetic trees, and correlation analyses, were performed. Seven wild species were identified: *Agave potatorum* Zucc., *A. seemanniana*, *A. nussaviorum* subsp. *nussaviorum*, *A. angustifolia* Haw., *A. marmorata* Roezl., *A. karwinskii* Zucc. and *A. americana* var. *Americana.* Also, a semi-wild unclassified specie *Agave* sp. was found. The values of the first four principal components in the PCA explain more than 89% of the total morphological variance. The dendrogram of the agglomerative hierarchical clustering (AHC) shown a high similarity between the species and divide them in two main cluster with one unassociated specie (*A. karwinskii* Miahuatlán shape). Following the different analyses done, we observed a very close relationship between *A. potatorum* and *A. nussaviorum*, and dissociated from *A. seemanniana*, which are belonging to the "Tobala" complex and never described before. The results obtained in this work suggest a great genetic diversity expressed in a wide morphological variety of agaves in Oaxaca; which can be used in futures molecular studies.

Keywords: Amatengo shape, breeding, ecotypes, genetic variability, genotypes, mescal, *Tobala* complex, Oaxaca, Mexico

Introduction

The *Agave* genus belongs to the subfamily Agavoideae, which in turn is integrated into the Asparagaceae family, but it had suffered some modifications in its classification. From 1998 to 2009, following the Angiosperm Phylogeny Group (APG) classifications I and II, the *Agave* genus was considered into the Agavaceae family (APG, 2002). In 2010, with the implementation of the APG III classification and based on molecular DNA sequences, the *Agave* genus still was considered into the Asparagales order, but the Agavaceae family was removed and disappears from the list of families, then the *Agave* genus started to become a member of the Asparagaceae family inside the Agavoideae subfamily (Chase *et al.*, 2009).

The *Agave* genus is nesting about 200 species, out of them more than 150 species are in Mexico (commonly known as "Magueyes"), which is a country considered as the center of origin for the high genetic diversity found in this genus (Good-Avila *et al.*, 2006; APG, 2009). Diverse forms and genotypes at interspecific and intraspecific levels can be discovered; due that the agave have played an outstanding role in developing cultural and economic issues in Mexico, with a slightly impact in others countries where some species were also found (Delgado-Lemus *et al.*, 2014; Félix-Valdez *et al.*, 2016). The agave is distributed from the southern of United States to the north of Argentina, with an altitude that can be ranged from the sea level up to 3000 meters over the sea level, and even more (García-Mendoza, 2010). While, their habitat and niches are more diverse and can be found in xeric shrub, deciduous forest, and in forests of pine, oak and pine-oak (García-Mendoza, 2010). Morphologically, most of the species are well

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represented by having long leaves armed with thorns and inserted into a stem or "pineapple" which is used to produce various traditional alcoholic beverages. Agaves have different forms of reproduction, mostly asexual, they are long-lived plants and only have one chance in a lifetime to reproduce through sexual manner; stochastic events produced naturally could avoid this opportunity, which is why they have evolutionarily developed alternative methods to preserve their species (García-Mendoza, 2007). The forms of reproduction so far known is through stolon, bulbils and seeds. Some species are involving specific pollinators in sexual reproduction, but in general, most of the species are including hummingbirds, bees and bats as main pollinators (Aguirre-Dugua and Eguiarte, 2013).

The wild phenotypes of agaves in Mexico are well known by most of the native people by its common name; whereas, inside the scientific community around the world is more frequently to acknowledge at the specie level and in some cases the subspecies or varieties can be recognized by its morphology (Ramsay, 2004). Thus, ancient and traditional knowledge was passed through generations from father to son, to whom empirically liked the biodiversity (Toledo and Barrera-Bassols, 2008), but nowadays this tradition is going down due to the low interest of the new generations to preserve the *in situ* genetic diversity.

In general, the taste, shape, size and related features have been selected in traditional breeding programs to be improved (Félix-Valdez *et al*., 2016). Also, agave plant species containing large leaves were serving in construction to build houses; the thorns were used as needles. Another use of the agave is in the Traditional Mexican cuisine, where the leaves cuticle, leaves and flowers were used to prepare various traditional dishes. In mescal, tequila and pulque productions – which are of alcoholic beverages – the most common used agave plant species are belong to *Agave potatorum*, *A. angustifolia*, *A. tequilana* var. *azul*, *A. karwinskii* and *A. mapisaga* (García-Mendoza, 2007). The *Agave* genus has by them the benefit to obtain the evolutionary advantages to survive and to generate enough phenotypic diversity in order to coexist and be distributed in parallel way to a large number of different plant species (García-Mendoza, 2007; Mora-López *et al*., 2011; Félix-Valdez *et al*., 2016).

There are a variety of methods to study the genetic diversity of the species, one of these methods is the karyological studies in which is possible to know the genome size, nuclear DNA content, the chromosome number and the shapes of chromosomes (Moreno-Salazar *et al*., 2007). Another method is the molecular markers, used to identify DNA deletions, insertions, mutations and inversions, moreover to develop the exact nucleotide sequences, as well as to the identification of genetic fingerprinting and isolation of genes, among other benefits (González-Hernández *et al*., 2012; Eguiarte *et al*., 2013).

On the other hand, the morphological or phenotypic markers are basically to quantify the tangible and visible expression of the genetic diversity, that for example, in ancient communities were using this characterization to select important economical characters (Eguiarte and González, 2007; Mora-López *et al*., 2011).

