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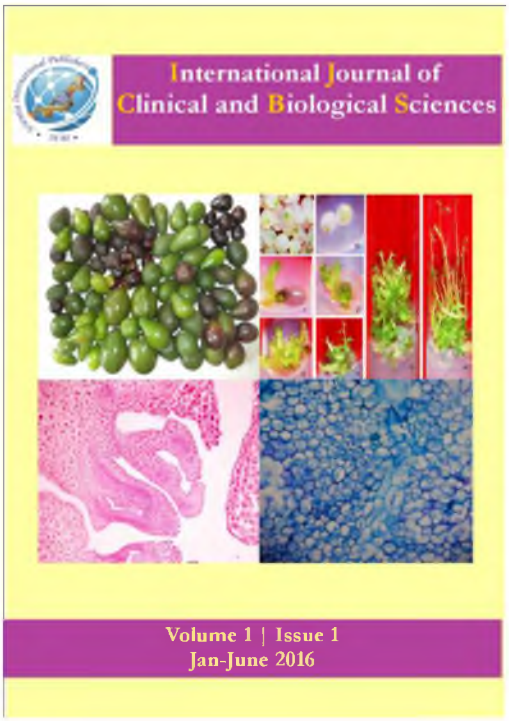

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Genetic Variability of Avocado Germplasm for Plant Breeding

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Abstract

Avocado (*Persea americana* Mill.), due to their physico-chemical and nutritional traits, and its productive characteristics has become a crop of high interest. For the purpose of breeding, it is necessary to know the genetic variability in this fruit crop. Thus in the present study, 182 samples from the avocado work collection belonging to the experimental farm Tumbaco from the National Institute of Agricultural Research (INIAP) together with 18 samples of avocado cultivar 'National' were collected from the valleys of southern Ecuador (Austro) to molecularly characterize and determine the diversity and genetic structure. In addition, an overall analysis using 48 other avocado samples (cultivar 'National') which were collected in the valleys of central-north of Ecuador was carried out. Ten microsatellites were used for this study by M13-Tailing technique. The samples were genotyped using a DNA analyzer LI-COR 4300. It was determined that a lack of genetic structure in the avocado samples exists through similarity analysis (UPGMA) and principal coordinate analysis (PCO) respectively. The overall analysis revealed that avocado samples of cultivar 'National' collected from 6 provinces of the Andean valleys of Ecuador, showed a genetic difference of 4%, while the avocado introduced cultivars from the INIAP's work collection showed a genetic difference of 17%.

Keywords: *avocado, cultivar, genetic variability, molecular breeding*

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Introduction

Avocado consumption has an annual increase of 1.4% [1]. In Ecuador, the production of avocado (*Persea americana* Mill.) has ceased from being a crop of purely local production to become a product with high export potential [2] because of its valuable features, such as flavor, high yield, quality, nutrition and production throughout the year [3,4]. In Ecuador, avocado producing areas are located in the inter-andean valleys in the Provinces of Carchi, Imbabura, Pichincha, Tungurahua, Azuay and Loja [5]. Almost all of the Ecuadorian production is destined to the local market; however, a small proportion is exported to Colombia, which in 2005 obtained 93.27% of total exports, while United States is the second destination for exports and to a lesser extent to European countries [2].

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Currently, there is an increasing concern for the conservation of biodiversity and agricultural sustainability. Therefore, the preservation and use of plant genetic resources have been important in maintaining this diversity issues. The Fruit Program of INIAP has a collection of 31 avocado cultivars that can be exploited for breeding programs for selection of materials showing fruit quality or/and resistance or tolerance to biotic and abiotic factors.

In recent decades, in the orchards of the Ecuadorian highland, the avocado from Mexican races called 'National' (which is tolerant to root diseases) [6] has been replaced by commercial varieties (Fuerte and Hass) which are accepted in the local and international market. In addition, an analysis of genetic diversity has been carried out to determine the use of avocado cultivars as rootstocks that are tolerant to *Phytophthora cinnamomi* Rands, 1922 [7], which is of great interest for breeding programs of this fruit.

