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Research Article

CHEMICAL AND MOLECULAR PROPERTIES OF SOTOL PLANTS (DASYLIRION CEDROSANUM) OF DIFFERENT SEX AND ITS FERMENTATION PRODUCTS

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ABSTRACT
Sotol (*Dasylirion Cedrosanum*) is a dioecious plant which has different uses, being alcohol production the most important. Sotol beverage is produced mostly in a traditional way, where farmers had the strong belief that use of female plants pineapples produces a drink of better quality so that only these plants are harvested. The aim of this study was to compare sotol plants (male and female) through chemical and molecular characterization. In addition, fermentation was carried out using pines from plants of different sex. DNA polymorphism between male and female plants was achieved using AFLP´s. Chemical characterization showed no significant differences between pines of male and female plants, while there was no difference for alcohol percentage between fermented male and female plants. Dendrogram showed that male and female populations were not different. Results of this study suggest that sotol plants of both sexes can be used equally for alcoholic production.

Key Words: *AFLP, Alcoholic Fermentation, Dioecious, DNA, Perenne, Sotol*

INTRODUCTION
Sotol (*Dasylirion ssp*.) is characterized as a perennial, poly carp, semi-cylindrical plant which the dying leaves fall on the stem as a form of protection and structure, sotol leaves are thin, narrow and stiff, sword-shaped about1 meter long for 2 to 3 cm in width and taper toward appendix flared at the base. It has a fibrous root, shallow and widely branching, which arises from the trunk (Bogler, 1998) The plant core is not very hard and it is known as "head or pineapple", which generally weight from 20 to 40 kg in mature plants (Cruz *et al.*, 2008). This plant survives both extreme summers and cold winters and can live 150 years. Its stem grows to some individuals up to 3 m, having cones weighing up to 100 Kg (Bogler, 1998).
The sotol belongs to Nonlinaceae familiy. In Mexico have been identified about 16 species, particularly in the state of Coahuila have been recognized around 6 or 7 species, but only some of them are economically important because of the pineapple increased diameter and carbohydrates amount. Only 2 species have characteristics and properties to be used in the alcohol industry: *Dasylirion cedrosanum* and *Dasylirion duranguensis* (Garza *et al.*, 2008). Liquor demand has increased, both domestically and internationally, due to wider circulation, production and consumption. The sotol production presents some advantages, these are increased revenues per hectare than those obtained with many traditional cultures, this profit does not require large investments and large-scale productions and sotol economic performance depends on the volumes of cones per hectare and these are controlled by farmers (Contreras and Ortega, 2005).
It is believed that sotol carbohydrates are of two types: the mass in the shell-shaped structures that form the stem, are mostly polysaccharides Fructan family (known as inulin) but also contains a certain amount of starch, glucose and fructose (Garza *et al.*, 2008). The liquor obtained from this species is also known as "sotol". *Dasylirion* plant throughout history has had a great significance since ancient times because

people gave multiple uses not only as food and feed but they used sotol to make paper, religious ornaments, and as material to construct house roof or fences, etc (Vazquez, 2001).

Liquor of sotol is obtained through a cooking of pineapples or heads, which are ground and subjected to alcoholic fermentation with yeast and then distilled. Sotol is a liquid according to its type is colorless or yellowish when matured in wooden containers (Cruz *et al.*, 2007). The producers of this auto chthonous beverage believe in the myth in which use of female plants pines produces a drink of better quality, so they harvest only female plants, jeopardizing the survival of this vegetal because this practice reduces seed source. Recently, the first description of sotol traditional fermentation process was reported as well as some technological improvements (Garza *et al.*, 2008). The aim of this study was to determine physical, chemical and molecular differences between sotol plants of different sexes as well as its fermentation products.

MATERIALS AND METHODS

Vegetal Material

The 20 samples of sotol pineapples and leaves from each plant sex in adult phase were collected on summer in the hills near to "Bañuelos" town, Coahuila. The samples were collected at random.

Chemical Characterization

Sotol pineapples were cleaned and then cut into small pieces. Moisture, ash, fat, protein, total and reducing sugars and crude fiber were determined using the small pieces of sotol pineapples following the methodologies recommended by AOAC (1980).