In recent years with the development of genetic engineering and the support of traditional or classical methodologies, the scientific society has relatively elucidated the complexity of this economically important genus. In Mexico, and especially in Oaxaca State, who has the first place in mescal production, there were few studies on the phenotype of wild and semi-wild agave species. Most of the information in agaves is coming from species and varieties under production conditions to obtain mescal and tequila. Despite this, is important to continue the studies about *Agave* species, which are not used to produce alcoholic beverages, in order to know the genetic diversity, present in wildlife as an important tool to carry out plant breeding programs in this genus and to preserve the gene pool that have an important role in Oaxaca ecosystems (Mora-López *et al*., 2011; León *et al*., 2013).

Due to the plasticity to environmental conditions described to the species of *Agave karwinskii* and *Agave macroacantha* reported by Flores-Maya *et al*. (2010) in previous studies to Zapotitlán Salinas region in Puebla, we assume that Oaxaca must to have a wide phenotypic variability in this genus. This study aims to characterize through phytogeographic traits the genetic diversity of the *Agave* genus and to evaluate the morphological variability under wild and semi-wild culture conditions of the *Agave* species co-existing in Maguey Largo community, in the municipality of San José del Progreso, belonging to Oaxaca state on Mexico country.

Materials and Methods

Study site

The study area was located in "Maguey Largo" community, in the municipality of San José del Progreso District of Ocotlán de Morelos, Oaxaca state in Mexico. The prospected area took part of the Sierra Madre del Sur (16° 41′ N and 96° 41′ W); with an altitude between 1600 and 2900 meters above sea level (MASL), the predominant vegetation was pasture and forest (INEGI, 2005).

Prospection, characterization and identification of collected accessions

During the second half of 2015 and first quarter of 2016, botanical explorations were conducted in the study area, the coordinates and height above sea level of each collection using a free browser were documented (Google, 2015). All evaluated plants were selected according to its age, following the criteria of morphologically mature age (close to the time to start flowering and calculated by the central core of leaves); therefore, this age was specie–specific to each collected accession. The morphological characteristics of the *Agave* accessions collected were recorded. First of all, the species were identified by the common names known in the region. In order to quantify the genetic diversity present in the prospected region, eleven phenotypic characteristics commonly used to describe the agave plants [Plants High (PH), Plants Diameter (PD), Leaf "D" Width (LDW), Leaf "D" Length (LDL), Leaf "D" Thickness (LDT), number of lateral spines (NLS), lateral spacing-thorns (LST), length of lateral spines (LLS), length of terminal thorn (LTT), diameter of terminal thorn (DTT) and number of leaves (NL)], were considered. By definition, in plants that define a rosette from the expanded leaves borne by the stem, the leaf "D" is the younger of the old leaves and the older of the young leaves.

The morphological characteristics mentioned above were measured with a green flexible meter biomaterials (WOLFOX trademark, model WF9492) and an electronic digital caliper (Starrett trademark, model 798A-6/150). In the laboratory, with the help of dichotomous keys and images, the botanical identification was carried out and after that were planted in the experimental area of the Centro Interdisciplinario de Investigaciones para el Desarrollo Integral Regional (CIIDIR) Unidad Oaxaca.

Data analysis

To identify the species were used the CONABIO (2006) catalog "Mescals and Diversity " and also the dichotomous keys published by García-Mendoza (2010); whereas to characterize the newest species from *Agave potatorum* complex (or Tobala complex) the packet 88 from "Flora del Valle Tehuacán-Cuicatlán" and the dichotomous keys published by García-Mendoza (2011), were used.

Spreadsheets with the measurements to each variable were prepared. The means as central tendency and standard deviation as dispersion were calculated. The coefficient of variation expressed as a percentage (CV%) was also calculated. In addition to a principal component analysis (PCA), a correlation matrix from the characters and between species, a dendrogram of similarity constructed with the agglomerative hierarchical clustering (AHC) based on Pearson correlation coefficient, and finally a heat map showing the relationships and significance between the *Agave* species and the phenotypic characteristics evaluated, were developed through the XLSTAT Software (version 2015.6.01.23865).

Results

Morphological characterization shows the existence of seven wild species: The *Agave potatorum* Zucc. (Fig. 1a), *A. nussaviorum* var. *nussaviorum* Garcia-Mend. (Fig. 1b), *A. seemanniana* Garcia-Mend. (Fig. 1c), *A. angustifolia* Haw. (with four shapes or varieties, called: Pasmo (Fig. 1d), Mexicano, Barril and Cuishe), *A. marmorata* Roezl. (Fig. 1e), *A. karwinskii* Zucc. (with two shapes: Miahuatlán and Amatengo) and *A. americana* var. *Americana* (Table 1). Also, a semi-wild unclassified specie the *Agave* sp. (Pulque), was found. In addition to that, the different agave species identified that were collected and evaluated at different altitudes (Table 1 and Fig. 1).