The genetic relationships among species of the genus *Persea* are not well defined and are based mainly on morphological parameters [8]. Avocado is characterized by a high level of heteregozidad resulting in unpredictable hybrid [9]. The variability in orchards of this fruit comes from its dicogamic hermaphrodite feature, namely, it has male and female flowers to the same tree, but they do not ripe at the same time [10]. For this reason, it is necessary to have at least two different cultivars of avocado in the same orchard, to allow the cross-fertilization genetic exchange.

Genetic relationships of Avocado have been analyzed with different molecular markers: SSRs [11], AFLPs [12], RFLPs [13], minisatellites and microsatellites [14, 15] and RAPDs [16]. There are also studies to develop a linkage map of this fruit [17], which would benefit in the future development of molecular markers to identify quantitative trait loci (QTL). These studies have shown a genetic complexity, because avocado are species with open pollination, and the genetic segregation is carried out resulting in greater variability [18]. The selection and multiple crosses between cultivars have not allowed the formation of defined groups with similar genetic material; on the contrary, differences have increased [19].

Materials and methods

Samples for the analysis of genetic variability were obtained from 214 avocado trees of 31 cultivars from the work collection of the Tumbaco Experimental Farm (INIAP), located in the Province of Pichincha (Ecuador). The site has the following characteristics: latitude 00°13'00 " S, longitude 78°24'00" O, altitude of 2348 MASL, an average temperature of 16 degree Celsius and a relative humidity of 70.86%. In addition, 20 samples of avocado cultivar 'National' were collected from the southern region of Ecuador.

Figure 1a and 1b: Variability of avocado fruit from different cultivars belonging to the INIAP's work collection of germplasm for breeding

50 g of young leaves of each tree were taken, using forceps and scissors which were previously disinfected with 75% alcohol. The samples were stored in plastic bags and dehydrated with silica gel (100 g/bag). Molecular characterization was performed at Department of Biotechnology laboratory, INIAP. For DNA extraction from the dried samples, DNA extraction protocol of Ferreira and Grattapaglia [20] was used with little modifications [21]. The integrity of the extracted DNA was assessed by 1% agarose gel electrophoresis and the bands were visualized using an ultraviolet transilluminator. DNA quantification was performed using Epoch™ Spectrophotometer (BioTek). DNA samples were diluted to 5 ng / µl for their use as template in PCR amplification. For PCR amplification, the primer AV1 [14] was used jointly with PCR cocktail as described in Table 1. PCR reaction was carried out using PTC-200 thermocycler and the PCR conditions are described in detail in Table 2.

Table 1: PCR cocktail for microsatellites amplification [21]

Reagent	Volume
DNA (5 ng/µl)	2,000 µl
$MgCl_2$ (25 mM)	0,600 µl
Buffer GoTaq (5 X)	1,500 µl
dNTP's (5 mM)	0,380 µl
Primer F (10 µM)	0,375 µl
Primer R (10 µM)	0,375 µl
Taq polymerase (5 U/µl)	0,100 µl
Ultrapure water	2,170 µl
Final volume	**7,500 µl**

A reaction cocktail for PCR (Table 3) was used for amplification of the samples using 10 primers reported by Ashworth et al. [14] by M13-tailing method. PCR reaction was carried out using PTC-200 thermocycler and the PCR programming conditions are described in detail in Table 4.