Pineapples Fermentation

Sotol pineapples were divided into 10 groups, 5 groups with female cones and 5 groups with male cones. After that, the sotol pineapples were grinded in a food processor "Black & Decker" pica-lica model, until sotol strips were obtained. Then, sotol stripes were cooked in 1:4 ratio, so it weighed 100 g samples and 400 ml of distilled water for each group, this mix was placed in plastic bags and closed. Cooking was performing during 30 min/15 lb/120 º Cusing a pressure cooker, which was then filled with water. After that, samples were left to rest for 24 hours under refrigeration, then, pineapples bagasse were removed from the bagsand pressed with a juice extractor to obtain the juice that could be trapped into the fibers, this juice and that from cocking were filtered through muslin cloth, then pH was adjusted to 4.5, and were added the following salts: $(NH_4)_2$ SO_4 1%, $KHPO_4$ at 0.1% and $MgSO_4$ 0.01% (w/v) in order to promote faster growth of yeast and ethanol production. The wort was transferred to clean, and labeled glass jars with screw cap, 6 for each group having a total of 60 vials, these were sterilized. After that, jars were left at room temperature to be inoculated.

Fermentation Kinetics

Fermentation was carried out under the following conditions: incubation temperature 30 °C for three days, recollecting samples every 12 hours, preventing aeration and contamination. At each kinetic point a fermented mash roast was taking and planted on a Petri dish. The media culture contained in Petri dishes consisted of malt extract (2 g L^{-1}) and bacteriological agar (18 g L^{-1}). The yeast strain used for fermentation was *Saccharomyces cerevisiae* which was previously isolated from sotol. This yeast strain was reported as a good ethanol producer (Garza *et al.*, 2008).

Analysis of Alcohols by High Pressure Liquid Chromatography (HPLC)

Products of fermentation (ethanol, monosaccharides and secondary products) were analyzed by Waters HPLC equipment, with an ion exchange column Phenomenex mark Rezex ROA Organic Acid H^+ (8 %) of dimensions 300 x 7.8 mm. A pre-column Phenomenex to promote the separation of carbohydrates ROA Organic Acid H + (8%) of dimensions 50 x 7.8 mm. Columns with ability to resolve separate peaks of glucose, and fructose (from inulin) were used as a detector of refractive index Waters model 2414. Using the platform developed by Waters EMPOWER ® for monitoring, control and quantification. It was employed a scheme to circumvent isocratic at a flow rate of 0.5 mL min^{-1}, using 0.005 N H_2SO_4 as mobile phase. Temperature of cooling system auto sampler was 6 °C, column temperature was 60 ºC and

temperature of the refractive index detector was 35 °C and injection volume 5-microliter (Tellez *et al.*, 2002). To perform the calibration curve, some standard solutions were used (10% ethanol, 1% methanol, 1% isopropanol, 1% isobutanol, n-propyl 1%, isoamyl alcohol 1 %, furfural to 5 ppm, glucose 80 g L^{-1} and fructose 80 g L^{-1}). From these standard solutions were done the dilutions. Each of the standards was placed in the HPLC to determine the calibration curve.

Statistical Analysis

Mean treatments of each response variable in the chemical characterization were analyzed using paired Student t test ($p < 0.05$). Data from fermentation kinetics was analyzing according to a complete randomized design with five replications using an ANOVA analysis ($p < 0.05$). Each statistical analysis was performed using InfoStat software (Rienzo *et al.*, 2008).

Amplified Fragment Length Polymorphism (AFLP)

Leaves from 15 female and 15 male sotol plants collected at the Bañuelos town. Plant leaves were selected based on absence of disease and or physical damage.DNA isolation was carried out with the method reported by Dellaporta (1985). The AFLP polymorphism (amplified fragment length) was performed according to Vos *et al.*, (1995) and following the LI-COR ® Biosciences protocol. DNA digestion was performed with Mse1 (frequent cutting) and EcoR1 (rare cutting) enzymes, following the steps of binding of specific adapters, pre-amplification and finally a selective amplification step, we used several sets EcoR1 and Mse1 primers, which were marked with IRDye 700 and IRDye 800. For polyacrylamide gel preparation were used the following reagents: 150 µl of Ammonium Persulfate 10%, 20 ml acrylamide-bisacrylamide and 15 µl TEMED. The selective amplification samples were denatured (3 min at 94 ° C) before were adding 5 ul of stop blue solution then AFLP bands were separated for 3 hours using a Li-COR sequencer.