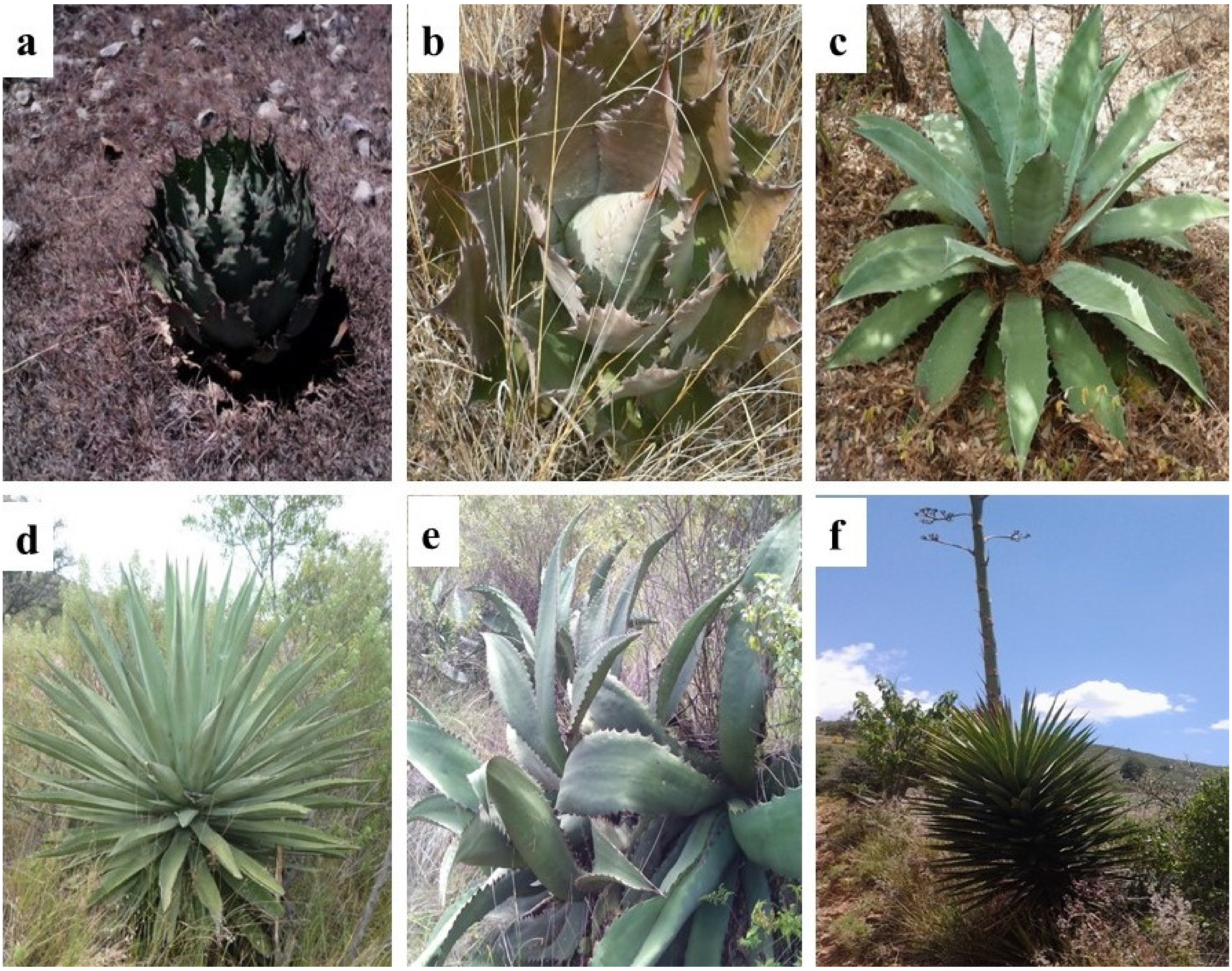

Fig. 1. Representation from different *Agave* species under wild culture condition in Maguey Largo region: (a) *Agave potatorum*; (b) *Agave nussaviorum* subsp. nussaviorum; (c) *Agave seemanniana; (d) A. angustifolia Haw. (Pasmo shape); (e) A. marmorata Roezl.; (f)A. karvinskii Zucc.* (Miahuatlan shape)

Table 1. Agave species by common and scientific names that were identified and evaluated at different altitudes in Maguey Largo region from Oaxaca state in México

Common name	Scientific name	Altitude (MASL)
Maguey Tobala	Agave potatorum Zucc.	1690 to 2350
Maguey Tobala	Agave seemanniana García-Mend.	1700 to 2400
Maguey Tobala	Agave nussaviorum García- Mend.	2200 to 2500
Maguey Tobasiche (Amatengo shapes)	Agave karwinskii Zucc.	1600 to 1900
Maguey Tobasiche, Maguey Largo or Madrecuishe (Miahuatlán shapes)	Agave karwinskii Zucc.	1600 to 1900
Maguey Mexicano	Agave angustifolia Haw.	1680
Maguey Barril	Agave angustifolia Haw.	1850
Maguey Pasmo	Agave angustifolia Haw.	1720
Maguey Cuishe	Agave angustifolia Haw.	1780
Maguey Tepeztate	Agave marmorata Roezl.	1700 to 1900
Maguey de Pulque	Agave sp.	1650
Maguey Coyote	Agave americana var. americana L	1750