Table 2: PCR amplification program [21]

	Temperature °C	Time	
Initial denaturation cycle	94	5 min	
Denaturation	94	45 sec	
Annealing	According to primer	1 min	30 cycles
Elongation	72	2 min	
Final cycle of elongation	72	7 min	
Stabilization	10	5 min	

Table 3: Cocktail for amplification of individual microsatellite - M13 [21]

Reagent	Volume
DNA (5 ng/µl)	2,000 µl
$MgCl_2$ (25 mM)	0,500 µl
Buffer GoTaq (5 X)	1,000 µl
dNTP's (5 mM)	0,200 µl
M13-700/800 (1 µM)	0,800 µl
Primer F-M13 (1 µM)	0,050 µl
Primer R (10 µM)	0,080 µl
Taq polymerase (5 U/µl)	0,050 µl
Ultrapure water	0,320 µl
Final Volume	**5,000 µl**

Table 4: Micro satellite amplification program by M13 tailing technique [21]

	Temperature °C	Time	
Initial Denaturation cycle	94	2 min	
Denaturation	95	4 min	
Denaturation	95	1 min	
Annealing	50-68	2 min	25 cycles
Elongation	72	2 min	
Final cycle of elongation	72	10 min	
Stabilization	4	5 min	

A database was created for genotyping using SAGA-GT Microsatellite software. Information for each primer was described, such as size, channel of amplification (700-800 nm), range of size of bands and the duplex formed.

The acrylamide gel was composed of 20 ml of KB Gel Matrix Plus 6.5%, 150µl of ammonium per sulfate at 10% and 15 µl of Tetramethylethylenediamine (TEMED) at 99%; it was placed between glass plates of LI-COR 4300 with the respective comb. After 1 hour of polymerization of the gel, it was placed in the LI-COR 4300 with the buffer 1X TBE (Tris-Borate-EDTA) KB Plus LI-COR. Before starting the loading of the samples, a pre-run of 25 minutes was performed at 1500 V to focus the laser at 700 to 800 nm. Finally, 0.8 µl of the amplified products were loaded, which were previously diluted with Blue Stop Solution LI-COR in a ratio of 1: 1 and denaturalized to 94 °C for 5 minutes. The run at 1500 V for one and half hour was started. The molecular weight marker used was IRDye 30-350 bp.

Results and discussion

Genetic diversity analysis revealed a total of 110 alleles in 10 locus, with an average of 11 alleles/locus. Locus AV5 was the most polymorphic. The polymorphic information content (PIC) was 0,73; the expected heterozygosity (He) was 0.77; and the observed heterozygosity (Ho) was 0.65; being indicators of high genetic diversity.

Ho for the characterized avocado samples from the south-central inter-andean valleys was 0.64 while He was 0.74; these results are consistent with data obtained in an earlier study from the north-central inter-andean valleys [22], where Ho was 0.65 and He was 0.77 have been granted; values that indicate high genetic diversity among avocado samples.

The average PIC for the 10 microsatellite markers used in the study in the center-south region was 0,70; value that depends on the number of alleles for this locus and their frequencies; whereas the average value in the characterization of avocado samples from the center-north was 0.73. The locus showing the most polymorphism for both the center-north and south-central was AV2 with a value of 0,90. In the present study, the locus AV10 was not very informative, obtaining a PIC of 0.37; while for the study of genetic diversity of north-central, the locus AV4 with a PIC of 0.52 was the lowest value. Loci showing a PIC higher than 0.5 reveal that there is heterozygosity, and they are highly polymorphic and informative to detect genetic variability.

According to cluster analysis (UPGMA) detailed by cultivars, it is inferred that due to cross-pollination in avocado**,** samples were not genetically structured (Figure 2), namely, clearly defined groups were not formed; nevertheless some collected samples grouped with 'National' avocados (Mexican origin). Studies by Ashworth and Clegg [23] mention that due to the large phenotypic variability and hybrids between different varieties of avocado, the identification of genotypes by microsatellites has been ambiguous, consequently, it has been difficult to designate the racial composition (breed).

According to Ashworth and Clegg [23] and Galindo and Milagro [19] the conditions for grouping avocado germplasm is explained by the complexity of the hybrid status, species hybridization of the different racial ecotypes, crop domestication, dichogamy and gene flow; which has resulted in a great diversity of genotypes. In addition, Chen et al. [24] mentioned that it is difficult to infer the genetic history of avocado breeding because of the role of the hybridization in contemporary cultivars.

