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Genetic variation and relationships among agaves related to the production of Tequila and Mezcal in Jalisco

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ABSTRACT

The study of evolutionary history allows us to examine diversification, selection and domestication processes. Mexico belongs to Mesoamerica, one of the world's most important centers of origin and diversification of plants. One of the plants that has sustained its peoples for over 10,000 years is the agave (*Agave* sp.). Mexico is the center of diversity of the genus, with 75% of the species. Two agave products, tequila and mezcal, are of great economic and biocultural importance for Mexico. The description of genetic diversity and the identification of the wild relatives of the agave species used to produce these emblematic beverages is fundamental information for their production and conservation. Previous studies have proposed wild populations of *A. angustifolia* in Jalisco as possible wild relatives of blue agave or tequila (*Agave tequilana*). We use microsatellite (eight loci) to study the genetic diversity and the relationships between wild populations of *A. angustifolia* and traditional cultivars of the *Agave* species utilized in the production of tequila and mezcal in Jalisco. The studied taxa present intermediate genetic variation, with the exception of *A. tequilana* "Azul" which had the same genotype. A Structure analysis indicates that the "Azul" is closely related to *A. angustifolia* mainly to wild populations from southern Jalisco. *Agave rhodacantha* and the cultivars of *A. tequilana* ("Sigüin" and "Chato") form a group separate from *Agave angustifolia* y *A. tequilana* "Azul".

1. Introduction

Mexico is a megadiverse country with complex evolutionary histories owing to the existence of different landscapes due to the presence of mountains, sierras and its geographical location both north and south of the Tropic of Cancer (Rzedowski, 1978). In addition, Mexico's biodiversity has increased as a result of the rich cultural interaction of its diverse indigenous groups (Hernández-Xolocotzi, 1993; Casas et al., 2007). One of the plants that has given Mexicans a number of resources for thousands of years is the maguey or agave (*Agave* sp.), making these plants important elements of Mexico's biocultural diversity (Gentry, 1982).

The genus *Agave* L. is endemic to the American continent and includes 200 species (García-Mendoza, 2011). Mexico, its center of diversity, has 159 species of which 119 are endemic (García-Mendoza, 2011). Agaves have served human communities in Mexico for the last 10,000 years (Callen, 1965); during that time the plant has been utilized for various purposes (Gentry, 1982). For food, its flowers, fruits, stalks and inflorescences' peduncles have been harvested. To produce beverages, the sap flowing to the inflorescence – called aguamiel – has

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been used; this sap, when fermented, becomes the pulque. Starting from either pulque or the fermented juice of the agave's cooked stalks, the distilled beverages known generically as mezcals (the well-known tequila being one of them) are made. From it leaves' fibers many goods have been produced such as ropes and dresses; its leaves and floral peduncles have been used as construction materials. For *A. angustifolia* Haw. alone, the species with the widest distribution, Colunga-GarcíaMarín and May-Pat (1993) reported the current use of all its morphological structures in 40 different ways.

In the nineteenth century and beginning of the twentieth the industrial production of tequila grew and with it a marked preference for producing it with blue agave (*Agave tequilana* F.A.C. Weber "Azul"), given its shorter life-cycle and higher sugar content compared to other traditional cultivars such as "Sigüín", "Chato", "Bermejo", "Pata de Mula", "Zopilote", "Mano Larga" and others (Pérez, 1887; Diguet, 1902; Gentry, 1982). In 1974, the Tequila appellation of origin was established (Diario Oficial de la Federación, 1974, 1997), according to which it can only be produced from the "Azul" variety and from a certain geographical area, which currently comprises five states: Jalisco, Guanajuato, Michoacán, Nayarit and Tamaulipas (Consejo Regulador del Tequila, 2016).

In 2016, 273.3 million liters of tequila were produced nationwide, starting from 941.8 metric tons of raw material (Consejo Regulador del Tequila, 2016). The production of tequila is increasing, though with strong phytosanitary challenges due to the susceptibility of agaves to diseases and pests. This is probably related to its low genetic variation –the result of 400 years of asexual propagation- requiring the use of large amounts of pesticides and fertilizers (Eguiarte et al., 2013). The reduced genetic diversity of the blue agave in Jalisco (0.8% polymorphism) has been reported by some authors (Gil-Vega et al., 2001; Vargas-Ponce et al., 2009). An alternative option for facing the latter problem is the increase the agave's adaptive potential against diseases and global climate change through an increase in genetic diversity. The genetic diversity of the "Azul" agave could be increased through the use of its wild relatives; these could present greater genetic diversity than the cultivar and be incorporated by means of genetic flow. The "Azul" agave, however, is only know in cultivation and its wild relatives are unknown. There are some hypotheses about the possible wild relatives of the blue agave which we describe here below.

First hypothesis: "*Agave tequilana* "Azul" is distinguished from its close relatives, *A. angustifolia* by its larger leaves, thicker stems and heavier, more diffusive panicles of relatively large flowers with tepals long in proportion to the relatively short tube. Since these differences are of degree rather than of distinct contrast, their separation as a species is nominal, but appear tenable for the Rigidae, where species are so difficult to define. Currently, the commercial trade with this economically important plant will profit by the maintenance of a simple binomial. Plants closely related to *A. tequilana* grow wild on the semiarid slopes west and south of Tequila, as along the road from Cocula to Tecolotlán, Jalisco (Gentry, 1982, pp. 583–584)."

Colunga-GarcíaMarín and Zizumbo-Villarreal (2007) propose that the use of Filipino distillers brought to the Pacific coast of the Mexican state of Colima for the distillation of coconut spread to the slopes of Colima volcano for the distillation of agaves, where a center of managed agave diversification for the production of mezcal was established. As for the origin of tequila, they propose:

Second hypothesis: "if the foothills of the volcanoes of Colima was the area in which the Filipino still was adapted to the distillation of traditional fermented drinks from agaves in west Mexico, then […] it is in this area that the ancestral populations of the native cultivars and their greater diversity can be found" (Colunga-GarcíaMarín and Zizumbo-Villarreal, 2007, pp. 1656).

Zizumbo-Villarreal and Colunga-GarcíaMarín (2008) reiterate that the selection of wild plants for the production of mezcal o tequila could have taken place in the foothills Colima volcano. Subsequently, Zizumbo-Villarreal et al. (2009b) point out that the distillation of agave could have ocurred in the same area, but since precolombian times. The distillation process would have been different (and for ceremonial purposes) by using a type of clay still called "Capacha still", which was developed from bean cooking pots.

