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ANÁLISIS AFLP DE LA VARIABILIDAD GENÉTICA EN TRES FORMAS DE REPRODUCCIÓN DE *Agave tequilana*

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SUMMARY

The *Agave* genus belongs to the Agavaceae family and contains around 200 species with diverse uses. *Agave tequilana* is one of the most important species at the industrial level in México since sugars accumulated in the stems of these plants are used to produce the spirit called tequila. Agave production is supported by intensive clonal propagation and the suppression of the sexual reproduction, leading to reduction in genetic variability and a greater susceptibility to plagues and diseases in commercial plantations. Nevertheless, this study with plants from a field in Guanajuato, México, carried out using AFLP markers shows that differences at the molecular level exist between offsets and bulbils produced asexually from the same mother plant (75.08 and 86.06 % polymorphic loci, respectively). Although a significant level of polymorphism is observed between rhizome offsets, levels are even higher between bulbils, reaching levels comparable to those found between plantlets produced from seeds (90.1 %). We propose a more detailed study of the factors causing genetic variability between asexually propagated individuals of *Agave tequilana*.

Index words: *Agave tequilana*, AFLP, genetic variability, asexual reproduction.

RESUMEN

El género *Agave* pertenece a la familia Agavaceae y cuenta con alrededor de 200 especies, con diversos usos. *Agave tequilana* es una de las especies más importantes a nivel industrial en México, ya que los azúcares que se acumulan en sus tallos son usados para producir la bebida alcohólica llamada tequila. La producción de agave es soportada por una intensiva propagación clonal y supresión de la reproducción sexual, lo cual ha conducido a reducción en la variabilidad genética y mayor susceptibilidad a plagas y enfermedades en plantaciones comerciales. No obstante, este estudio con plantas de un campo de cultivo del Estado de Guanajuato, México, llevado a cabo con marcadores AFLP, muestra que existen diferencias a nivel molecular entre hijuelos y bulbillos producidos asexualmente provenientes de la misma planta madre (75.08 % y 86.06 % de loci polimórficos, respectivamente). Aunque se observa un nivel significativo de polimorfismo entre hijuelos de rizoma, los niveles son aún más altos entre bulbillos con valores comparables a los encontrados entre plantas producidas por semilla (90.1 %). Por ello se propone un estudio más detallado de los factores que están causando la variabilidad genética entre individuos de *Agave tequilana* propagados asexualmente.

Palabras clave: *Agave tequilana*, AFLP, variabilidad genética, reproducción asexual.

INTRODUCTION

The Agavaceae family has nine genera and around 300 species, is endemic to America and its center of origin and diversity is located in México where 76 % of all known species are found (García-Mendoza, 2007). Currently the economic importance of these species is based on the extraction of fibers and production of alcoholic beverages such as tequila and mezcal (Valenzuela, 1997), but they are also of ecological importance (Good-Avila *et al*., 2006). The requirements of the tequila industry and restrictions of origin denomination have caused an intensive clonal selection and severe reduction of sexual reproduction of *A. tequilana* Weber, var. Azul (Palomino *et al*., 2003). This has resulted in a reduction in genetic variability, leading to concerns of susceptibility to plagues and diseases in commercial plantations (Gil-Vega *et al*., 2001).

In general, *Agave* species are monocarpic, semelparous, and can be propagated by seeds, rhizome offsets and inflorescence bulbils. *Agave tequilana* Weber var. 'Azul' may be propagated by all three forms, although reproduction is mainly achieved through rhizome offsets (Granados, 1993). Crossing and recombination between genotypes of *Agave* with desirable agronomic traits has been limited by asexual reproduction and the long life

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cycle of the plants. It is important to determine the degree of genetic variability between populations. If it is enough high it could be possible to select plants with economically important characteristics.

