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Article

Genetic analysis of Hass avocado variants using SSRs and EST-SSRs

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Abstract

The research was carried out at the Plant Genetic Resources Laboratory of the 'Presidente Juárez' Faculty of Agrobiology, Michoacán University of San Nicolás de Hidalgo, in 2020 and 2022. The objective was to determine the viability of molecular markers of the SSR and EST-SSR types and their usefulness for the discrimination of avocado variants of the Hass variety. For this purpose, seven variant genotypes of Hass avocado and the Hass variety were analyzed using 19 SSR markers and nine EST-SSR markers. The InfoGen 2016 program helped determine the polymorphism information content (PIC) and the following genetic parameters: genetic diversity (I), heterozygosity (He), and number of effective alleles (Na) per locus. Genetic distances were estimated using the Jaccard index criterion applying the Neighbor-joining hierarchical clustering method. The analysis determined a total of 757 bands, an average of 27.03 alleles/marker locus and an average PIC value of 28.61%. In terms of genetic parameters, the EM2HG genotype had the highest genetic diversity value (I= 0.47), a PIC of 0.36 and a Na= 1.92. The Hass variety and the EM6HG variant showed the greatest genetic similarity (43%). These results show the viability of SSR and EST-SSR markers for the discrimination of closely related Hass avocado genotypes.

Keywords:

Persea americana, genetic diversity.

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Introduction

The Hass avocado is the main variety marketed worldwide due to the high quality of its pulp, among other qualities. Mexico is the largest producing country with almost 2.6 million tonnes (SIAP, 2023) and ranks as the world leader in exports with 47.5% of the value of world exports (ITC-Trade Map, 2023). In Mexico, the state of Michoacán is the main producer, with a share of 74.21% of the national production, a place it obtains for the 183 385 ha planted (SIAP, 2023). In this region, there is a wide variety of agroclimatic conditions that have favored the identification of genotypes with morphological differences compared to the Hass variety (Gutiérrez-Contreras *et al*., 2010).

The evaluation of avocado tree variability has been done mainly based on morphological traits; however, this descriptive level is limited since the expression of genes is subject to environmental factors; in this regard, the use of molecular techniques offers the advantage of working directly with the genetic base of individuals to make a more precise identification and accelerate the selection work for genetic improvement and use of existing diversity.

Previous studies on genetic variability in avocado crops have been aimed at understanding and maintaining existing genetic diversity. Restriction fragment length polymorphisms (RFLPs)-based markers (Botstein *et al*., 1980) have been used in evolutionary, phylogenetic, and genealogical studies (Galindo-Tovar *et al*., 2011).

Markers based on random amplified polymorphic DNA (RAPD) (Robarts and Wolfe, 2014) and microsatellites or simple sequence repeats (SSRs) (Litt and Luty, 1989) have been used for similar studies in all three horticultural races and in varieties of commercial interest (Abraham and Takrama, 2014; Ferrer-Pereira *et al*., 2017; Sánchez-González *et al*., 2019; Liu *et al*., 2020). SSR markers detect repeated sequences of (GT)n or (CT)n types with a high degree of variation in the number of repeats in different individuals (Litt and Luty, 1989); specific primers are used, which hybridize in conserved regions flanking the specific region of DNA containing the repeated sequences (Masuelli, 1999).

These markers are considered to be a more robust and informative technique than RFLPs, RAPDs and AFLPs. They are of simple Mendelian inheritance, codominant, easy to measure and analyze, have 100% reliability, are reproducible and automatable. The rapid rate evolution of microsatellites allows them to be reliable markers when studying individuals with close genetic relationships. In addition, nuclear loci data are essential to obtain an overview of the evolutionary potential and history of the avocado (Galindo-Tovar *et al*., 2011).

On the other hand, expressed sequence tags-single sequence repeat (EST-SSR) markers are short sequences obtained from complementary DNA (cDNA) clones and serve as small gene identifiers. These primers are able to provide a rough estimate of the genes that are actively expressed in the genome to which they are linked, so they represent potentially functional markers (Dillon *et al*., 2014), they have the advantage that they can be easily generated from different types of cells, are highly polymorphic and codominant in nature.