In Jalisco, one of the main sources of mezcal plant germoplasm are the plants, obtained by peasants searching in ravines in their vicinity, that can be transplanted in their plots and be useful for the production of mezcal. Subsequently, the collected plants propagate by offsets (asexual reproduction). The constant recruitment and propagation of wild plants has generated various cultivars as well as plots containing a variety of them. In these plots, cultivars are safeguarded and maintained. However, if these varieties are left unexploited, they can disappear, as is the case of some *A. tequilana* cultivars (Vargas-Ponce et al., 2007).

One of the ways of identifying wild relatives and the genetic relationship between traditional cultivars is through the use of molecular markers (Gepts, 1993; Morrell et al., 2012). In the case of the agaves, it has not been an easy task (Good-Avila et al., 2006; Eguiarte et al., 2013). One phylogenetic hypothesis, generated with some genes and intergenetic cDNA, indicates that the genus *Agave* is very recent (6–10 million years, Good-Avila et al., 2006). Additionally, the genetic flow between agave populations, mediated by pollinators such as bats, which can fly over 100 km in a single night, is very high. This obscures the resolution of the genetic relationships between the species (Good-Avila et al., 2006; Eguiarte et al., 2013).

In various studies, different molecular markers have been used to study kin relationships between the "Azul" agave and other *Agave* species. Some have tested directly and indirectly Gentry's (1982) hypothesis on the relationship between the "Azul" agave and *A. angustifolia*, as well as its relationship with populations of this species in Jalisco and other Mexican state where it is naturally distributed. The results have not been consistent, even when the same molecular markers were used. Nevertheless, the species that most frequently turn out to be close to the "Azul" agave are *A. angustifolia* and *A. rhodacantha* (Gil-Vega et al., 2001, 2006; Torres-Morán et al., 2008; Vargas-Ponce et al., 2009; among others). In these works, markers that analyze DNA sections with a high mutation rate, which could change from one generation to the next, have been utilized (Zietkiewicz et al., 1994). Microsatellite markers, which have a much slower mutation rate than the markers used so far, have not been used (Slatkin, 1995). The microsatellites could reflect a time window long before the transplantation of plants to plots.

Recently, the use of microsatellites (SSR) in *Agave* for the study of intra- and interspecific genetic variations (Parker et al., 2010; Lindsay et al., 2012) has been reported. Microsatellites were also used to track the wild populations from which cultivated agaves come from (Parker et al., 2010). By means of microsatellites to carry out analyses of kin relationships (genealogies) and the analysis of genetic flow, it is possible to identify the origin (wild populations) of the cultivars or the wild relatives. Based on eight SSR molecular markers, we carried out the first approximation of the analysis of genetic variation and relationships of the agaves in Jalisco, Mexico, which are used in the production of tequila and mezcal in that state.

2. Material and methods

2.1. Study system

We analyzed traditional cultivars used in the production of tequila and mezcal in central and southern Jalisco belonging to three species: *A. angustifolia*, *A. tequilana* and *A. rhodacantha*; as well as wild populations of *A. angustifolia* that might be related to the blue agave, following the hypotheses of Gentry (1982) and Colunga-GarcíaMarín and Zizumbo-Villarreal (2007). Not all the traditional Mezcal and Tequila cultivars that have been reported (Pérez, 1887; Diguet, 1902; Gentry, 1982) were included, due to their limited or null presence in the field.