The AFLP banding pattern was coded as follow: absence (0) and presence (1) of bands. After that, it was estimated the genetic diversity measures (Nei'sunbiasedheterozygosity, polymorphic index content (PIC) and effective alleles). In addition, a cluster analysis was performed using the InfoGen software. For dendrogram construction, the Euclidean distance was calculated with the Mathematical software, distance values were used to elaborate the dendogram using the Phylip software.

RESULTS AND DISCUSSION

Chemical Analysis

Chemical analysis showed no significant differences between male and female plants for each of the analyzed parameters (Table 1).

Table 1: Chemical characterization of sotol plant with different sex

Parameter (%)	Plantgender		Average
	Female	Male	
Moisture	69.27 ± 6.54 a	66.22 ± 6.16 a	69.24 ± 6.29
Ash	0.88 ± 0.21 a	0.96 ± 0.31 a	0.91 ± 0.26
Fat	0.88 ± 0.47 a	0.65 ± 0.25 a	0.78 ± 0.40
Crude fiber	9.31 ± 1.97 a	10.03 ± 1.66 a	9.63 ± 1.85
Protein	0.49 ± 0.21 a	0.51 ± 0.24 a	0.49 ± 0.22
Total sugars	5.58 ± 1.74 a	6.24 ± 1.54 a	5.89 ± 1.67
Reducing sugars	2.98 ± 1.42 a	4.17 ± 1.61 a	3.53 ± 1.61

Means with the same letter, in the same row, are not significantly different according to paired Student t test ($p < 0.05$).

The amount of water present the sotol cones (69 %) is lower compared to other plants living in the desert such as *Agave angustifolia* (90.8 %) and the paddle cactus with 97 % (Gallegos *et al.*, 2006) that due to its

morphology is characterized by having more fiber. In this study, male plants showed similar amount of ashes than those of female plants (Table 1).

The fiber was similar in male and female plants. Fiber is the insoluble organic residue that is edible and non-available carbohydrates in plant and food (Kirk *et al.*, 1996). The ether group is formed by fats, oils and fat-soluble substance whose function in plants is to form part of cells, and not as an energy reserve (Badui, 1999). Fat, protein, reducing sugars and total sugars were similar in female and male plants (Table 1).

In the chemical characterization, sotol plants were collected regardless of their age so small differences in the chemical composition between plants of different sexes could be due to plant age and not necessarily to sex. For example, older plants have more fiber regardless the plant gender.

Alcoholic Fermentation

After hydrolysis of inulin by heating sotol pineapples, monosaccharides present in juice, glucose and fructose were fermented by *S. cerevisiae*. The initial quantities of reducing sugars were 48.13 g/L and 75.88 g/L for male and female must respectively, which were reduced after 72 hours of fermentation to 21.83 g/L for female fermented must and 7.16 g/L for male (Figure 1).

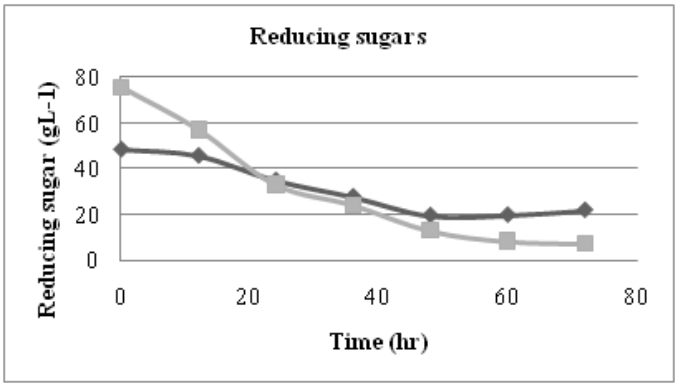

Figure 1: Consumption of reducing sugars in sotol samples fermented ♦Female sample and ■Male sample