The primers that flank EST-SSR sequences are derived from relatively conserved sequences, so null alleles are likely to be a minor problem for these types of markers in contrast to SSR markers (Ellis and Burke, 2007). EST-SSR markers developed for a specific species can be successfully used for diversity studies, comparative mapping, and marker-assisted selection. Nonetheless, this technique is not as efficient as SSRs due to the lower level of polymorphism they detect and therefore, they are not as efficient at discriminating closely related genotypes.

Microsatellite markers, due to their codominant nature and high mutation rate, allow the estimation of genetic diversity within and between races, as well as genetic mixing between races, even if they are closely related (Sunnucks, 2000); this characteristic makes them very useful for analyzing the degree of relationship between individuals or groups (Al-Samarai and Al-Kazaz, 2015). These types of markers have been successfully tested in genetic diversity studies in avocado genotypes with close levels of kinship, such as hybrids of the Mexican race (Sánchez-González *et al*., 2019),

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as well as in other crops such as rice (Pérez-Almeida *et al*., 2011), cotton (Abdurakhmonov *et al*., 2008) and grapes (Cipriani *et al*., 2008), among other species.

The research aimed to evaluate the feasibility of SSR and EST-SSR markers for genetic diversity studies in closely related genotypes, in this case, variants of the Hass variety identified in the avocado-growing belt of the state of Michoacán, Mexico.

Materials and methods

The research was carried out at the Plant Genetic Resources Laboratory of the Advanced Agrobiotechnology Research Unit (UIAA, for its acronym in Spanish) of the 'Presidente Juárez' Faculty of Agrobiology under the Michoacán University of San Nicolás de Hidalgo from 2020 to 2022.

Genetic material

The seven variant genotypes of the Hass variety included in the research are from different areas of the avocado-growing belt of the state of Michoacán and are established in the Avocado Germplasm Bank of the 'Presidente Juárez' Faculty of Agrobiology, located in the locality of Santa Rosa, Uruapan, Michoacán, between the coordinates 19° 22' 35" north latitude and 102° 01' 38" west longitude. The genotypes were selected for the analysis because they present differences in productivity, earliness, and plant height compared to the Hass (HASS) variety, these were identified as follows: EM1HG, EM2HG, EM3HG, EM4HG, EM5HG, EM6HG, and EM7HG. The Hass variety was included as a control.

Total genomic DNA isolation

DNA was obtained based on the procedure described by Huang *et al*. (2013); lyophilized and ground tissue was obtained without the application of liquid nitrogen, the incubation time was extended to 90 min and the first centrifuge stage was extended to 15 min. To avoid the high concentration of phenolic compounds in the DNA solution, chloroform was replaced by dichloromethane and the second centrifugation stage was extended to 10 min like the rest of the centrifugations. DNA quality was verified by 1% agarose gel electrophoresis composed of 0.5 g agarose dissolved in 50 ml of 1X TBE (Tris, Borate and Edta) buffer solution and 2 µl of SYBR® Safe DNA gel stain (10 000X) and by means of an ultra-low volume spectrophotometer (ThermoScientific® NanoDropND-000V3.7).

Amplification conditions

A total of 28 primers: 19 of the SSR type and 9 of the EST-SSR type (Gross-German, 2013), except for the bLMAV.01 locus, were used in the present work. The reaction mixture for DNA amplification was prepared in 10 µl composed of 0.8 x Red Taq (2x), 0.5 µM forward primer (10 µM) and 0.5 µM reverse primer (10 µM) and 40 ng DNA (10 ng μl^{-1}). The amplification program consisted of an initial cycle of 94 °C for 5 min, 30 denaturation cycles of 94 °C for 40 s, 57 °C for 40 s of annealing with the primer, and 72 °C for 19 s of extension. Finally, an extension cycle of five min at 72 °C. The thermal cycler used was a Techne model: FTC41H2D. The separation of the fragments was done in 8% polyacrylamide gels in 200 ml vertical electrophoresis systems (Enduro™ Power Supplies 300 V) and the identification of the amplified fragments was achieved by staining with silver nitrate ($AgNO_3$).

Determination of the level of polymorphism

To determine the level of polymorphism detected by each of the primers, polymorphic bands were considered to be those that were absent in at least one of the genotypes evaluated; with these data, a binary matrix of absence/presence was generated for each allele, where the presence of the band was translated as 1 (one) and the absence as 0 (zero).