For molecular marker analysis, the AFLP (Amplified Fragment Length Polymorphism) technique is useful and has the advantages that no previous knowledge of the genome under study is necessary, little DNA is required and differences at level of species and subspecies may be detected (Vos *et al*., 1995). Normally, for plant genome analysis between 50 and 100 markers are obtained for each combination of oligonucleotide primers (Simpson, 1997).

Until now, reports for *Agave tequilana* have only described studies in which the genetic variability between rhizome offsets was determined, but not of bulbils or plants produced from seeds. Recently the level of polymorphism between nine *A. tequilana* varieties and rhizome offsets from different plants was reported (Gil-Vega *et al*., 2006). This report also showed that the 'Manso', 'Azul listado', 'Moraleño' and 'Sigüin' cultivars are closely related genetically to *A. tequilana* Weber var. Azul. These results agree with another report based on the levels of ploidy and DNA nuclear content (Palomino *et al*., 2003) in which eight varieties of *A. tequilana* were analyzed, and with a more recent analysis using the transposon display technique (Bousios *et al*., 2007) where the same grouping of varieties reported in Gil *et al*. (2006), was obtained.

Genetic variability in other asexually propagated *Agave* species such as *A. fourcroydes* (Infante *et al*., 2003) and *Agave angustifolia* (Barraza-Morales *et al*., 2006; Sánchez-Teyer *et al*., 2009), has also been reported in addition to other asexually propagated plants including banana (Loh *et al*., 2000) and also between plants propagated by somatic embryogenesis such as *Coffea arabica* (Sánchez-Teyer *et al*., 2003).

In this work the levels of genetic variability within offsets, bulbils and seed-propagated *Agave tequilana* Weber var. 'Azul' plants were compared using AFLP analysis.

MATERIALS AND METHODS

Plant material

Samples were taken from leaf tissue of offsets, bulbils and germinated seedlings (15 individuals of each type) obtained from the same mother plant located in a commercial plantation at Ex-Hacienda de Silva, Romita, Guanajuato, México. Samples were frozen in liquid nitrogen and stored at -70 ºC until processed.

DNA extraction

The CTAB DNA miniprep method was used (2.5 % CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris HCl pH 8, 0.02 % of β- mercaptoethanol) (Edwards, 2001).

AFLP analysis

The Li-cor Biosciences IRDye™ Fluorescent AFLP® Kit for Large Plant Genome Analysis was used based on Vos *et al*. (1995). In the selective amplification four primer combinations were used, where primers EcoRI were fluorescently labeled: MseI + CAG/EcoRI+ ACC, MseI + CAG/EcoRI + AGG, MseI + CAA/EcoRI + ACC and MseI + CAA/EcoRI + AGC. Bands were detected using a Li-cor model 4200 DNA analyzer™, (Lincoln, Nebraska).

Data analysis

Amplified fragment patterns were analyzed with SAGA^{MX} software to produce binary matrix with (+) for presence and (-) for absence of bands, and then were analyzed in the software Free Tree (Hampl *et al*. 2001) using the Simple Matching coefficient (Skroch *et al*., 1992). A dendrogram was constructed using the unweighted pair-group method with arithmetic averages (UPGMA), and the bootstrap values were calculated based on 1000 resampled trees (Felsenstein, 1985) using Free Tree. Levels of polymorphism were determined as described Infante *et al*. (2003) and Gil *et al*. (2006) (Method 1), where the number of polymorphic fragments is divided by total number of fragments analyzed and expressed as a percentage. The second was reported by Newbury *et al*. (2000) (Method 2), in which the number of polymorphic fragments is divided by the number of amplified fragments and multiplied by the number of individuals and expressed as percentage.

RESULTS AND DISCUSSION

This is the first study in *Agave tequilana* that describes the levels of genetic variability found between offspring produced by two different methods of asexual reproduction (offsets and bulbils) from the same mother plant and comparing them with plants sexually produced.