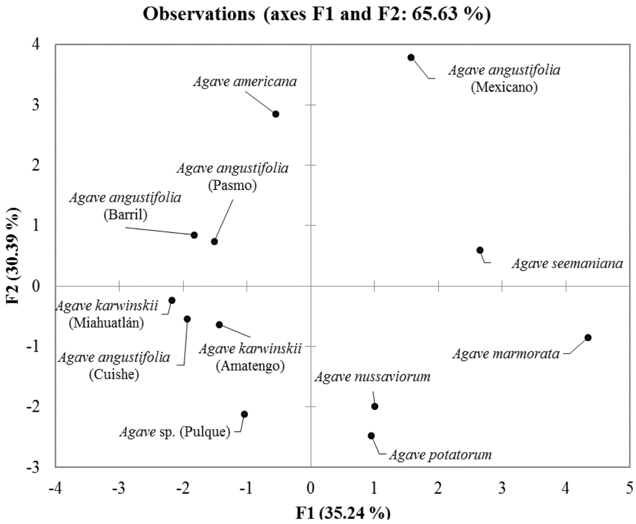

Fig. 2. The principal component analysis (PCA) plot of the eleven phenotypic variables evaluated in the eight *Agave* species, under wild culture conditions in Maguey Largo region. The analysis of factors extraction from the PCA was performed by Pearson (n) method. The percentage of variation explained by each factor is given between parentheses in the axis legend, whereas, the percentage of total variation from both factors are given in parenthesis in plot legend

Analyzing the phenotypic features, we observed that *Agave karwinkii* (Miahuatlán shape, Fig. 1f) was the highest (up to 259 cm), while *Agave* sp. (Pulque) shown the lowest size with only 43 cm high (Table 2). Four out of eleven evaluated characters the specie *A. marmorata* obtained the highest values in: width (19.8 cm) and thickness (27.9 mm) of leaves, lateral spacing-thorns (4.78 cm) and length of lateral spines (8.5 mm; Table 2). This specie was one of the evaluated agave species that shown a large age to reach its biological maturity under wild culture conditions, this could be one of the reasons why some character average means were superior to the rest of the species. Another particular characteristics of *A. marmorata* were the leaf mechanical resistance to support its proper weight and the homogenous distribution of the lateral spines (Fig. 1e).

The specie *A. angustifolia* (Mexicano shape) follow the specie *A. marmorata* with higher values in three characters: diameter of the plant (257 cm), length of leaves (153 cm) and diameter of the terminal thorn (10.0 mm, Table 2).

Table 4. Eigenvectors of the first four components of quantitative characteristics evaluated in the eight *Agave* species found in Maguey Largo region in Oaxaca, Mexico

Features/components	F1	F2	F3	F4
Plant High (PH)	-0.144	0.436	0.120	0.447
Plant Diameter (PD)	0.078	0.529	-0.003	-0.069
Leaf "D" Width (LDW)	0.468	0.090	-0.125	0.138
Leaf "D" Length (LDL)	0.071	0.523	-0.141	-0.052
Number of Leaves (NL)	-0.376	0.010	0.184	0.577
Leaf "D" Thickness (LDT)	0.290	-0.216	-0.097	0.633
Number of Lateral Spines (NLS)	-0.324	0.280	-0.255	0.049
Lateral Spacing-Thorns (LST)	0.329	0.092	-0.478	0.040
Length of Lateral Spines (LLS)	0.470	-0.045	0.133	0.158
Length of Terminal Thorn (LTT)	0.142	0.006	0.669	-0.078
Diameter of Terminal Thorn (DTT)	0.264	0.336	0.384	-0.067

Interesting results were obtained in *A. potatorum* complex (or Tobala complex), where the common name of "Tobala" have been applied indistinctly to the three species (*A. potatorum*, *A. nussaviorum* and *A. seemanniana*) belonging to the complex, but they have differences in some characters (high of the plant, number of leaves and length of the terminal spine) for the rest of the characters its behavior was pretty similar (Table 2). The specie of *A. seemanniana* shown superior mean values (compared inside the complex) in most of the characters, except for the number of leaves (NL = 34), the number of lateral spines (NLS = 35.5) and length of terminal thorn (LTT = 3.95 cm), where *A. potatorum* got the higher values with 46, 65.3 and 5.3 cm, respectively. While, *A. nussaviorum* had an intermedia behavior for those three evaluated parameters with NL = 36, NLS = 43.8 and LTT = 4.22 (Table 2). The length of terminal thorn was considered an important characteristic, in order to get the agave plants protection (mechanical protection) against the predators, mainly ruminants (Table 2).

The principal component analysis (PCA) was used to identify the most outstanding characters (Fig. 2). Inspection of the first two principle components, which account for 65.63% of the total variance within the data set allowed the classification and association of the species according to the global analyses of the characters; for example, the species of *A. potatorum* and *A. nussaviorum*, were clustered in the same quartile, and *A. seemanniana* was includes relatively close to the others two species belonging to the complex; or in the case of the accessions from *A. karwinskii* that were clustered in the

Table 2. Phenotypic characterization of *Agave* species collected in Maguey Largo region from Oaxaca state, Mexico. Range of the measurements recorded to the main characteristics (plant, leaf "D", lateral spines and terminal thorn) with its calculated standard deviation (SD) and coefficient of variation (CV) to the eight-species found in the prospected area