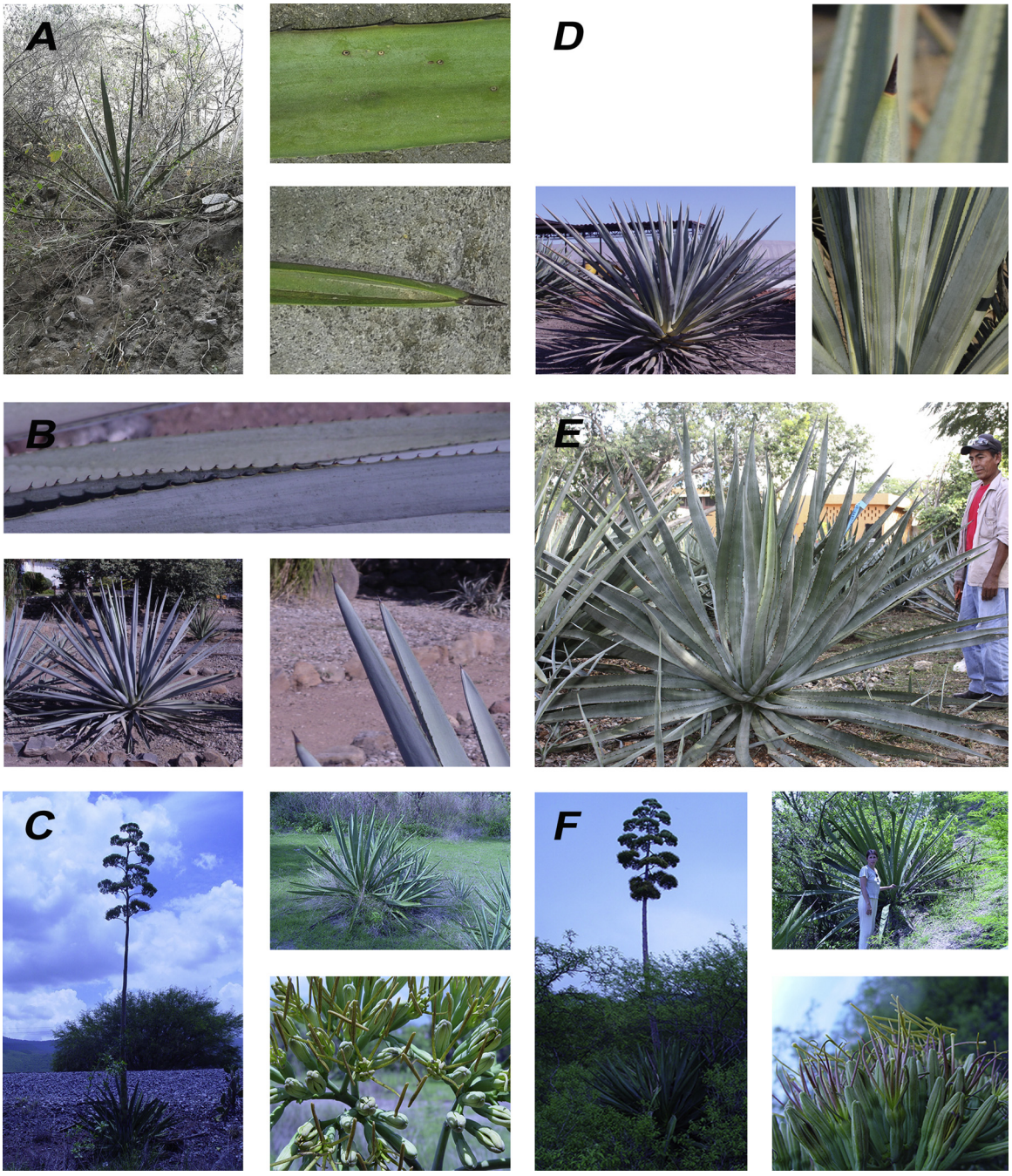

Fig. 1. Individuals of A) *Agave angustifolia*, B) *A. tequilana* "Azul", C) *A. tequilana* "Sigüín", D) *A. tequilana* "Chato", E) *A. angustifolia* "Ixtlero Verde", F) *A. rhodacantha* "Ixtlero Amarillo", collected in Jalisco.

2.1.1. Wild A. angustifolia populations

Agave angustifolia is the species with the largest distribution of any *Agave* species, ranging from the Mexican states of Sonora and northeast Tamaulipas to Panama along the Pacific and Atlantic coasts. It is distributed in tropical, pine and pine-oak forests, as well as xerophilous scrubland between 300 and 1800 m above sea level (Gentry, 1982; García-Mendoza, 2011). It has been considered to represent a species complex that shows morphological variation throughout its range (Gentry, 1982), that has not been studied in detail (Fig. 1A). The plants form rosettes and offsets, are from 1.50 to 2.00 m high and 1.5–2.0 m wide. Leaves: 40–120 per individual, 60–120 cm long and 3.5–10.0 cm wide; lanceolate, rigid, concave, light green to glaucous (Gentry, 1982; García-Mendoza, 2011).

2.1.2. Agave tequilana F.A.C. Weber "Azul" (tequila, blue agave)

It is worth noting that the taxonomic identity of traditional cultivars such as *Agave tequilana* F.A.C. Weber "Azul", from which tequila is produced, does not formally exist under the rules of the International Code of Nomenclature for Cultivated Plants. For this reason, we use in this work a nomenclature that would have to be evaluated later through taxonomical studies.

Known only in cultivation (Fig. 1B), it features cespitose rosettes which can reach 1.2–1.8 m high and 1.3–1.8 m wide. Its leaves are lanceolate, striate, concave, 90–125 cm long and 8–12 cm wide. A peculiar characteristic of this species is its bluish or greyish green color (Gentry, 1982).

2.1.3. *Agave tequilana F.A.C. Weber "Sigüín"*

Diguet (1902) reports it as both wild and cultivated. This traditional cultivar is the most similar to the blue agave (Fig. 1C); it is distinguished from it by its narrow and thick leaf bases, a similar number of leaves with a more open angle and a spherical head. Cespitose rosette, 5–6 m high and 4–5 m wide. Leaves: 80–90 per individual, greenish blue to glaucous grey, lanceolate, rigid, 1–1.2 m long and 8–12 cm wide (Valenzuela and Nabhan, 2003).

2.1.4. *Agave tequilana F.A.C. Weber "Chato"*

This cultivar is also known in Jalisco as "Sahuayo", in reference to the town in Michoacán where it supposedly originated (Fig. 1D) and where it is processed to produce mezcal. As with "Sigüín" it was used to produce tequila in the namesake valley before the establishment of the Norma Oficial Mexicana (NOM). Acaulescent rosette, cespitose, surculose, 1.70–2.0 m high. Each individual can have 80–100 rigid leaves, 150–200 cm long and 15–16.5 cm wide, light green to greyish blue-green (Valenzuela and Nabhan, 2003).

2.1.5. *Agave angustifolia Haw. "Ixtlero Verde"*

This species is characterized by its 180–250 cm wide rosette; numerous long and narrow leaves, 130–182 cm long, 7–11 cm wide; Green color (Fig. 1E). It produces a large amount of fiber and can live up to 25 years. It is also known as mezcal bravo or ixtlero and is one of southern Jalisco's oldest traditional cultivars (Vargas-Ponce et al., 2007; Carrillo-Galván, 2011).

2.1.6. *Agave rhodacantha Trel. "Ixtlero Amarillo"*

One of southern Jalisco's most widely used traditional cultivars for the production of mezcal (Fig. 1F), it is also popular as living fences and for its long and strong fibers (Vargas-Ponce et al., 2007). They are large (211–250 cm high) and robust plants; their leaves are long (160–200 cm) and narrow (9–13 cm), abundant, yellowish-green (Carrillo-Galván, 2011).

2.2. Sampling

In this study three species were analyzed: *A. angustifolia*, *A. tequilana* and *A. rhodacantha*, represented by a total of 237 individuals, including plants from eight wild *A. angustifolia* populations (158 individuals) in central and southern Jalisco, plants representing three traditional cultivars traditionally used in the Tequila valley for the production of tequila ("Azul", "Sigüín" and "Chato"; 45 individuals) in intensive fields of Tequila (central Jalisco); and two traditional cultivars used to produce mezcal in southern Jalisco ("Ixtlero Verde" and "Ixtlero Amarillo"; 34 individuals) (Fig. 2; Table 1) in traditional plots. The plant material was collected between 2003 and 2014 and it was either frozen or dried in silica and sent to the Centro de Investigación Científica de Yucatán (CICY), where they were stored in ultrafreezers at −80°C.

2.3. Study area

The study area is located in western Mexico, in the state of Jalisco, where a warm and sub-humid climate prevails; the locality is dominated by igneous rocks, and derived soils, substrates that have been reported as favorable for the establishment of agaves (Vázquez-García et al., 2007). In the central part of Jalisco (between 20°–21 °N, 103°–104 °W and altitude between 1050–1709 m) the predominant vegetation is mixed pine-oak forest and tropical deciduous forest, in the southern part of Jalisco (19 °N, 103 °W and altitude between 793–1709 m), where mescal are made, the tropical deciduous forest is predominant.