The selective amplification using four primer combinations produced 293 amplified fragments in each group of analyzed plants ranging in size from 50 to 500 bp. Levels of polymorphism determined by two different

methods appear in Table 1. Although the levels of polymorphism determined by the different methods are numerically very different, the relationship between the three different types of samples is the same. Plants produced from seeds showed the highest levels of polymorphism, bulbils intermediate and offsets the lowest levels.

Figure 1 shows the dendrogram obtained using the simple matching coefficient and UPGMA. Two main groups supported by boostrap values are observed. The nodes defining each group showed high bootstrap values indicating that the topology of the dendrogram shown in the figure is robust. Group A contained all samples asexually reproduced and group S all samples from germinated seedlings. Group A showed two sub-groups, R and B, which included samples from offsets and bulbils, respectively. Levels of similarity within groups in the dendrogram confirm the results obtained by calculating the levels of polymorphism that individuals reproduced by seeds are genetically more diverse than those reproduced asexually.

Detailed analysis of the AFLP images revealed markers that are unique to one of the three groups of plants and therefore specific to a single reproductive group. An example is shown in Figure 2, where a 102 pb band in the M-CAA, E-ACC primer combination is present in the bulbil samples but absent in offsets and seed derived plants. This suggests that the mechanisms producing the genetic variability in bulbils and offsets act independently in different parts of the mother plant.

Several previous reports have described the occurrence of genetic variation in different asexually propagated plant species (Crouch *et al*., 2000; Loh *et al*., 2000; Pornon *et al*., 2000; Infante *et al*., 2003, Rottenberg and Parker, 2004; Wang *et al*., 2004; Gil-Vega *et al*., 2006; Barraza-Morales *et al*., 2006 and Sánchez-Teyer *et al*., 2009) as shown in Table 2. Comparison of the levels of polymorphism observed in different studies is hampered by the different population types and different methods used to calculate variation. But by comparing polymorphism level in *Agave* species we can observe that in *A. fourcroydes* and *A. tequilana* these are lower than in *A. angustifolia,* and these differences could be because populations analyzed in *A. angustifolia* are wild, so being heterogeneous populations. Although the two methods used in this study to calculate the level of polymorphism produced by each reproductive strategy lead to numerically large different results, the relative levels of polymorphism between each group remained constant. This suggests that both methods are informative, although care should be taken to apply the same method when comparing different reports. In general, levels of polymorphism were higher than other reports in *Agave*.

Of the two methods of asexual reproduction, offsets showed lowest levels of variability. These results were reflected in the similarity values of the dendrogram where the samples also grouped according to reproductive mechanism.

Although it was expected that the sexually propagated individuals would be genetically distinct to those produced asexually, it is interesting that the bulbil and offset samples also form discrete groups. These groups are determined by markers which are specific to or highly represented within each. This may be related to the developmental processes of the plant. Offsets are produced throughout the life cycle of the plant on rhizomes or directly from the stem, whereas bulbils are only formed on the mature floral scape when sexual reproduction has failed. Different mutations may accumulate in certain cell lineages throughout the long life cycle of the plant leading to differences in genotype when bulbils and offsets develop on different plant tissues.

Preliminary data (Abraham-Juarez *et al*., unpublished observation) suggest that new meristems form to produce bulbils on the floral scape and many hundreds of these may form. This implies a massive developmental reprogramming and initiation of cell division which may lead to errors during replication or mitosis, resulting in an increase in detectable changes at the DNA level. These changes may be single base substitutions, deletions or insertions or involve rearrangement of larger fragments or whole chromosomes.

This study confirms previous reports of variation in asexually propagated *A. tequilana* plants and shows that significant variation may also be found between individuals obtained from the same mother plant. These results suggest that genotype variability could be maintained and even increased in *A. tequilana* by inducing and propagating bulbils on selected plants, but at the same time avoid the much higher levels of variability produced by sexual recombination which could produce undesirable phenotypic traits. Since *in vitro* propagation is being exploited as an alternative to produce plants for commercial plantations, it will be essential to determine the levels of variability produced by this process. It will also be of interest to determine the molecular nature of the underlying mutations and the mechanisms which cause them.

