Agro-morphologic traits, isoenzyme and DNA markers for estimating the polymorphism levels, discriminating capacity and informativeness in avocado

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RESUMEN. Se compararon los niveles de polimorfismo, capacidad de discriminación e informatividad de caracteres morfoagronómicos y de los marcadores AFLP, ISTR, SSR e isoenzimas, empleando 17 genotipos procedentes de la colección de germoplasma de Alquízar, Cuba. El poder de discriminación *D* utilizado para caracteres morfoagronómicos fue útil para la identificación de genotipos. Sólo cuatro variables fueron necesarias para distinguirlos: Forma del fruto, época de cosecha y color y espesor de la corteza del fruto. Los marcadores SSR, ISTR y AFLP constituyeron técnicas poderosas para la discriminación y certificación varietal, pero los marcadores dominantes resultaron los más eficientes. Con una simple combinación de cebadores AFLP o ISTR se identificaron todos los individuos. Las isoenzimas resultaron además, técnicas de bajo costo útiles para este propósito en el germoplasma local. Los niveles más elevados de heterocigosidad esperada se detectaron con marcadores codominantes, pero el valor determinado con los microsatélites superó en dos veces o más los obtenidos con isoenzimas y marcadores dominantes. El índice de diversidad morfológica resultó un buen estimador de la diversidad de las accesiones de aguacatero cuando se utilizaron variables de gran heredabilidad, y a su vez, comparable con la heterocigosidad esperada determinada con las isoenzimas y los marcadores de ADN. El valor de este índice fue similar a los obtenidos con ISTR y AFLP. Los índices de eficiencia del ensayo (A_i) y del marcador (*MI*) tuvieron el mismo patrón de variación que *D*, *I*, I_u y *P* para todos los marcadores moleculares, lo que sugiere que ambos índices probablemente son indicadores de la capacidad de discriminación en el aguacatero.

ABSTRACT. Agro-morphologic traits and molecular markers were compared in terms of their discriminating power and informativeness among 17 genotypes assembled in the Cuban avocado germplasm. *D* parameter adopted for agro-morphological traits was useful for genotype identification. Only four morphological traits were necessary for distinguishing all the individuals analyzed: fruit shape, fruit skin color in mature fruits, harvest season and fruit skin thickness. SSR, AFLP and ISTR markers were powerful techniques for avocado discriminating and varietal identification, but the high level of polymorphic loci detected by dominant markers highlights the discriminating capacity of these genetic markers. With a single AFLP or ISTR primer combination all the individuals were identified. Also, isoenzymes were a low cost technique useful for this purpose in local germplasm. The higher values of expected heterozygosity were detected in codominant markers, but the value for microsatellites doubled or more the ones obtained with isoenzymes and dominant markers. The morphological diversity index was a good estimator of diversity among avocado accessions when variables of high heritability are used and comparable with the expected heterozygosity scored with isoenzymes and DNA markers. The value of this index was very close to those obtained with ISTR and AFLP. The assay efficiency index (A_i) and marker index (*MI*) had the same pattern of variation than *D*, *I*, I_u and *P* for all molecular markers. Then, both indexes are probably indicators of the discriminating capacity in avocado.

INTRODUCTION

Latin America is one of the most important regions on the richness of tropical fruit trees, and it takes the first position in the production of oranges (*Citrus sinensis* (L.) Osb.), papaya (*Carica papaya* L.), guava (*Psidium guajava* L.) and avocado (*Persea americana* Mill.).[1] In

this way, avocado has particular importance since it is native from the tropical areas of America and constitutes an essential part of the human diet supplying minerals and vitamins.[2] This species is a perennial evergreen fruit tree which belongs to Lauraceae and can be recognized into Mexican, Guatemalan and West Indian horticultural races which are regarded as geographical ecotypes.[3] A new race was also described as *Persea americana* var. Costaricensis.[4]

In the case of fruit trees, *ex situ* conservation is the most used way for germplasm collection.[5] At the foundation in 1965 of the Tropical and Subtropical Genebank of Fruit Trees of Cuba, the greatest collection of avocado of the country was established at the Research Institute on Tropical Fruit Crop (IIFT).[6] It is constituted mainly by plants of West Indian race and in a little proportion by Guatemalan race and hybrids of Guatemalan X West Indian.[1,6] Characterization and evaluation of the gene pool have permitted to recommend some of them for commercial purposes, and nowadays new varieties have been introduced.