2.4. DNA extraction, SSR amplification and gels

DNA was extracted by following the CTAB (Cetyl Trimethylammonium Bromide) protocol (Doyle and Doyle, 1987), modified with silica (Echevarría-Machado et al., 2005). We used seven microsatellite primers (SSR) designed for *A. parryi* as reported by Lindsay et al. (2012), and five for *A. tequilana* as developed by Cabrera-Toledo of Guadalajara University (Unpublished). For this study were eliminated three pairs (P1027, P1659 and P7741) described by Cabrera-Toledo because they were unspecific in amplified bands; and one pair (APARLC21) designed by Lindsay also was eliminated because it was monomorphic in all populations.

For the amplification, we used a GeneAmp PCR system 9700 thermocycler (Applied Biosystems) with the following parameters: denaturation at 95 °C/5 min, followed by 35 cycles at 95 °C/1 min; 59 °C/90 s for APARLC11, APARLC12 and APARLC35; 60 °C for APARLC20, P1448 and P1763; 57 °C for APARLC28; 58 °C for APARLC28 and 72°/1 min, and finally an elongation period of 72°/7 min. The final volume of the amplification mixture was 25 µl; each reaction contains 10–100 ng of DNA, 5 µl 10× of buffer, 0.5 µl of 10 µmol/L dNTPs in an equimolar ratio, 1 µl of each of the primers, 50 mM $MgCl_2$, and 1.5 unit of Taq polymerase. The PCR products were run in a 1% agarose gel (0.75 agarose in 75 ml of TBt buffer 0.5×), stained with 3.5 µl ethidium bromide and run for 30 min at 100 V. After running, the bands were observed in a UV transilluminator.

Subsequently, 5% vertical acrylamide gels were used to separate the fragments obtained through PCR, for which a 1 min 95 °C denaturation cycle was used. The gel was run at 2000 V, 50 mA and 60 W for 1:15–1:30 h, depending on band size. A 10pb molecular weight marker was added. Once the gel ran, it was fixed by immersion in 10% acetic acid in agitation for 20 min. The gel was then washed twice for 5 min with distilled water; subsequently, it was stained with silver nitrate (2 g in 2000 ml distilled water plus 3 ml formaldehyde) and shaken gently for 20 min. The gel was developed with a sodium carbonate solution (60 g Na_2CO_3, 3 ml formaldehyde and 60 µl sodium thiosulfate). The band size was determined by reading with a white light transilluminator.

2.5. Diversity and genetic differentiation analysis

For each species, a homozygote (one band) or heterozygote (two bands) matrix was developed per individual, all being diploid. With these data, we obtained the polymorphic loci and allelic frequencies through the POPGENE 32 ver. 1.32 program (Yeh and Yang, 1999).

The observed heterozygosity (H_O) was estimed using the adegenet library in R. For the wild populations, the expected (H_S) and total expected heterozygosity (H_T), was estimated by using the Hickory ver. 1.1 program (Holsinger and Lewis, 2002). For this program, which uses Bayesian statistics to estimate the parameters use the *f* model indicated co-dominant markers and the lowest Deviance Information Criterion (Spiegelhalter et al., 2002). The run parameters were: 5000 burn-in iterations, followed by 25,000 iterations and sampling values every 5th iteration. We carried out a total of 10 runs which proved to be convergent.

We estimated the Weir and Cockerham's (1984) Θ genetic differentiation estimate and its 95% confidence interval by using Hickory ver. 1.1 (Holsinger and Lewis, 2002). We also estimated the paired R_{st} by means of the Arlequin ver. 1.1 program (Schneider et al., 1997).

We carried out a molecular variance analysis (AMOVA) by using Arlequin ver. 1.1 (Schneider et al., 1997) to determine whether the genetic variation distribution of *A. angustifolia* is structured between the central and southern regions of the state of Jalisco. To determine the existence of an isolation by distance pattern, a Mantel test was carried out by using the ade4 package (Dray and Dufour, 2007) in R. The geographical distances were calculated with the fossil package in R (Vavrek, 2011).