Table 1. Determination of levels of polymorphism using two different methods.

Analyzed form of reproduction	Number of analyzed bands	Number of polymorphic bands	Level of polymorphism Method 1	Level of polymorphism Method 2
Offsets	293	220	75.08 %	9.3 %
Bulbils	287	247	86.06 %	10.7 %
Seedlings	293	264	90.10 %	11.3 %

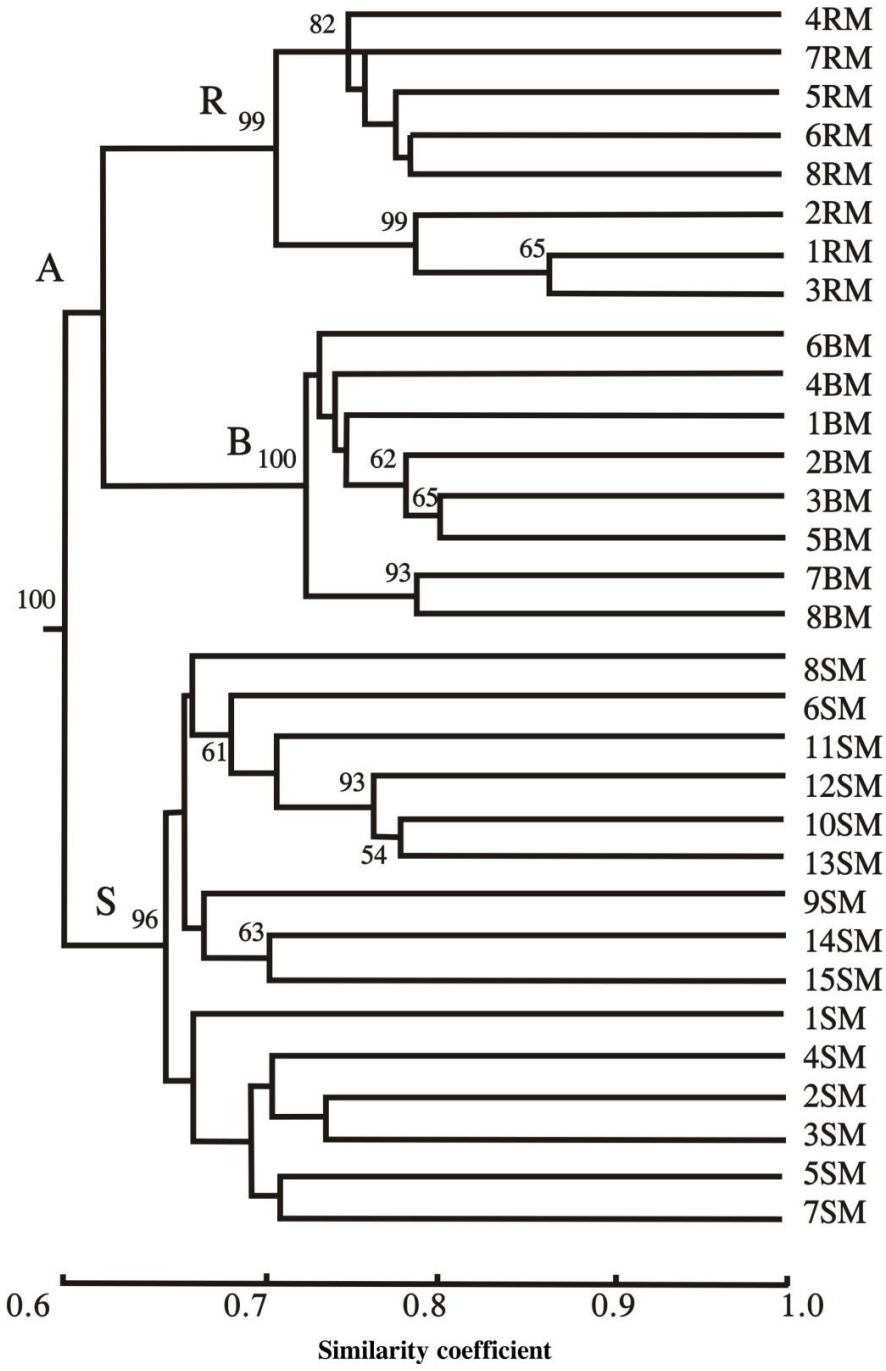

Figure 1. Dendrogram showing the genetic relationships between the analyzed individuals. The numbers indicate the percentage of Bootstrap repetitions obtained at each node from 1000 resampled trees. R = offsets; B = Bulbils; S = Plants grown from seed.

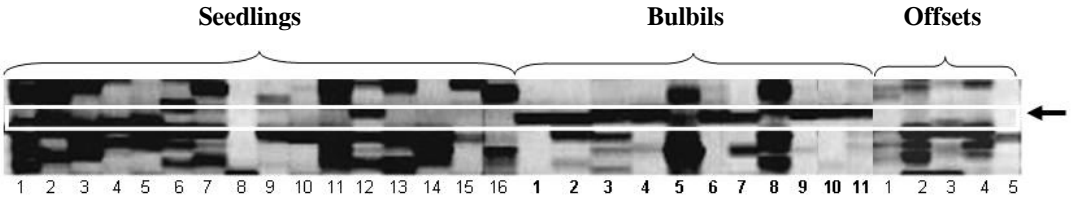

Figure 2. AFLP banding patterns obtained from all three reproductive groups of plants of *A. tequilana*. The arrow indicates a marker specific to bulbils.

Table 2. Comparison of polymorphism levels in asexually propagated plant species.

pecies	Level of Polymorphism	Marker type	References
Musa spp. group AAB	68 %	RAPD	Crouch *et al.,* 2000
Cvs Musa spp.	89 %	AFLP	Loh *et al.,* 2000
Rhododendron ferrugineum	60-70 %	AFLP	Pornon *et al.,* 2000
Oxalis pes-caprae	70.2-87.6 %	AFLP	Rottenberg *et al.,* 2004
Titanotrichum oldhamii bulbils	77.7 %	RAPD	Wang *et al.,* 2004