Species	Plants		Number of leaves	Leaf "D"			Lateral spines			Terminal thorn	
	High (cm)	Diameter (cm)		Length (cm)	Width (cm)	Thickness (mm)	Number	Spacing-thorns (cm)	Length (mm)	Length (cm)	Diameter (mm)
Agave seemaniana. ("Maguey Tobala" with high size and open shapes*)											
Range	96-122	187-208	30-38	73-84	15-17	8-12	33-38	1.5-1.8	5-9	3.4-4.5	9-10
Mean± SD	109±13	197.5 ±10.5	34±4	78.5±5.5	16±1	10±2	35.5±2.5	1.65±0.15	7±2	3.95 ±0.55	9.5±1
CV%	11.92	5.31	11.76	7	6.25	20	7.04	9.09	18.18	13.92	10.52
Agave nussaviorum subsp. *nussaviorum* ("Maguey Tobala" with small size and opened shapes*)											
Range	44-62	56-105	32-40	32-50	7.5-11	10-16	29-60	1.1-2.5	4-11	2.5-5	3-7
Mean± SD	55.2 ±6.87	78.2 ±18.72	36±3	36.6±7.5	8.72 ±1.43	12.8 ±2.77	43.8 ±11.7	1.76±0.57	6.2±2.77	4.22 ±1.01	5±1.41
CV%	12.44	23.91	8.33	20.49	16.39	21.64	26.73	32.38	44.67	23.93	28.2
Agave potatorum ("Maguey Tobala" with intermedia size and closed shapes*)											
Range	64-97	72-113	29-60	37-53	6-14	13-23	38-114	0.5-2.7	3-15	3.5-6	1-6
Mean± SD	77.11 ±10.6	90.3 ±12.9	46±10	45.44 ±5.43	9±3.37	19.22 ±4.9	65.3 ±29.1	1.6-0.65	6.22±3.52	5.3 ±1.02	4.33±1.41
CV%	13.85	14.38	21.73	11.94	37.44	25.80	44.56	40.62	56.59	19.24	32.56
Agave marmorata Roezl ("Maguey Tepeztate")											
Range	83-118	80-182	12-14	69-116	8-26	25-33	36-54	4-5.7	4-15	1.5-2.6	3-7
Mean± SD	94.1 ±12.4	127 ±32.9	13.25 ±0.8	83.6 ±17.05	18.9 ±5.4	27.9 ±2.23	45.9 ±6.40	4.78±0.74	8.5±2.87	2.03 ±0.43	4.8±1.03
CV%	13.17	25.9	6.2	20.39	28.57	7.99	13.94	15.48	49.48	21.18	21.45
Agave americana ("Maguey Coyote")											
Range	108-221	167-320	37-58	109-166	7.7-11	2	92-140	2-4	2-6	2.2-3.7	4-6
Mean± SD	169.5 ±29.6	244.7 ±43.6	47±7.38	133 ±16.70	9.34 ±1.03	2±0	110 ±13.67	2.88±0.53	4.61±1.2	2.99 ±0.51	4.76±0.59
CV%	17.46	17.81	15.7	12.55	11.02	0	12.42	18.40	26.03	17.05	12.39
Agave karwinskii Zucc ("Maguey Tobasiche, Maguey largo or Madrecuishe" with Miahuatlán shape and the tallest sizes*)											
Range	122-259	46-140	135-162	41-91	3.3-7	2-17	30-88	0.5-2.6	2-4	2-5	2-7
Mean± SD	207.5±38	106.7 ±22.2	148±8.5	58.72 ±3.8	5.20 ±1.28	9.13 ±4.9	63.5 ±15.2	1.71±0.67	3.13 ±0.83	3.38 ±0.68	4.54 ±1.18
CV%	18.31	20.8	5.74	6.47	24.61	53.66	23.93	38.18	26.51	20.11	25.99
Agave karwinskii Zucc. ("Maguey Tobasiche" with Amatengo shape and intermedia sizes*)											
Range	109-131	93-152	97-120	56-73	5.5-11	19-28	56-108	1-2.5	3-7	1.5-6	3-6
Mean± SD	122.83 ±8.0	119 ±29.03	103 ±12.2	61.33 ±7.28	7.41 ±2.28	22.33 ±3.2	66.66 ±20.6	1.66±0.6	4.16 ±1.47	3.86 ±2.8	4.16±1.32
CV%	6.57	24.39	11.84	11.87	30.760	14.6	30.9	36.14	35.33	72.53	31.73
Agave angustifolia Haw. ("Maguey Barrilito")											
Range	125-192	140-213	102-110	76-150	5-10.2	6-11	76-114	1.4-2.5	3-3	2.1-2.3	4-5
Mean± SD	154.1 ±23.8	173.3 ±25.8	106.5 ±2.9	102 ±26.75	7.03 ±1.77	9±1.67	91.3 ±14.1	1.73±0.40	3±0	2.18 ±0.07	4.16±0.40
CV%	15.5	14.88	2.77	26.22	25.17	18.55	15.44	23.12	0	3.44	9.15
Agave angustifolia Haw. ("Maguey de Pasmo")**											
Range	175	170	112	79	10	15	104	1.4	3	2.5	5
Mean ±SD	175	170	112	79	10	15	104	1.4	3	2.5	5
CV%	-------	--------	--------	--------	--------	--------	--------	--------	--------	--------	--------
Agave angustifolia Haw. ("Maguey Mexicano" with mexican shapes)											
Range	182-215	244-270	12-14	142-165	10-14.5	3-3	74-78	3-3	5-5	4-4.5	10-10
Mean± SD	198.5 ±23.3	257 ±18.38	13.05 ±0.78	153.5 ±4.03	12.25 ±1.7	3±0	76±1.68	3±0	5±0	4.25 ±0.59	10±0
CV%	11.75	7.15	6.1	2.62	14.53	0	2.2	0	0	13.88	0
Agave angustifolia Haw. ("Maguey Cuishe")											
Range	69-115	101-136	58-75	58-82	5-6	6-6	86-108	1-1.4	3-4	1.8-2.1	4-5
Mean± SD	94.2±19	120.6 ±16.4	65±5.8	71.6 ±9.86	5.8 ±0.44	6±0	93.6 ±8.87	1.2±0.15	3.2±0.44	1.9 ±0.15	4.2±.44
CV%	20.16	13.6	8.92	13.77	7.58	0	9.47	12.5	13.75	7.9	10.47
Agave sp. ("Maguey Pulque")**											
Range	43	82	12	45	8	3	64	2.7	2	1.7	2
Mean± SD	43	82	12	45	8	3	64	2.7	2	1.7	2
CV%	--------	--------	--------	--------	--------	--------	--------	--------	--------	--------	--------