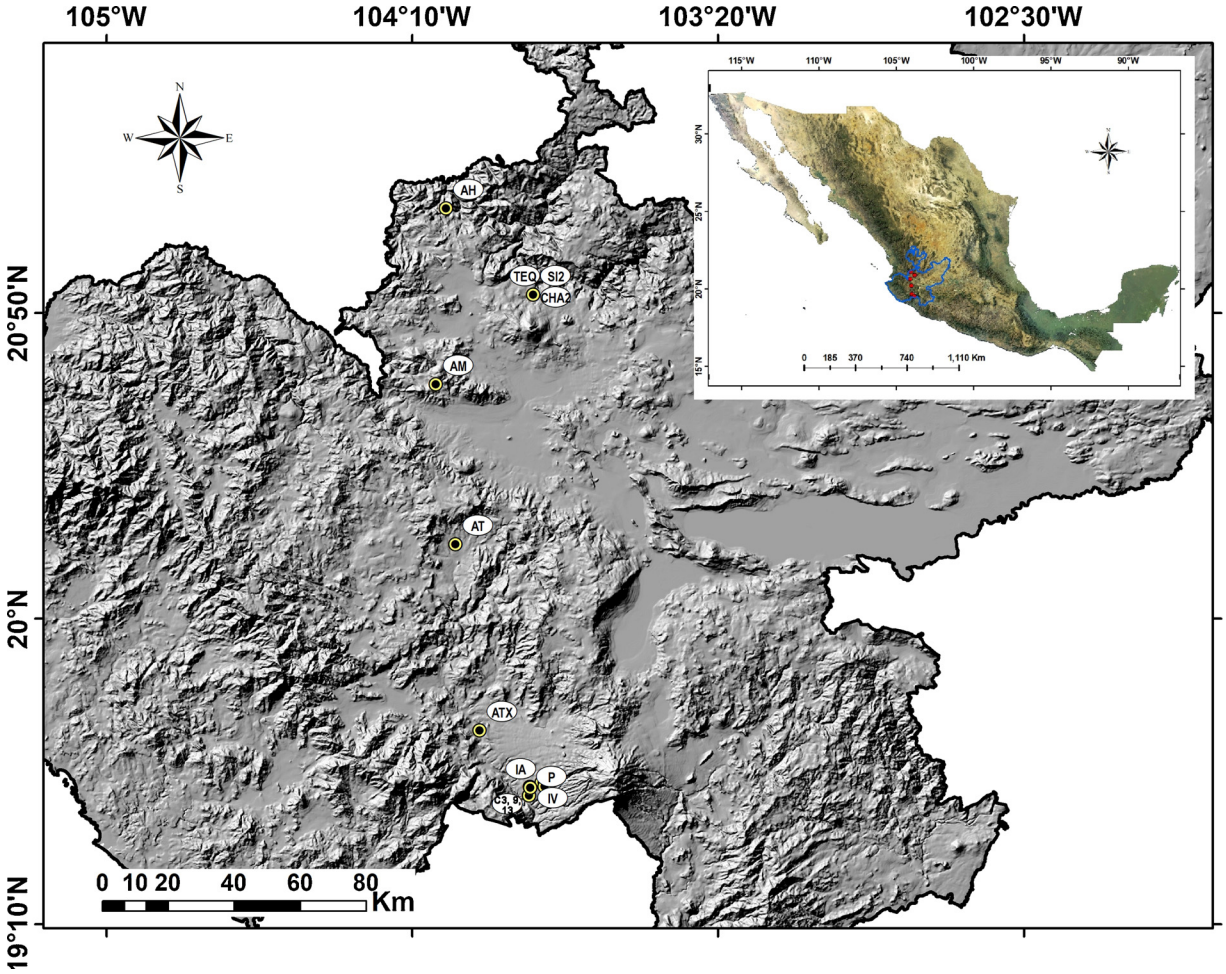

Fig. 2. Populations of *Agave angustifolia*, *A. tequilana* "Azul" and *A. rhodacantha* analyzed in this work.

2.6. Genetic relationships

The paired Nei (1987) distances between populations were calculated with adegenet package in R (Jombart, 2008). A dendrogram was generated by using the UPGMA method and the obtained distances using the ape and adegenet pakages in R base package was used (R Core Team, 2013; Jombart, 2008; Paradis et al., 2004).

Genetic relationships were further analyzed using a principal components discriminant analysis from the allelic frequencies. For this, we use six principal components (87.5% of the variation) and three discriminant functions with the adegenet package (Jombart, 2008).

An ancestry analysis was conducted by means of Bayesian statistics using the STRUCTURE ver. 2.2 program (Pritchard et al., 2000; Falush et al., 2003, 2007). We used the admixture model because the constant carrying of pollen between populations by bats cause the agaves to be in constant genetic interchange. We ran 200,000 Markov Chain Monte

Table 1
Collecting data and genetic diversity indices.

Species	Wild/ Cultivar	Location	Code	Latitude N	Longitude W	Altitude (msnm)	N[a]	H_S	P	Number of alleles
A.angustifolia	Wild	Hostotipaquillo	AH	21°07'06"	104°04'28"	1050	30	0.453 ± 0.008	100	19
A.angustifolia	Wild	Ameca	AM	20°38'18"	104°06'08"	1600	30	0.455 ± 0.015	100	18
A.angustifolia	Wild	Tecolotlán	AT	20°12'10"	104°02'56"	1709	24	0.453 ± 0.012	100	21
A.angustifolia	Wild	Tuxcacuesco	ATX	19°41'39"	103°58'58"	793	20	0.389 ± 0.011	87.5	16
A.angustifolia	Wild	Zapotitlán	C3	19°33'12"	103°48'50"	1124	10	0.376 ± 0.021	100	16
A.angustifolia	Wild	Zapotitlán	C9	19°32'28"	103°50'43"	959	10	0.372 ± 0.021	75	14
A.angustifolia	Wild	Zapotitlán	C13	19°32'32"	103°48'22"	1138	9	0.361 ± 0.011	62.7	14
A.angustifolia	Wild	Perempitz	P	19°31'01"	103°50'52"	1709	25	0.359 ± 0.019	87.5	15
H_T								0.455 ± 0.006	100	23
A.angustifolia	"Ixtlero Verde"	Zapotitlán	IV	19°32'18"	103°50'40"	965	20	0.459 ± 0.013	100	17
A. tequilana	"Azul"	Tequila	TEQ	20°52'58"	103°50'12"	1180	23	0	0	8
A. tequilana	"Sigüin"	Tequila	SI2	20°52'58"	103°50'12"	1180	10	0.409 ± 0.024	100	17
A. tequilana	"Chato"	Tequila	CHA2	20°52'58"	103°50'12"	1180	11	0.435 ± 0.018	100	18
A. rhodacantha	"Ixtlero Amarillo"	Zapotitlán	IA	19°32'18"	103°50'40"	965	14	0.490 ± 0.005	100	16

N[a] = Number of individuals, Hs = Average expected heterozygosity per population, P = Percentage of polymorphic loci, H_s = Average expected heterozygosity ± standard deviation (SD), H_O = observed heterozygosity ± SD, H_T = total heterozygosity ± SD.

Carlo (MCMC) with 100,000 burn-in periods for each run and 10 runs were designed to estimate from 1 to the populations total (13). The optimum number of clusters (*K*) was obtained by using the *ad hoc* ΔK statistic (Evanno et al., 2005), calculated with the STRUCTURE HARVESTER program (Earl and vonHoldt, 2012).

3. Results and discussion

3.1. Genetic diversity and population structure

The agaves utilized for the production of mezcal in Jalisco present intermediate genetic diversity values compared with other agaves, with the exception of the "Azul" agave (Eguiarte et al., 2013). The genetic diversity between the cultivars and the wild populations of *A. angustifolia* is similar, consistent with the findings of Vargas-Ponce et al. (2009). Within the populations of *A. angustifolia* n Jalisco, a low population structure can be observed. The peasants' selection pressures on the wild populations they collect, transplant and propagate asexually in their plots hasn't had a significant effect on the genetic variation of the studied cultivars, with the exception of the "Azul" agave.

In *A. angustifolia*, a total of 24 alleles were detected, with an average of three per locus. The total heterozygosity ± standard deviation for *A. angustifolia* was $H_T = 0.45 \pm 0.006$; the highest expected heterozigosity in a population was observed in the Ameca population ($H_s = 0.455 \pm 0.015$) and the lowest in Perempitz ($H_s = 0.359 \pm 0.019$, Table 1). The populations from the central area presented values higher than $H_s = 0.453$. In contrast the southern populations had values lower than $H_s = 0.389$. For *A. tequilana* "Azul", all individuals shared the same genotype. The other traditional tequila and mezcal cultivars presented average genetic variation values close to those observed in *A. agustifolia* populations from the central area ($H_s = 0.409 \pm 0.024$–0.490 ± 0.005). Of all the analyzed groups, *A. rhodacantha* exhibited the highest heterozygosity ($H_s = 0.490 \pm 0.024$–0.490 ± 0.005) (Table 1).