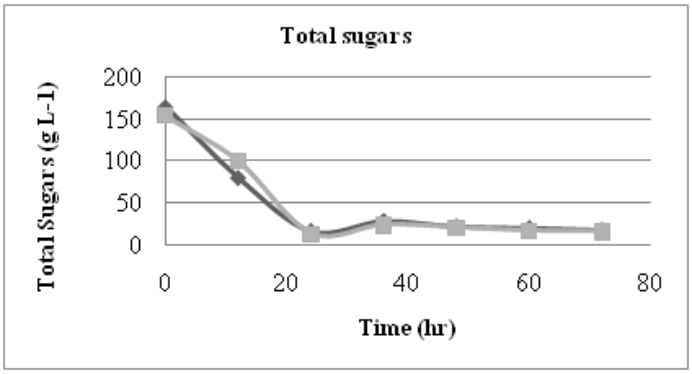

Figure 2. Total sugarconsumption in fermentedmash of sotol pineapples ♦Female sample and ■Male sample

On the other hand, the values of total sugars were 154.43 g/L form female must and 164.71 g/L for male, such amounts were reduced approximately 89 % in both cases obtaining values 16.28 g/L in male fermented must and 17.22 g/L for female (Figure 2).

The percentage of sugar consumption in the two plant populations was 89.46% and 89.45% respectively (Figure 1), while the ethanol yield per gram of sugar consumed was 3.94% for female fermented

pineapple while for male fermentedpineapplewas3.75% (Figure 1). In this case, *S.cerevisiae*metabolized glucose and fructose together which are in greater proportion in the must, transforming these sugars to ethanol and CO_2 (Owen, 1989) as shown in Figure 3.

There is not enough information about fermentation of sotol pineapples, but it can find information about similar plants as maguey (*Agave salmiana*) which produces mescal. Bagasse mescal (traditional fermentation) has a similar behavior, with an initial concentration ofsugarsaround7% and endingat95 hourswith2% sugar, while mescal filtering bagasse show eda concentration of about12% and ended at 140hours with 2% sugar. In both cases, 1Lof water per kg of bagasse was added (Soto *et al.*, 2009) unlike bagasse sotol which initially 4 L of distilled water per kg were added. The end product of the fermentation was analyzed by ethanol content. Ethanol amounts produced from both types of plants (females and males) was very similar in which the maximum ethanol production was presented at 72hours, and within the first50 hours is produced the most important amounts of ethanol (Figure 3).

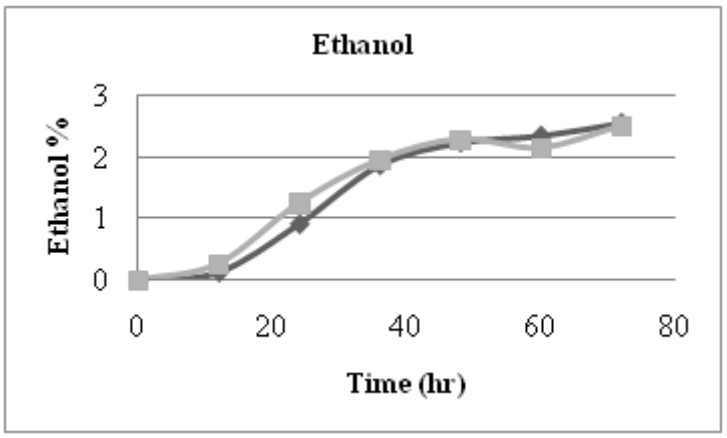

Figure 3: Production of etanol from fermented samples of sotol pineapples
♦Female sample and ■Male sample

The theoretical yield of 1g of glucose is 0.51gethanoland 0.49g of CO_2. However, in practice, approximately 10% of the glucose is converted into biomass and yield of ethanoland90% of the theoretical value to CO_2 (Owen *et al.*, 1989), in this case, it wasproduced0.40gofethanolfor every gram of sugar consumption offer men table sugars of stool female plant must while for male sotol must was 0.32gper gram of ferment able sugar, taking into account that at the beginning, the male plant must sotol presented greater amount of reducing sugars, the ethanol values produced from males and females plants are statistically similar(Table 2), such behavior was similar during all the fermentation process (Figure 1 and 2).