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Estimation of genetic parameters

For each marker locus, the following parameters were estimated: polymorphism information content (PIC), genetic diversity (I), heterozygosity (He) and number of effective alleles (Na) per marker locus. These determinations were made through the InfoGen 2016 program (Balzaniri and Di Rienzo, 2016); this program was also used to obtain point estimates of Bootstrap with 1 000 repeats for the parameters of genetic variability.

From the binary matrix, a genetic similarity matrix was obtained with the criterion of the Jaccard index a/(a+b+c) and the square root transformation of (1-similarity) (Jaccard, 1908), developed for binary data, which allowed obtaining and observing the similarity and dissimilarity that exists between individuals. The dendrogram was constructed by applying the Neighbor-joining hierarchical grouping method recommended to generate groups by similarity, on the matrix of distances formed from the Jaccard similarity index (sqrt (1-S)) with the help of the MEGA5 program (Tamura *et al*., 2011).

Results and discussion

The 28 primers tested detected a total of 757 bands, mostly polymorphic (91.94%). The number of alleles detected by marker locus ranged from 12 (LMAV.20) to 46 (LMAV.31), these results confirm the high level of information of the selected markers. The overall mean obtained was 27.44 alleles per marker locus: 26.32 of the SSR type and 28.56 of the EST-SSR type. These results are 14.06 average allele higher than those reported in other studies that have used these same microsatellites (Guzmán *et al*., 2017; Boza *et al*., 2018; Sánchez-González *et al*., 2019).

These works used *Persea* genotypes of the three botanical avocado races, as well as other genera and hybrids of the Mexican race (Sánchez-González *et al*., 2019); in contrast, the materials analyzed in this research work are variant genotypes of the Hass variety. It is important to note that the microsatellites used here come from *Persea americana* Mill. and that the genetic relationships between the three botanical races of avocado are not completely deciphered (Gross-German, 2013). This may limit the explanation of the greater number of alleles per marker locus determined in this work, and this may be reflected in the polymorphism information content.

In relation to the PIC detected, the results indicated that it fluctuated from 0.257 (LMAV.06) to 0.332 (LMAV.14). In general, the average PIC was 0.286, which is considered low compared to the results obtained in different studies (Abraham and Takrama, 2014; Boza *et al*., 2018; Sánchez-González *et al*., 2019) in hybrids of the Mexican race, of 0.72, 0.71 and 0.47, respectively, which can be explained by the high level of kinship presented by the individuals analyzed; in contrast to those evaluated by the cited authors.

A PIC greater than 0.5 revealed the existence of He (Botstein *et al*., 1980); although the average PIC obtained in the present study is below this index, these markers have demonstrated their usefulness in detecting genetic variability (Gross-German, 2013; Guzmán *et al*., 2017) in species such as avocados. These results are a reflection of the material used with respect to that included in other studies on this species: avocado races (Guzmán *et al*., 2017; Boza *et al*., 2018) or varieties (Ferrer-Pereira *et al*., 2017; Guzmán *et al*., 2017) have been compared and, therefore, they are genetically more diverse. The estimated values of the genetic parameters for each of the genotypes are shown in Table 1.

Table 1. Genetic parameters estimated in seven avocado variant individuals and for the Hass variety.

Parameter	EM1HG	EM2HG	EM3HG	EM4H	EM5HG	EM6HG	EM7HG	Hass
Polymorphic Loci (95)	1	1	1	1	0.964	1	1	1
Genetic diversity (I)	0.458	0.477	0.416	0.432	0.429	0.391	0.458	0.391

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Parameter	EM1HG	EM2HG	EM3HG	EM4H	EM5HG	EM6HG	EM7HG	Hass
Average heterozygosity (He)	1	1	1	1	0.964	1	1	1
Effective # of alleles (Na)	1.868	1.918	1.756	1.784	1.795	1.707	1.861	1.697

The I ranged from 0.39 (Hass and EM6HG) to 0.48 (EM2HG), with Hass and EM6HG being the genotypes with the lowest values. The estimated He= 0.99 confirms the high level of information of the markers used. The higher values obtained of He compared to the limit value of 0.5 for all the marker loci analyzed favored the idea that these markers can be used for studies which require information on closely related genotypes and their genetic diversity which allowed the non-conservation of duplicates in germplasm banks, especially in perennial species in which large areas and high maintenance costs are required.