Compared to other *Agave* species, *A. angustifolia* populations have intermediate values of genetic variation (Table 2), and similar ones than those observed in traditional cultivars, with the exception of the "Azul" agave (Table 2). Vargas-Ponce et al. (2009), using the ISSR (Inter Simple Sequence Repeats), a dominant molecular marker, found genetic variation levels in wild populations of *A. angustifolia* in Jalisco (Table 2) that are similar to those reported by us. It has been observed in some traditional cultivars that diversity can be greater than in wild populations due to the different origins of the germoplasm introduced into cultivation and to non-strict subsequent selection (Vargas-Ponce et al., 2009; Parker et al., 2010).

Table 3
Hierarchical analysis of genetic variance (AMOVA) in *A. angustifolia*, * = $P < 0.05$.

Variance/origin	Degrees of freedom	Sum of squares	Estimated variance	Variance percentage
Between groups	1	47.496	0.12121	5.56308*
Between populations within groups	9	57.047	0.25429	11.67138*
Within populations	3140	551.798	1.80326	82.76554*

The previous reported levels of genetic diversity for "Azul" agave plants ranges from *Hs* = 0.001 to 0.118 (Table 2). This suggests that, although the "Azul" agave's diversity is low, this depends on the analyzed sample: that it, whether it comes from intensive cultivation fields –where we would expect a stricter clonal selection- or the traditional plots where a less strict selection would be likely to occur.

Agave angustifolia exhibited relatively low, but significant population structuring ($\Theta = 0.169 \pm 0.0232$; 0.1289–0.2186). The AMOVA analysis (Table 3) indicated that most of the genetic variation is found within the populations (82.7%); with low differentiation between central and southern Jalisco ($\varphi f_{st} = 0.172$), while there is no isolation by distance pattern in *A. angustifolia* within Jalisco (r = 0.0813, p = 0.5834). The paired R_{ST} are presented in Table 4.

The levels of population structure in *A. angustifolia* in our study in Jalisco are lower (*Fst* = 0.169) than those of wild *A. parryi* (*Fst* = 0.76) populations, but similar to those found in cultivated *A. parryi* (*Fst* = 0.148). Similar results have been reported in wild *A. angustifolia* populations of Sonora, México, by Sánchez-Teyer et al. (2009) (*Fst* = 0.182) (Table 2).

3.2. Genetic relationships

Agave tequilana "Azul" is closely related to *Agave angustifolia*; mainly with the wild populations of *A. angustifolia* from the south of Jalisco

Table 2
Genetic diversity and population structure values obtained by means of different molecular markers for *A. angustifolia*, *A. tequilana* "Azul" and other agaves. Modified from Eguiarte et al. (2013).

Species	Marker	H_S	F_{ST}	*P*	Number of loci	Number of populations	Individuals per population average	Reference
A. angustifolia								
Sonora	AFLPs	0.259	0.175	0.735	155	3	45.33	Sánchez-Teyer et al. (2009)
Sonora	AFLPs	0.260		0.782	226	1	6	Rivera-Lugo et al. (2018)
Espadín	AFLPs	0.310		0.958	277	1	13	Rivera-Lugo et al. (2018)
Jalisco	ISSRs	0.350	0.182	0.899	69	9	25.77	Vargas-Ponce et al. (2009)
Cultivado, Jalisco	ISSRs	0.275	0.357	0.734	69	1	21	Vargas-Ponce et al. (2009)
Jalisco	SSRs	0.474	0.158	0.833	30	8	23.3	Trejo et al.,
Ixtlero Verde	SSRs	0.459		1	8	1	20	Trejo et al.,
A. angustifolia* var. *angustifolia	AFLPs	0.313		0.972	281	1	14	Rivera-Lugo et al. (2018)
A. angustifolia* var. *rubescens	AFLPs	0.310		0.969	280	1	10	Rivera-Lugo et al. (2018)
***A. tequilana* "Azul"**								
Jalisco	RAPs	0.001		0.008	124	4	10	Gil-Vega et al. (2001)
Jalisco/Guanajuato	$AFLP_S$			0.26	78			Gil-Vega et al. (2006)
Guanajuato	$AFLP_S$	0.205		0.799	231	1	5	Rivera-Lugo et al. (2018)
Jalisco	ISSRs	0.118		0.246	69	1	22	Vargas-Ponce et al. (2009)
En este trabajo	SSRs	0		0	8	1	23	Trejo et al.,
Others agaves								
A. rhodacantha	$AFLP_S$	0.234		0.824	238	1	7	Rivera-Lugo et al. (2018)
A. rhodacantha	SSRs	0.490		1	8	1	14	Trejo et al.,
A. palmeri	SSRs	0.658		0.857	52	2	30	Lindsay et al. (2012)
A. parryi silvestre	SSRs	0.621	0.76	1	4	8	24	Parker et al. (2010)
A. parryi cultivado	SSRs	0.433	0.148	0.786	4	7	22.57	Parker et al. (2010)

Table 4
R_{ST} calculated between pairs of populations de *Agave angustifolia*.

Population	Hostipaquillo (AH)	Ameca (AM)	Tecolotlán (AT)	Tuxcacuexco (ATX)	Perempitz (P)	Zapotitlán (C3)	Zapotitlán (C9)	Zapotitlán (C13)
Hostipaquillo (AH)	0							
Ameca (AM)	0.20161	0						
Tecolotlán (AT)	0.04247	0.18763	0					
Tuxcacuexco (ATX)	0.09132	0.31652	0.10791	0				
Perempitz (P)	0.45011	0.53784	0.40385	0.38713	0			
Zapotitlán (C3)	0.41215	0.42518	0.31484	0.40323	0.30665	0		
Zapotitlán (C9)	0.42804	0.4522	0.32969	0.40856	0.32293	0.02501	0	
Zapotitlán (C13)	0.39674	0.5298	0.32407	0.35333	0.1402	0.20705	0.20185	0

p-values are 0.0 ± 0.0 except C9-C3 (0.22168 ± 0.0150).