Agave fourcroydes	83 % between populations, 20.67 % between offsets of same plant.	AFLP	Infante *et al.,* 2003
Agave tequilana	61 % between varieties, 26 % between offsets of different plant.	AFLP	Gil *et al.,* 2006
Agave angustifolia	82.8 %	AFLP	Barraza-Morales *et al*., 2006
Agave angustifolia	73.5 %	AFLP	Sánchez-Teyer *et al*., 2009

REFERENCES

Barraza-Morales A, F L Sánchez-Teyer, M Robert, M Esqueda, A Gardea (2006) Variabilidad genética en *Agave angustifolia* Haw. de la Sierra Sonorense, México, determinada con marcadores AFLP. Rev. Fitotec. Mex. 29:1-8.

Bousios A, I Saldana-Oyarzabal, A G Valenzuela-Zapata, C Word, S R Pearce (2007) Isolation and characterization of Ty1-*copia* retrotransposon sequences in the blue agave (*Agave tequilana* Weber var. azul) and their development as SSAP markers for phylogenetic analysis. Plant Sci. 172:291-298.

Crouch H K, J H Crouch, S Madsen, D R Vuylsteke, R Ortiz (2000) Comparative analysis of phenotypic and genotypic diversity among plantain landraces (*Musa spp*., AAB group). Theor. Appl. Gen. 101:1056-1065.

Edwards K J (2001) Molecular Tools for Screening Biodiversity. Karp, P G Isaac, D S Ingram (eds). D S Kluwer Academic Publishers. pp: 22-23.