The discriminative power of the markers used was evaluated by calculating the PIC for each individual, which fluctuated from 0.31 to 0.36. The individuals with the lowest PIC were Hass and EM6HG, with a score of 0.31; this is consistent with the values determined for I. Both results were confirmed by the Na by locus. This parameter varied from 1 697 to 1 918; the Hass variety presented the lowest Na, this variety together with the EM6HG variant (1.707) are the genotypes that presented the lowest level of genetic variability of all the germplasm analyzed. The EM2HG genotype was identified with the highest percentage of variability, with 0.49% of I, a result ratified with a PIC of 0.36 and Na of 1.918.

This genotype is peculiar within the germplasm analyzed, suggesting that it is an important material within the collection for conservation purposes because it carries different information. Figure 1 shows the graphs of I, PIC, and Na; it was observed how this genotype (EM2HG) is separated from the rest of the individuals analyzed, mainly of the Hass variety.

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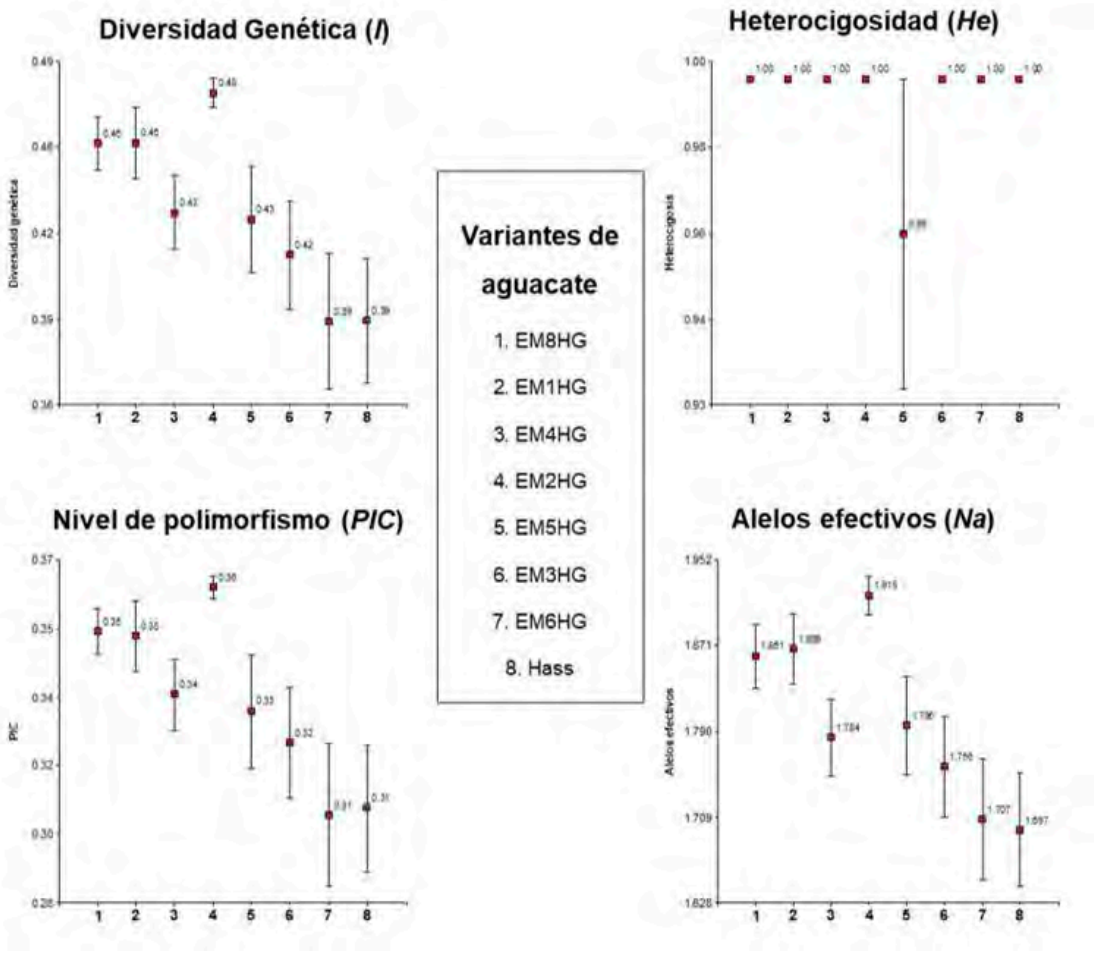
Figure 1. Averages (± SE) of genetic diversity (I), heterozygosity (He), polymorphism information content (PIC) and number of effective alleles (Na) determined in seven variants and in the Hass variety.