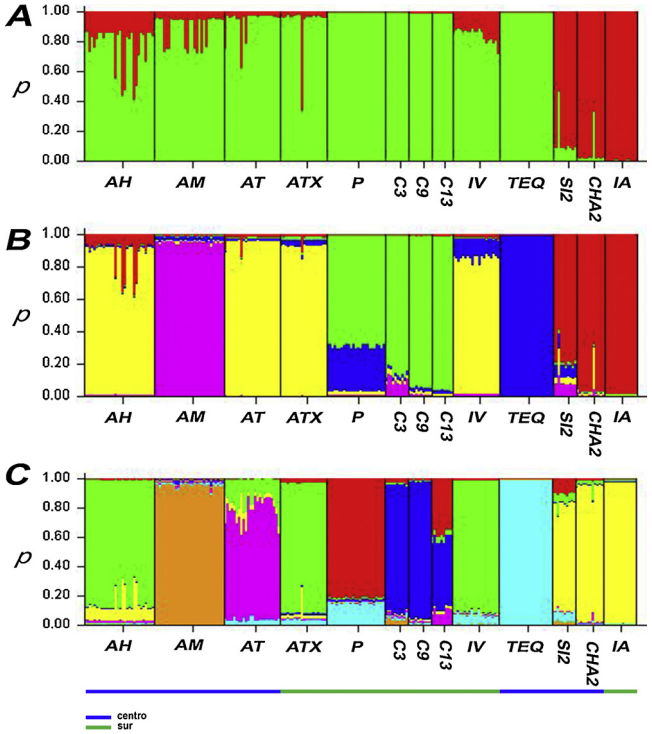

Fig. 3. Genetic relationships between *Agave angustifolia, A. tequilana* and *A. rhodacantha*. A) Neighbor-Joining (Saitou and Nei, 1987) similarity tree, B) Discriminants of principal components.

(Fig. 3). The "Azul" agave could represents a small sample of the total genetic diversity of wild populations of *A. angustifolia* from the south of Jalisco. The wild populations of *A. angustifolia* present different ancestry associations. The cultivars "Sigüin", "Chato" and Ixtlero Amarillo are the closest to one onother.

In the Bayesian wild populations assignment analysis for *A. angustifolia* and *A. tequilana*, two gene pools corresponding to $K = 2$ (Fig. 3A), the highest K value, based on the ΔK statistical test (Evanno et al., 2005) can be observed. These correspond mainly to a group comprised by *A. angustifolia* and *A. tequilana* and another which encompasses "Sigüín", "Chato" and "Ixtlero Amarillo". The second highest value of ΔK was $K = 5$ (Fig. 3B). The five groups correspond mainly to: 1. Hostotipaquillo, Tecolotlán, Tuxcacuexco and "Ixtlero Verde"; 2. Ameca; 3. Perempitz, Zapotitlán; 4. "Azul" and 5. "Siguín", "Chato" and "Ixtlero Amarillo". We also observed that when estimated Ln probability of data vs. *K* was plotted, an asymptote begins to form at $K = 7$ (Fig. 3C). The seven groups correspond mainly to: 1. Hostotipaquillo, Tuxcacuexco and "Ixtlero Verde"; 2. Ameca; 3. Tecolotlán; 4. Perempitz; 5. Zapotitlán; 6. "Azul" and 7. "Siguín", "Chato" and "Ixtlero Amarillo".

In the Nei distances dendrogram (Fig. 4A) it can be observed that the traditional "Azul" cultivar appears as a group distant from the rest of the agaves in which mainly two groups were observed: one is comprised by "Sigüín", "Chato" and *A. rhodocantha* ("Ixtlero Amarillo") and the other by *A. angustifolia* wild populations and traditional cultivars. The latter group is in turn divided into two groups in which central and southern Jalisco populations intermix.

The discriminant analysis of principal components (Fig. 4B) shows three groups with complex genetic relationships: one is comprised by "Ixtlero Amarillo" and it is separated from the other groups; another group is comprised only by some wild *A. angustifolia* populations (Zapotitlán and Ameca populations; far right of Fig. 4B) and a last group is comprised by all the other *A. tequilana* ("Azul", "Siguin" and "Chato") and *A. angustifolia* populations and traditional cultivars.

Our results concur with those of Torres-Morán et al. (2013), which recognized *A. angustifolia* as the species closest to the "Azul" agave by using molecular ISTR markers. Additionally, our work supports Colunga-GarcíaMarín and Zizumbo-Villarreal's (2007) ethnobotanical hypothesis, which identifies the wild populations of the south of Jalisco as the most closely related to the "Azul" agave.

Other studies have attempted to elucidate the genetic relationships of "Azul" agave with other taxa (Table 5) but we consider that previous results were not clear, though frequently *A. tequilana* is in a group distinct from the rest of the agaves, in others, *A. angustifolia* is one of the species showing greatest proximity to *A. tequilana*, even though the same type of molecular markers are used.

In other studies, in which kin relationships between *A. angustifolia, A. tequilana* and *A. rhodacantha* were analyzed, and in which different molecular markers were used, it has been observed that "Sigüín", "Chato", and *A. rhodacantha* belong to different groups of *A. angustifolia* and *A. tequilana*. There might be the possibility that "Sigüín" and "Chato" were different species closely related to *A. rhodacantha*.

3.3. Integration of molecular evidence into a multidisciplinary hypothesis

The molecular evidence analyzed in the present work supports Colunga-GarcíaMarín and Zizumbo-Villarreal's (2007) ethnobotanical hypothesis, which proposes the populations of the south of Jalisco as the plants most related to the "Azul" agave. This was reiterated by