Table2: Statistical analysis of sugars and ethanol content in the final fermented juice sotol

Variable	Female	Male
Total sugar	17.22 ± 4.77 a	16.28 ± 4.66 a
Reducingsugar	5.06 ± 0.46 a	7.16 ± 2.75 a
Ethanol	2.55 ± 0.20 a	2.05 ± 0.31 a

Means followed with the same letter in the same file are not significantly different according to the test according to Tukey test ($p \leq 0.05$).

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AFLP Analysis

The combination of primers for selective amplification that showed more banding was marked with fluorescent primers E-AGC and E-AAC, for the panels 800 and 700 nm respectively, combined with the first M-CTG. Figure 4 shows the amplified bands by the AFLP technique in both groups of plants.

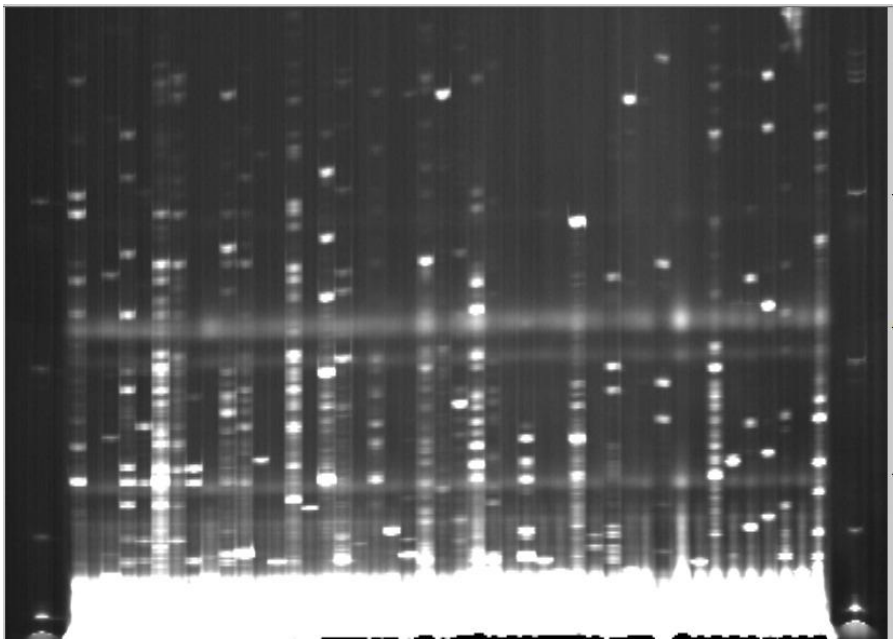

Figure 4: Banding pattern obtained by AFLP's in a group of female and male plants sotol .

Genetic Diversity

The values of genetic diversity within populations of female plants (0.418) and male plants (0.406) were statistically similar (Table 3) indicating thatin both populations the effects of evolutionary forces (mutation, selection , migration, genetic drift, etc.) are similar.

Table 3: Wilcox on average values for different genetic means female and male sotol plants

	Female	**Male**
Genetic diversity	0.418 a	0.406 a
Neiun biased heterocygosis	0.433 a	0.422 a
PIC	0.328 a	0.319 a
EffectiveAlleles	1.746 a	1.727 a

Means followed with the same letter in the same file are not significantly different according to the test according to Tukey test (p≤0.05).

Cluster Analysis

For the cluster analysis, Euclidean and Dice distances were calculated. Dice showed the highest cophenetic correlation value, by this reason, the Dice distance was used to perform the cluster analysis. This analysis was performed to separate the male and female plant populations (Figure 5). In this case, it was not possible to separate both populations indicating that they share most of the genetic content.

The genetic diversity of a population includes genetic variation within a species, both among geographically separated populations and among individuals within populations. Other genetic diversity measures are heterozygosity and polymorphism (Astorga *et al.*, 2006).