According to the results obtained, all the markers used detected high levels of polymorphism, which indicates their potential usefulness for the characterization of avocado variant genotypes that present close levels of kinship, from the genetic point of view. Some authors indicate the use of this type of marker for the identification of landrace materials (Guzmán *et al*., 2017; Boza *et al*., 2018) and cultivated varieties (Ferrer-Pereira *et al*., 2017). On the other hand, the estimated genetic distances indicate that the materials with the greatest genetic similarity were Hass and the EM6HG variant (0.43), in contrast to the EM3HG and EM4HG genotypes.

The genetic similarity analysis of the materials showed the formation of two perfectly defined groups (Figure 2). Group I, made up of the EM7HG, EM2HG, EM4HG and EM5HG genotypes, with a genetic similarity of 93%. Group II consisted of the EM1HG, EM3HG, EM6HG and Hass genotypes, with a genetic similarity rate of 86%. Within this group, the Hass and EM6H genotypes were the ones with the greatest similarity with 43%.

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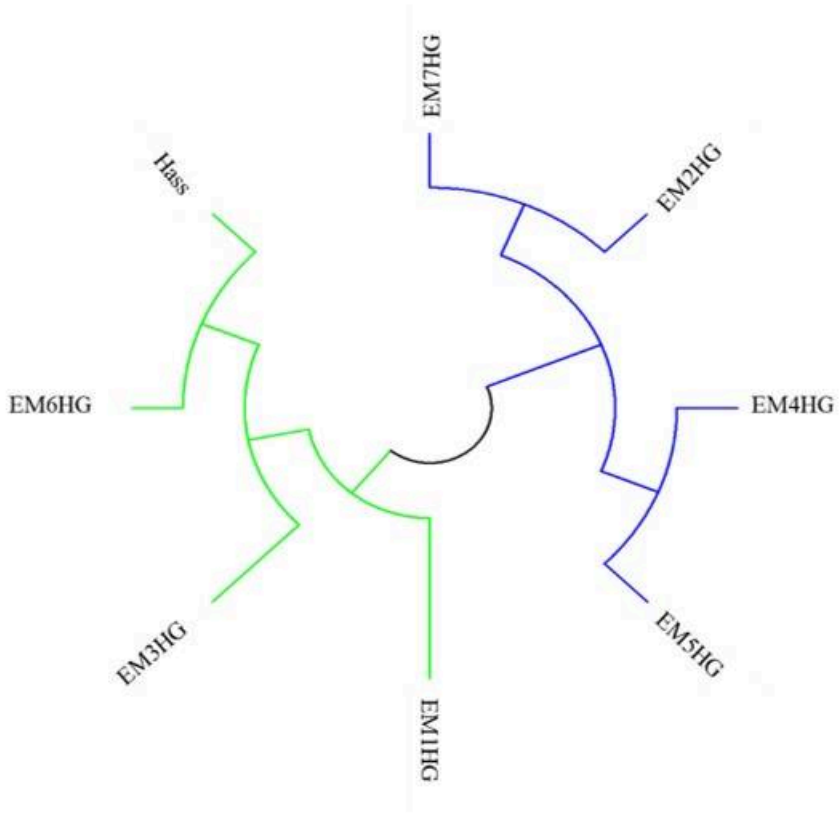
Figure 2. Dendrogram obtained by the Neighbor-joining method based on data from 28 molecular markers (SSRs and EST-SSRs) and genomic DNA samples from seven variant genotypes and the Hass variety (*Persea americana* Miller).



The grouping condition of the materials is due to the complexity of the hybrid state (Ashworth and Clegg, 2003) of avocado genotypes, domestication of the crop (Galindo-Tovar *et al*., 2011), gene flow and dichogamy, reflecting a great diversity of closely related genotypes. The low genetic distances observed are a response to the genetic closeness of the individuals due to the fact that they are variant genotypes of the Hass variety.

Conclusions

The SSR and EST-SSR markers showed their viability to discriminate closely related avocado genotypes; they allowed the estimation of genetic parameters for decision-making on the material to be conserved. The EM2HG variant had the greatest genetic similarity to the Hass variety.

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