In Figure 2, the principal component analysis indicated that avocado collected samples from cultivar 'National' were grouped at coordinate 1, while avocado samples from the INIAP's work collection were forming a gene pool showing the most genetic diversity.

Wu et al., [25] found in their study that the avocado analyzed samples were grouped into three groups (Antillean, Guatemalan and Mexican) based on their origin and volatile compounds from their leaves. Rodriguez et al. [26], by cluster analysis found the formation of five groups with high similarity in the cultivars of Antillean origin and larger variability for cultivars of Guatemalan origin and certain hybrids. In this study, according to cluster analysis, cultivars of Mexican origin formed a single group; where as those from Guatemalan and Antillean origin did not form defined groups. In addition, the principal component analysis revealed that traits such as the anise smell, texture and pulp fibrousness are relevant characteristics to determine materials of Mexican origin. The same analysis, in global way, applied to 6 Provinces (Carchi, Imbabura, Pichincha, Tungurahua, Azuay and Loja) of the inter-andean valleys distinguished a grouping of the Provinces where the samples of cultivar 'National' were collected, while the samples obtained from the avocado work collection of INIAP (Pichincha) differed from those collected. This is because in the work collection contains introduced germplasm from distinct races, which differ genetically (Figure 3). However, studies have shown that although the avocado is a subtropical crop and has predominantly cross-pollination, the overall level of genetic variation is not exceptionally high [27].

The identification of locations is useful for genetic resource management and development of breeding programs. The populations who showed the highest genetic distance were Pichincha and Tungurahua, considering suitable candidates in targeted crosses.

Data obtained from the variance molecular analysis (AMOVA) are summarized in Table 5, showing a differentiation of 17 % from the different provinces, and 83% of the observed variation among individuals. These results are due to genetic differences that exist in the avocado work collection because there are 31different cultivars.

Figure 2: Dendrogram formed from the Jaccard distance matrix with the different avocado samples