*Comparing accessions belonging from the same specie or 'Tobala' complex

**Only one plant of this specie was found in whole prospected region

Table 3. Total variance explained to each Eigen factor (component)

Component	Initial eigenvalues		
	Total	Variability (%)	Cumulative (%)
1	3.524	35.24	35.24
2	3.039	30.39	65.63
3	1.499	14.99	80.62
4	0.916	9.16	89.78

Table 5. Correlation matrix of the eleven phenotypic characteristics evaluated in the eight *Agave* species under wild culture conditions in Maguey Largo region in Oaxaca, Mexico

Variables	PH	PD	LDW	LDL	NL	LDT	NLS	LST	LLS	LTT	DTT
PH	1										
PD	0.661	1									
LDW	-0.113	0.322	1								
LDL	0.665	0.942	0.271	1							
NL	0.533	-0.134	-0.612	-0.169	1						
LDT	-0.253	-0.324	0.553	-0.291	0.160	1					
NLS	0.488	0.424	-0.467	0.479	0.346	-0.376	1				
LST	-0.038	0.208	0.667	0.376	0.578	0.336	-0.203	1			
LLS	-0.260	0.066	0.802	0.034	0.552	0.642	-0.615	0.477	1		
LTT	0.046	0.039	0.040	-0.069	0.099	0.034	-0.352	-0.256	0.424	1	
DTT	0.381	0.672	0.534	0.551	0.284	-0.050	-0.244	0.071	0.454	0.462	1

DTT – Diameter of Terminal Thorn, LST – Lateral Spacing-Thorns, LDL – Leaf "D" Length, LDT – Leaf "D" Thickness, LDW – Leaf "D" Width, LLS – Length of Lateral Spines, LTT – Length of Terminal Thorn, NLS – Number of Lateral Spines, NL – Number of Leaves, PD – Plant Diameter and PH – Plant High.

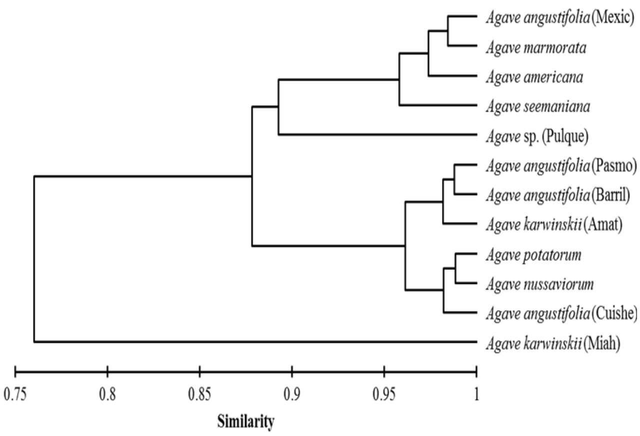

Fig. 3. Dendrogram of the eight Agave species and its shapes constructed with the agglomerative hierarchical clustering (AHC) from genetic distances based on similarity of Pearson correlation coefficient using the agglomeration method of unweighted pair-group average, and shown the relationships between them. Genetic distances were based on the eleven phenotypic characteristics evaluated

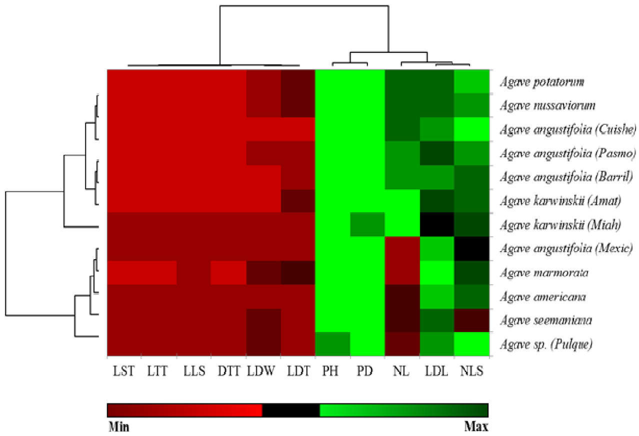

Fig. 4. Hierarchical clustering analysis showing the relationships and significance between the Agave species and the phenotypic characteristics evaluated in Maguey Largo region. Abbreviations following the scientific names means: Amat = Amatengo, Mexic = Mexicano and Miah = Miahuatlán. While the characteristics mean: DTT (Diameter of Terminal Thorn), LST (Lateral Spacing-Thorns), LDL (Leaf "D" Length), LDT (Leaf "D" Thickness), LDW (Leaf "D" Width), LLS (Length of Lateral Spines), LTT (Length of Terminal Thorn), NLS (Number of Lateral Spines), NL (Number of Leaves), PD (Plant Diameter) and PH (Plant High)

same quartile (Fig. 2). The analysis shown that the first component (F1) explains 35.24% of the total variance (Table 3), and was mainly influenced by the length of lateral spines (LLS = 0.470 eigenvalue) and leaf "D" width (LDW = 0.468 eigenvalue: Table 4). The second component (F2) explain a 30.39% of the total variance (Table 3), and was subjective to the plant diameter (PD = 0.529 eigenvalue) and the leaf "D" Length (LDL = 0.523 eigenvalue; Table 4). The third and fourth components (F3 and F4) contributed to explain a 14.99% and 9.16% of the total variance (Table 3), and were mainly determined by the length of terminal thorn (LTT = 0.669 eigenvalue) and the leaf "D" thickness (LDT = 0.633 eigenvalue), respectively (Table 4). Total statistical contribution of the four components above described was 89.78% of the total variance explained (Table 3).