The facts related to the identification of great number of plants in a fruit trees collection and in commercial nurseries, as well as, those concerned with the protection of varietal names, recommend the use of efficient methods with highly discriminating capacity. In this way, molecular markers were used for varietal identification in grapes (*Vitis vinifera* L.).[7] Also, the comparison of discriminating capacity and informativeness by means of DNA markers in olive (*Olea europaea* L.) have been reported using different indexes.[8] In avocado, morphological data have been traditionally used for germplasm characterization and variety identification,[1,6,9,10] but also have been supplemented by isoenzymes,[11-13] and DNA markers.[13-16] Avocado descriptors published by the International Plant Genetic Resource Institute (IPGRI),[17] suggested the use of morphologic traits and molecular markers to establish fingerprint of individual accessions.[18] In addition, UPOV (International Union for the Protection of New Varieties of Plants) is the driving force for a distinct, uniform and stable (DUS) testing, the introduction of new test methods, and the legal implications of such changes for plant variety protection.[19]

The objective of this paper was to compare the polymorphism levels, discriminating capacity and informativeness of agro-morphologic traits, isoenzymes and DNA markers including Amplified Fragment Length Polymorphism (AFLP),[20] Inverse Sequence-Tagged Repeat (ISTR)[21] and Simple Sequence Repeat (SSR)[22-24] for varietal identification and estimating variability of avocado.

MATERIAL AND METHODS

Plant materials

A total of 17 avocado accessions assembled in the Research Institute on Tropical Fruit Crop (IIFT) germplasm bank at Alquízar, Havana, Cuba were used: 'Amado Gómez', 'California', 'Casimiro Soledad', 'Catalina', 'Centro América No. 3', 'CH-1 No. 3', 'Choquette', 'Duke-7', 'Hass', 'Itzamná', 'Jaruco No. 1', 'José Antonio', 'Los Moros', 'Lula', 'Monroe Estación', 'Sicilia No. 6' and 'Suardía Estación'. This field collection was located at 22° 47' of North latitude and 82° 31' of West longitude, at 11 m over sea level with flat topography. These varieties were evaluated for 14 qualitative traits recommended as highly discriminating descriptors:[17] trunk surface, color of young twig, surface of young twig, leaf shape, petal pubescence, sepal pubescence, fruit shape, fruit skin color, pedicel shape, fruit skin thickness, flesh texture, harvest season, seed shape and cotyledon surface.

Isoenzyme analysis

Fully expanded, young leaves from 15 years old trees were harvested, wiped clean and stored at 2 - 3 °C . until used.[12] A system of vertical electrophoresis and discontinuous buffers were employed using polyacrylamide gel (8,5 %) and 0,04 mol/L tris-glycine buffer at pH = 8,3.[25] The detection techniques for peroxidases (PX, E.C. 1.11.1.7), polyphenol oxidases (PPO, E.C. 1.10.3.1.) and ascorbate oxidases (AO, E.C. 1.10.3.3.), were those reported by some authors.[12,13]

Isolation, purification and PCR amplification of genomic DNA

Before DNA extraction, fresh young leaves were cleaned with 70 % ethanol. Total DNA was extracted from leaf material by a variation of the CTAB method,[26] described by Doyle and Doyle in1990.[27]

The following DNA marker techniques were used under standard reaction conditions with ^{33}P-labeled PCR primers: Amplified Fragment Length Polymorphism (AFLP),[20] Inverse Sequence-Tagged Repeat (ISTR),[21] and Simple Sequence Repeat (SSR).[22-24] The amplification reactions were made on a MJ Research model PTC-100 thermocycler (MJ Research, Watertown, Mass).