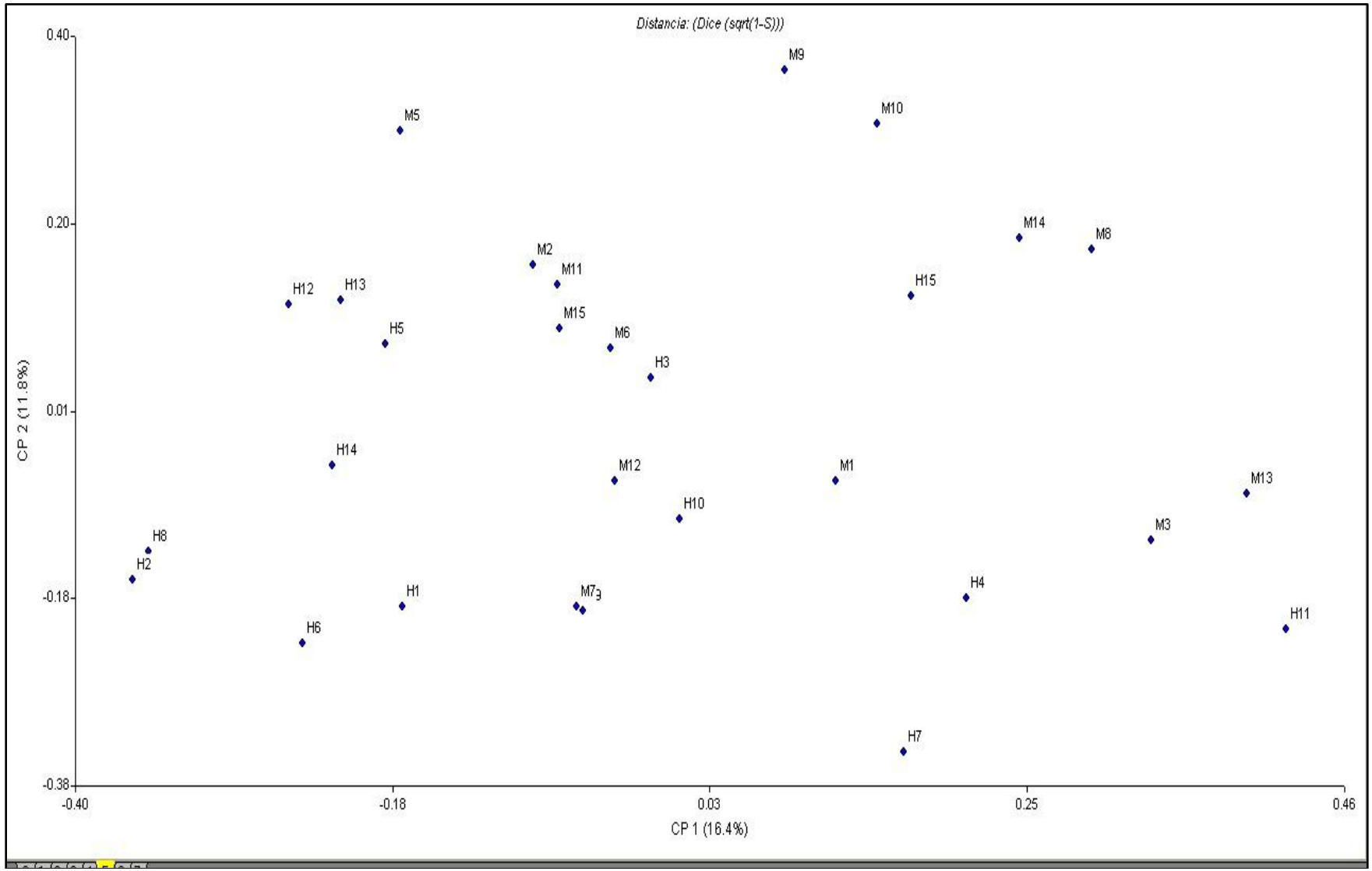

Figure 5: No separation of male (m) and female (h) sotol populations using a cluster analysis with the Dice distance