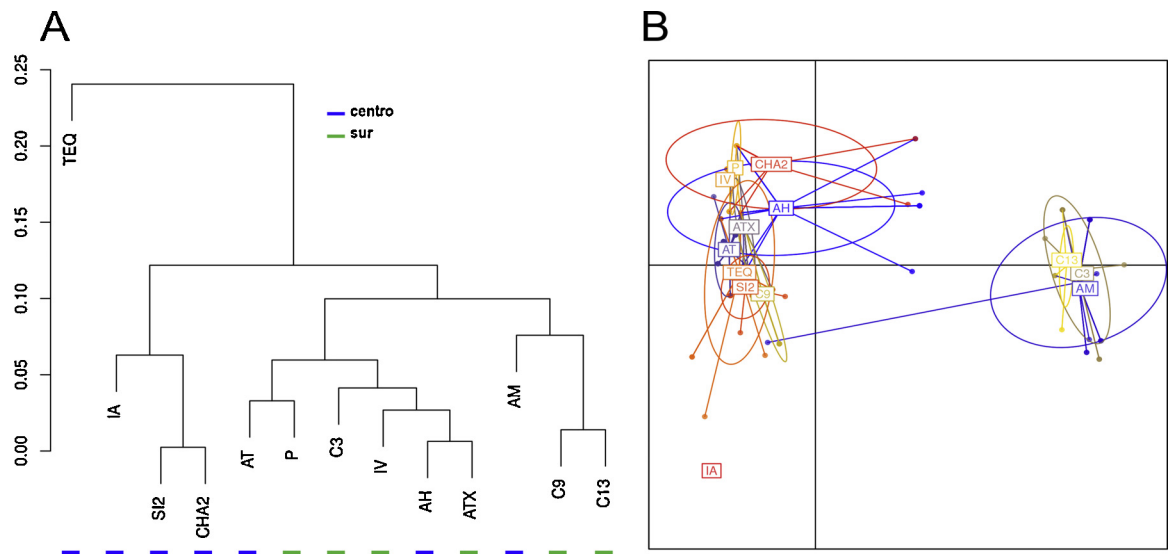

Fig. 4. Assignment analysis. A) K = 2, and B) K = 7, of individuals using Bayesian statistics with the P co-ancestry coefficient in the STRUCTURE ver. 2.2 program (Pritchard et al., 2000).

Zizumbo-Villarreal and Colunga-GarcíaMarín (2008) based on botanical, toponymic, archeological and ethno-historical evidence. They point out that the coconut palm was introduced between 1569 and 1571 and was cultivated to produce coconut liquor (1580). The technology for the production of coconut liquor was implemented by Filipinos established in Colima between 1580 and 1600. As suggested by Bruman (1940), the native people could have easily learned the distillation process and applied it to traditional fermented beverages like the ones obtained from cooked agave. The ethno-historical and archeological record provided by Zizumbo-Villarreal and Colunga-GarcíaMarín (2008) indicates that coconut and agave spirit production might have occurred simultaneously. The presence of Filipino distillers and *A. angustifolia* throughout the volcanos region suggests that the distillation process spread north from Colima to the Nayarit Sierra. The factors that impelled their dispersal include the richness of *Agave* species, the use of agaves as food and beverage since precolombian times, the practice of fermentation in the area, the ready acceptance of the Filipino distiller and an extensive commercial network. But two additional factors led mezcal and tequila production to the south of Jalisco: Firstly, it was one of the areas with the greatest richness in *Agave* species and secondly, it was the 1612 prohibition of the sale and consumption of liquors that weren't the Crown's. For this reason, coconut and mezcal spirits were clandestinely produced upriver on the isolated and remote slopes of Colima volcano, in southern Jalisco (Colunga-GarcíaMarín and Zizumbo-Villarreal, 2007; Zizumbo-Villarreal and Colunga-GarcíaMarín, 2008).

The use of agaves for the production of distillates in the vicinity of the Colima volcano could also have taken place since precolombian times, by using the clay "Capacha stills" which would have been developed from bean cooking pots (Zizumbo-Villarreal et al., 2009b). The

Table 5
Polymorphism and relationship data, obtained by different molecular markers for *Agave tequilana* "Azul".

Marker	Number of localities	State(s)	Individuals	Polymorphism, %	Genealogical analyses	Closely related taxa	Reference
RAPD[1]	4	Jalisco	40	0.8	UPGMA with bootstrap	*A. tequilana* var. *Sigüin*	Gil-Vega et al. (2001)
AFLP[2]	8	Guanajuato	26	26	Dice index and UPGMA with bootstrap	*A. tequilana* var. *Sigüin* and *Azul Listado*	Gil-Vega et al. (2006)
AFLP[2]	2	Jalisco Jardín Botánico, UNAM	81		UPGMA with genetic distances of $_1$Jaccard$_{xy}$	*A. tequilana* var. *Azul Listado* *A. angustifolia*	Cuevas-Figueroa and Flores-Berrios (2006)
AFLP[2]		Jalisco	2		UPGMA with genetic distances of Jaccard and Nei	*A. rhodachanta, A. pedunculifera* and *A. inaequidens*	Hernández et al., 2007
SSAP[3]		Sussex botanical collection			Nei´s distance and bootstrapping	*A. tequilana* var. *Azul Listado* *A. rhodachanta* y *A. americana*	Bousios et al. (2007)
ISTR[4]		Collection of Universidad de Guadalajara			UPGMA	*A. durangensis*	Torres-Morán et al. (2008)
RAPD[1]	4	Jalisco, Tamaulipas, Guanajuato and Michoacán	49	35			Rodríguez-Garay et al. (2009)
ISSR[5]	1	Jalisco	22	24.6			Vargas-Ponce et al. (2009)
ISTR[4]	14	Jalisco	100		UPGMA ρ and Δ*K*	*A. angustifolia*	Torres-Morán et al. (2013)

[1] Random Amplified Polymorphic DNA.
[2] Amplified Fragment Length Polymorphism.
[3] Sequence Specific Amplification Polymorphism.
[4] Inverse Sequence Tagged Repeat.
[5] Inter Simple Sequence Repeat.

production processes will be difficult to recognize, but the identification of the cultivars´ germoplasm source can become increasingly specific if higher resolution SNIPs (single nucleotide polymorphism) type molecular markers are used, and if analysis of more cultivars and wild populations throughout *A. angustifolia*'s distribution area are carried out.

4. Conclusions

The cultivars represent small proportions of the wild populations´ genetic diversity. In the case of the "Azul" agave, reproductively isolated through asexual reproduction, the selection pressures on a very small proportion of the genetic variation have been intense. *Agave tequilana* "Azul" and *A. angustifolia* belong to the same genetic pool; the "Azul" agave is most closely related to the southern Jalisco populations of *A. angustifolia*. This supports the hypothesis of Colunga-GarcíaMarín and Zizumbo-Villarreal (hypothesis two) that it is in this area where the ancestral populations of the native cultivars and their greatest diversity can be found. This is related to the fact that it was in this area that the Filipino distillers adapted to the production of agave distillates; distillation in this area could even have occurred earlier, by using the precolombian "Capacha" type stills.

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