Following the correlation coefficient among the evaluated variables was possible to infer that high phenotypic variability occur, with some interactions between them; although, few of them were influencing negatively in one character over the other (Table 5). The higher positive correlation (0.942) value between plant diameter (PD) and leaf "D" length (LDL), was observed. In the opposite extreme, the leaf "D" width (LDW) and number of leaves (NL) recorded the higher negative correlation (- 0.612) value (Table 5), meaning that the species with high number of total leaves decrease the size of the leaves, e.g., *A. potatorum* (NL = 46 & LDW = 9 cm) vs. *A. seemaniana* (NL = 34 & LDW = 16 cm).

A high level of similarity between species was observed. The higher similarity correlation (0.995) based on Pearson correlation coefficient between *A. marmorata* and *A. angustifolia* (Mexicano), was perceived. While, the lower similarity correlation (0.601) based on Pearson correlation coefficient between *A. karwinskii* (Miahuatlán) and *Agave* sp. (Pulque), was observed. But in general, the similarity among the evaluated agave species was high, showing a representation of the phenotypic diversity observed in the prospected area, with a special emphasize in the *A. potatorum* complex, where *A. potatorum* and *A. nussaviorum* revealed 0.971 coefficient of similarity; however, the correlation between *A. nussaviorum* and *A. seemanniana* was 0.889 of similarity.

Those similarities also were expressed in the relationship between species with the dendrogram (Fig. 3), forming specific clades according to the similarity matrix.

Two principal clades were found in the agave species. The first clade was integrated by four species: *A. angustifolia* (Mexicano), *Agave marmorata*, *A. Americana* var. *americana* and *A. seemanniana*. The second clade was recognized by two sister clades of four agave species too; one sister clade integrated by two species with three accessions of the *A. angustifolia* (accessions: Barril and Pasmo) and *A. karwinskii* (Amatengo), and the second sister clade was composed by *A. potatorum*, *A. nussaviorum* and *A. angustifolia* (Cuishe) (Fig. 3). The second clade was nesting three out of the four accessions from *A. angustifolia*, and two species belonging to the *A. potatorum* complex (*A. potatorum* and *A. nussaviorum*). Finally, two unclustered species the *Agave* sp. (Pulque) and *A. karwinskii* (Miahuatlán). Although, the *Agave* sp. (Pulque) could be included in the first mentioned cluster as a relative far species (Fig. 3).

A great variability within the *Agave angustifolia* was observed, with four different phenotypes, which were not grouped into the same clade despite belonging to the same taxon, just as occurs with both types of *A. karwinskii* (Fig. 3). Grouping the species was according to their phenotype and each formed clade coincides with the morphological similarity observed. This information reveals a large phenotypic variation in a considerably small area as compared to the Oaxaca state.

A heat map grouping the characters and species based on Euclidean distances is summarized in Fig. 4. Between columns were forming two main clusters, and the colors was ranged from red to green through black, meaning that information from color scale was from lowest to highest values, respectively. In this analysis, the agave species were clustered in three clades pretty similar to the dendrogram, but with some variations that can be translated as an astringency of the analysis (Fig. 4). Paid attention the case of the specie *A. karwinskii* (Miahuatlán), which almost was unclustered, but with the goodness that both accessions from this species were clustered in the same clade (Fig. 4).

To further explore global effects of the phenotypic characteristics evaluated on the *Agave* species that were sorted by hierarchical clustering analysis (Fig. 4). In the green part of the cladogram were associated the variables that provide more information and in red part those with less representation and influence without any difference between them (Fig. 4). The diameter and height of the plant (PH and PD), along with the number of lateral spines (NLS), leaf "D" length (LDL) and number of leaves (NL) were the ones who provided more information with high incidence over the agave species (Fig. 4). The cluster formed by the rest of the characters (red part of the cladogram) the phenotypic information was dispersed with less (or null) incidence over the agave species (Fig. 4). With the information obtained from the principal component analysis, the dendrogram of similarity and the heat map we can confirm the high phenotypic variation found in Maguey Largo region to agave species that coexist under the same habitat conditions in wildlife.

Discussion

The *A. potatorum* complex mentioned by García-Mendoza (2010), is comprised by three different species [*A. potatorum*, *A. nussaviorum* (var. *nussaviorum* and var. *deltoidea*) and *A. seemanniana*], with similar morphological characteristics, which were called in Maguey Largo region as the same common name of "Tobala" (Table 1). In this work were identified and evaluated those three species (Table 2), except for *Agave nussaviorum* var. *deltoid*ea, who was not possible to observe its presence in the prospected region.