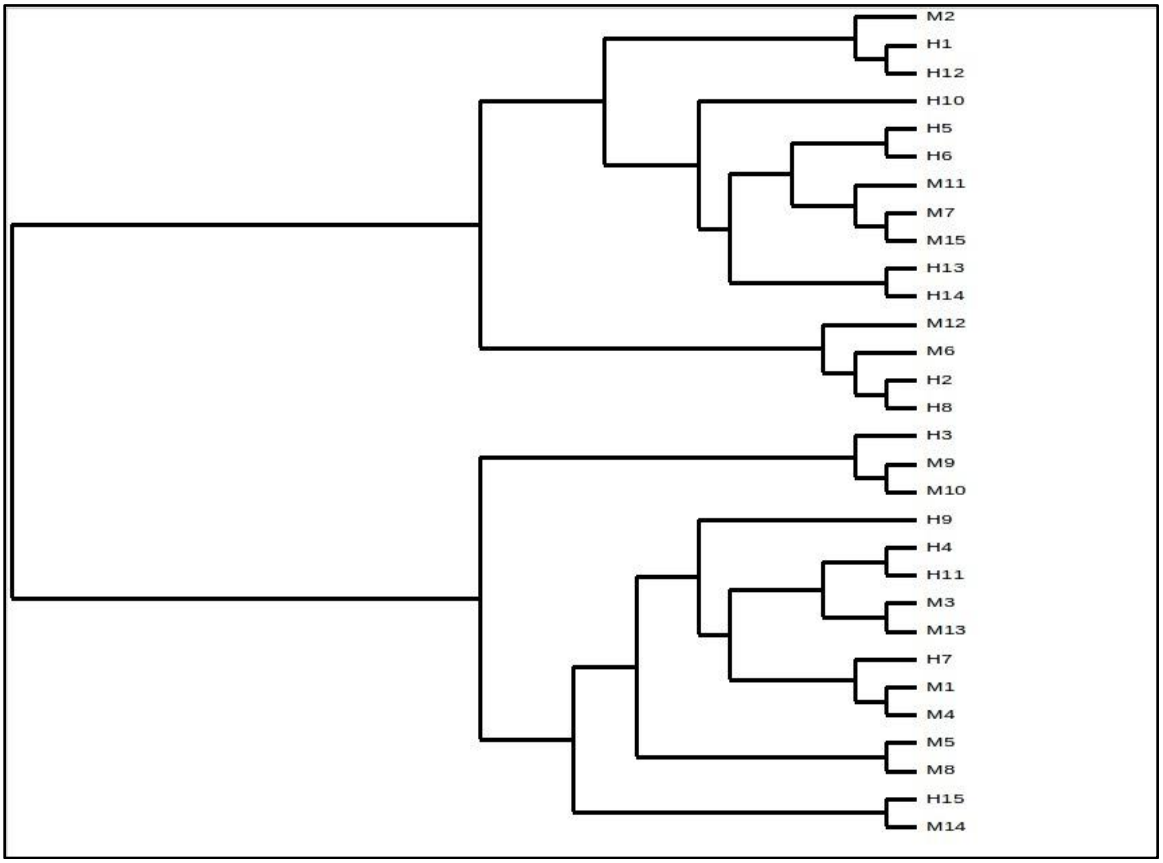

Figure 6: Dendrogram of individuals from two populations of sotol plants (females (H) and males (M)) using molecular markers (AFLP's) (800).

Heterozygosity is the expected frequency of random mating conditions or the probability of finding two different alleles in a population according to the data, the populations of male and female plants are very similar. Genetic polymorphism is a variation of the genome that displayed by mutations in some individuals, is transmitted to off spring and acquires some frequency in the population after many generations, the values of polymorphic information content for females and males respectively were similar (Table 3). The number of effective alleles was similar between the two plant populations, suggesting little difference between females and males. This parameter indicates the number of alleles that may be present in a population as well as the ability to compare different distributions in allele frequency (Pistorale *et al.*, 2008).

Dendrogram was performed with the observed banding pattern data (AFLP's), however it was not possible to separate the two populations, this also suggests that both populations share the same genetic background (Figure 6). Interest in gly, the dendrogram groups the plants regardless of sex into two groups which may suggest a process of subdivision in the sotol of Bañuelos town.

Conclusion

Al though there are small differences between female and male sotol plants in chemical composition this is not reflected in the amount of alcohol obtained by fermentation, which makes it unnecessary the exclusive use of female plants for the alcoholic beverage production. The determined genetic diversity measures show no differentiation between the two groups of plants indicating that male and female plants have the same evolutionary path and the differences between these two populations are minimal.

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