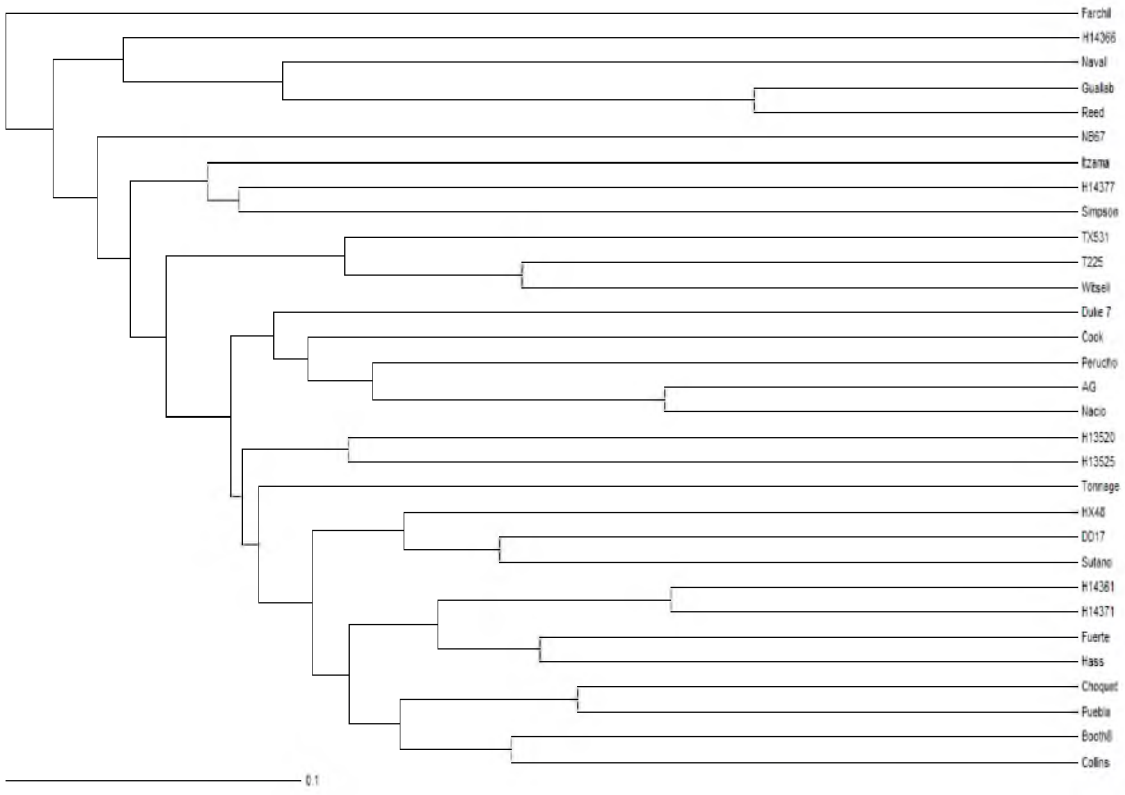

Figure 3: Principal component analysis by Provinces

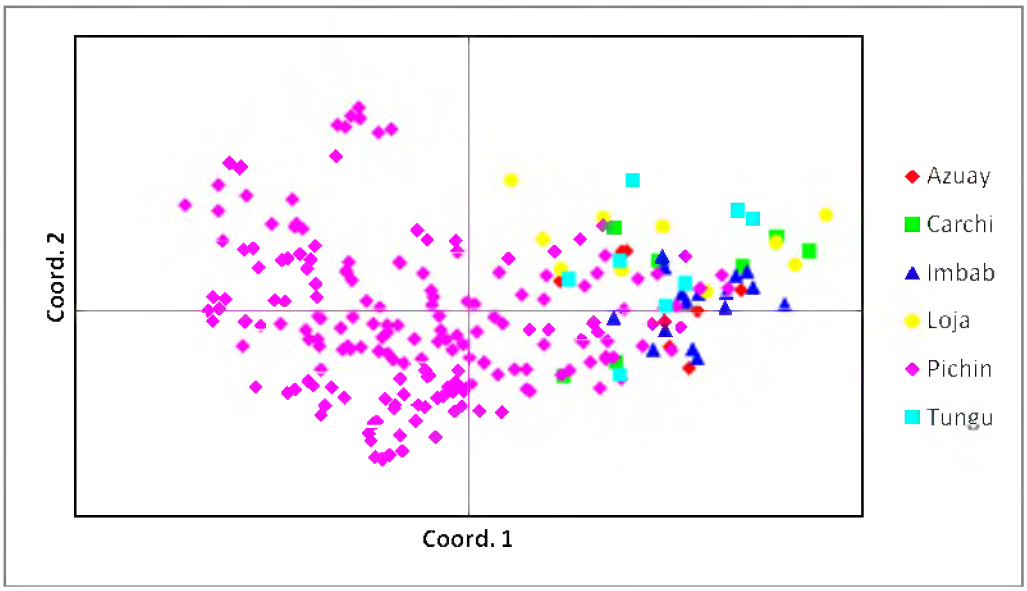

Table 5:Variance molecular analysis (AMOVA) for avocados samples collected in Ecuador

Origin	Degrees of freedom	Sum of squares	Variance Compounds	Standard Deviation	Genetic Variation (%)
Between Provinces	5	201.2	40.2	1.8	17
Within Provinces	242	2204.6	9.1	9.1	83
Total	247	2405.8		10.9	100

Conclusion

Molecular characterization of 200 avocado samples, using 10 microsatellites revealed an allelic richness of 111 locus with an average of 11 allele/locus. Genetic diversity or expected heterozygosity (0.74) indicated that the selected microsatellites are a useful set of great information for the characterization of avocado germplasm. The collected avocado samples for the cultivar 'National' in the north-central and southern region showed a low genetic variability. On the other hand, the variation between Provinces was high; therefore, it is inferred that the genetic variability in this study was due to the 31 avocado cultivars from the INIAP's work collection.

Conflict of Interest

We declare that we have no conflict of interest

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