León *et al.* (2013) working in phenotypic characterization of agave species in the municipality of San Miguel Tilquiapam in Oaxaca state, reported the presence of three species with four accessions: *Agave karwinskii* (accessions: Miahuatlán and Amatengo shapes), *A. marmorata* (accession: Maguey Becuela) and *A. potatorum* (accession: Maguey Tobala), similar to the ones we found and evaluated in this study to the region of Maguey Largo. Main differences of the *A. karwinskii* accessions previously reported and ours was the height of the plants. For example, to Amatengo shape (reported PH = 43.62 cm vs. ours PH = 122.83 cm) and Miahuatlán shape (reported PH = 57.13 cm vs. ours PH = 207.5 cm), respectively; which was a noticeable difference in this phenotypic characteristic, we assumed that this difference was due to the altitude and ecological adaptation. Even though, to the leaf "D" width, lateral spacing-thorns and number of lateral spines followed similar pattern in the reported in León *et al.* (2013) that in our study. Flores-Maya *et al.* (2010) conducted a phenotyping and karyological analyses, measuring six variables in *A. karwinskii* from the Tehuacan-Cuicatlán Valley; and the values to the evaluated variables were plant diameter (66.6 cm), number of leaves (62.4), length and width of the leaves (37 cm and 3.6 cm), length of terminal and lateral thorns (2.6 cm and 0.42 cm), pretty different almost twice in this work (Table 2). These differences for those characteristics can be inferred by the genome plasticity of *A. karwinskii* and/or the presence of different genotypes (sometimes called ecotypes) or varieties (Colunga-García *et al.*, 2007).

In the case of *Agave marmorata* reported by León *et al.* (2013), the length and width of the leaves was 142 cm and 32.3 cm, respectively, contrary to the accession of this species that we described in Maguey Largo region (Table 2) that scored significantly lower values for these characteristics, and the opposite occurred in the lateral spacing-thorns, they reported 1.5 cm, while in this study was 4.78 cm; and the means of the number of lateral spines remained relatively constant in both studies, around 45.

León *et al.* (2013) reported 12 lateral spines per leaves in *A. potatorum* low to the amount we informed in this work (Table 2), while the rest of parameters evaluated were in ranges. García-Mendoza (2010), in a taxonomic study of the *A. potatorum* complex mentioned that the measure of terminal thorn was 3-4 cm long, and the value we obtained here was 5.3 cm, this deference was mainly due to the study location and the genotype (ecotype) evaluated.

Flores *et al.* (2014) conducted a three-year study to produce seedling from *Agave cupreata* Trel et Berg., in order to obtain a selective population that exceed the mean average, and were carrying principal component analysis and correlation matrix with some of the morphological characteristics also described in our study; and concluded that the most vigorous agave plants were selected specifically for their phenotype (i.e., number of leaves, plant diameter, plant high, and length and width of the leaves). This type of improvement from phenotype (large and vigorous healthy plants) has been made by ancient cultures for centuries in a wide variety of taxa, which has led many cultivated species to its current state and will be our next step.

Morales *et al.* (2008) conducted a principal component analysis (PCA) in two populations of *Agave tequilana* Weber var. Azul and reported the first and second components for both populations, concluding that there were an intra- and inter-population differences, with the leaf area, leaf length, plant height and number of leaves the characters with more descriptive values for both populations. In the present study the variables that provided more descriptive value were the high and diameter of the plant, length of leaves and number of lateral spines (Table 3 and 4). These and other characters' match those mentioned by the scientists or experts to explain during the field trips how to identify the phenotypes or variants.

To obtain a global view of the differences between the plant species and across the treatments, Kazachkova *et al.* (2013), applied the principal component analysis (PCA), and the inspection of the first two components accounted for 78.5% of the total variance that allowed classification of samples by species. Our PCA showed that species was the predominant factor explaining differences between them with 65.63% of the total variance explained (Fig. 2).

In many parts of the world, scientists have been studied morphology coupled with genetic studies in species of economic importance for agronomic improvement, identifying characters of importance. Hajibarat *et al.* (2014), in a study of genetic diversity characterization by microsatellite and phenotypic characterization with seven quantitative variables of a local chickpea varieties in Iran, found that the morphological variability was very high in one local variety, which was useful for the improvement and to develop the phenotypic diversity of the specie. In correlation with this work where the diversity in *A. angustifolia* specie was pretty high and gave different relationships between its accession (Fig. 3). A similar study was conducted by Rana *et al.* (2015) where they observed that the ratio of length and diameter of the fruits, persistent calyx in mature fruit, fruit surface and texture of the flesh were important in detecting the morphological variations between pear species.

The global effects of the phenotypic characteristics evaluated on the *Agave* species, were sorted by hierarchical clustering analysis (Fig. 4), and the species were clearly clustered according to the main phenotypic trait. However, hierarchical clustering analysis demonstrated that within each agave species could be separated on the basis of the phenotypic characteristics evaluated (Fig. 4). Because all these species were under the same wild culture condition and evaluated at the same time and similar age. Like in *Arabidopsis taliana*, Kazachkova *et al.* (2013) studied different treatment and growth platform and determined that the samples were clearly clustered according to the species first, and also clear subclades were observed separated by growth platform. Also, similar results were reported in *Eutrema salsugineum* by Guevara *et al.* (2012).

According to the above and with the results we can conclude that there is great genetic diversity expressed this in a wide morphological variety of agaves in the community of "Maguey Largo", this due to the great native diversity that has characterized Mexico and specifically Oaxaca and can be applied in the handling and use of these species in traditional breeding or for its usefulness and complementarity with molecular markers as the exploitation of the species analyzed and their genetic improvement in one of the states with the highest production of mescal in Country. The results obtained in this work suggests the need to take actions in order to protect and preserve the genetic diversity of these species.

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