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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.