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:793-791.

arcía-Mendoza A (2007) Los agaves de México. Ciencias 87:14-23.

Gil-Vega K, C M Gonzáles, O Martínez de la Vega, J Simpson, G Vandemark (2001) Analysis of genetic diversity in *Agave tequilana* var. Azul using RAPD markers. Euphytica 119:335-341.

Gil-Vega K, C Díaz, A Nava-Cedillo, J Simpson (2006) AFLP analysis of *Agave tequilana* varieties. Plant Sci. 170:904-909.

Good-Avila S, V Souza, B Gaut, L Eguiarte (2006) Timing and rate of speciation in *Agave* (Agavaceae). Proc. Nat. Acad. Sci. (PNAS) 103:9124-9129.

Granados S D (1993) Los Agaves en México. Universidad Autónoma Chapingo, México. pp:31-35.

Hampl V, J Pavlícek, J Flegr (2001) Construction and bootstrap analysis of DNA fingerprinting-based phylogenetic trees with the freeware program Free Tree: application to trichomonad parasites. Internatl. J. Syst. Evol. Microbiol. 51:731-735.

Infante H D, E L Peraza, H J Herrera, O G González, L M Keb, M Robert (2003) Asexual genetic variability in *Agave fourcroydes*. Plant Sci. 164: 223-230.

Loh J, R Kiev, O Set, L Gan, Y Gan (2000) Amplified fragment length polymorphism fingerprinting of 16 banana cultivars (Musa cvs.). Mol. Physiol. Evol. 7:360-366.

Newbury J H, E C Howell, J H Crouch, B V Ford-Lloyd (2000) Natural and culture-induced genetic variation in plantains (*Musa* spp. AAB group). Austr. J. Bot. 48:493-500.

Palomino G, J Dolezel, I Mendez, A Rubluo (2003) Nuclear genome size analysis of *Agave tequilana* Weber. Caryologia 56:37-46.

Pornon A, N Escaravage, P Thomas, P Taberlet (2000) Dynamics of genotypic structure in clonal *Rhododendron ferrugineum* (Ericaceae) populations. Mol. Ecol. 9:1099-1112.

Rottenberg A J, S Parker (2004) Asexual populations of the invasive weed *Oxalis pes-caprae* are genetically variable. Proce. Royal Soc. Lond. B (Suppl.) 271:206-208.

Sánchez-Teyer L F, F Quiroz-Figueroa, V Loyola-Vargas, D Infante (2003) Culture-induced variation in plants of *Coffea Arabica* cv. caturra rojo, regenerated by direct and indirect somatic embryogenesis. Mol. Biotechnol. 23:107-115.

Sánchez-Teyer L F, S Moreno-Salazar, M Esqueda, A Barraza, M L Robert (2009) Genetic variability of wild *Agave angustifolia* populations based on AFLP: A basic study for conservation. J. Arid Environ. 73:611-616.

Simpson J (1997) Amplified fragment length polymorphisms (AFLP). Bol. Soc. Bot. Méx. 60:119-122.

Skroch P, J Tivang, J Nienhuis (1992) Analysis of genetic relationships using RAPD marker data. *In*: Joint Plant Breeding Symposia. Crop Science Society of America- American Society for Horticultural Science- American Genetic Association, Minneapolis, Mn. pp 26-30.

Valenzuela A (1997) El Agav e Tequilero su Cultivo e Industria. Litteris Editores. México. pp:31-51.

Vos P, R Hogers, M Bleeker, M Reijans, T V D Lee, M Hornes, A Frijters, J Pot, J Peleman, M Kuiper, M Zabeau (1995) AFLP: A new technique for DNA fingerprinting. Nucleic Acids Res. 23:4407-4414.

Wang C N, M Moller, Q C B Cronk (2004) Population genetic structure of *Titanotrichum oldhamii* (Gesneriaceae), a subtropical bulbiliferous plant with mixed sexual and asexual reproduction. Ann. Bot. 93:1.