Amplified Fragment Length Polymorphism

Genomic DNA was restricted with *Eco*RI and *Mse*I followed by the ligation of the corresponding adaptors and pre-amplification in the presence of universal *Eco*RI and *Mse*I primers (E01: GACTGCGTACCAATTCA; M01: GATGAGTCCTGAGTAAA). Ten selective AFLP primer combinations were used for final amplification: E-AAC x M-AAC (AA1), E-AAC x M-ACC (AA2), E-AAC x M-ACT (AA3), E-AAC x M-AGA (AA4), E-AAC x M-ATC (AA5), E-AAC x M-ATG (AA6), E-AAC x M-ATT (AA7), E-AAC x M-CAA (AA8), E-AAG x M-ACC (AA9), E-AAG x M-AGT (AA10).[20] PCR amplifications followed were performed in a final volume of 20 μL containing 1 ng of preamplified genomic DNA, 0.2 mmol/μL dNTPs, 1.5 mmol/μL $MgCl_2$, 10X PCR buffer (GIBCO-BRL, Groningen, Netherlands), 10 pmol/μL each of ^{33}P-labelled AFLP primers and 5 U/μL of *Taq* DNA polymerase (GIBCO-BRL). The amplification program consisted of the following steps: 94 °C, 30 s; 65 °C (−0.7 °C/cycle), 30 s and 72 °C, 60 s during 12 cycles, until reaching the optimal annealing temperature of 56 °C . At this temperature, 24 more cycles were performed to complete the amplification.

Inverse Sequence-Tagged Repeat

Primers for forward (F_3) and backward reaction (B_2B) previously designed were used in PCR amplification.[21] Both F_3 and B_2B primers were labeled using polynucleotide kinase and (γ-^{33} P) ATP (Amersham-Pharmacia-Biotech). PCR reactions were performed according to standard protocols in a final volume of 25 μL containing 25 ng of genomic DNA, 200 μmol/μL dNTPs, 2.5 mmol /μL $MgCl_2$, 10X PCR buffer (GIBCO-BRL), 2.5 pmol/μL of each primer, and 1 U/μL of Taq DNA poymerase (GIBCO /BRL).[16] The amplification program consisted of the following steps: 95 °C, 3 min; 95 °C, 30 s; 45 °C, 30 s; 72 °C, 2 min; 72 °C, 10 min, with 40 cycles of steps 2 to 4.

Simple Sequence Repeat

Fifteen SSR primer pairs previously designed were used:[28] AVAG01 (AM1), AVAG02 (AM2), AVAG03 (AM3),

AVAG05 (AM4), AVAG06 (AM5), AVMIX01 (AM6), AVAG08 (AM7), AVAG09 (AM8), AVMIX02 (AM9), AVAG10 (AM10), AVMIX03 (AM11), AVMIX04 (AM12), AVAG11 (AM13), AVAG13 (AM14) and AVAC01 (AM15). The reaction mixtures contained 30 ng of template DNA,1.5 μmol/μL of each primer (with the reverse primer 5' end ^{33}P-labeled), 100 μmol/μL of dNTPs, 1.5 mmol/μL $MgCl_2$, 10X *Taq* buffer (containing 50 mmol/μL TRIS-HCl, pH 9; 0.1 % Triton X-100) and 1 U/μL of *Taq* DNA polymerase in a total volume of 10 μL . The reaction was processed at 94 °C for 30 s, followed by 32 cycles consisting of 94 °C, 15 s; 45 °C to 50 °C, 25 s (depending on the primers); 68 °C, 25 s, and a final extension step of 68 °C, 2 min .[29]

Gel electrophoresis analysis

After the reactions, the amplified AFLP, ISTR and SSR fragments were processed for analysis by polyacrylamide gel electrophoresis (PAGE) by adding sequencing loading buffer (Promega, Mannheim, Germany) and denaturation by heating at 94 °C. Aliquots of 2 - 3 μL were loaded onto a 4 % polyacrylamide sequencing gel run in 1X TBE buffer, pH 8.9 at 40 W . After running, the gel was fixed in 10 % acetic acid, washed with water, dried for one hour at 80 °C and exposed to X-rays films at room temperature for 1 to 3 d .

Data analysis

With qualitative traits, the analysis was made on the basis of the presence (1) or absence (0) of the stages of each variable and data processed apart from the number of loci and the inheritance type involved in these characters. Similarly, intense and reproducible AFLP and ISTR bands were scored by the same 1/0 system. Because of the codominance of the markers, isoenzymes and microsatellites (SSR) were scored as homozygotic and heterozygotic genotypes.[7,8,29]

To compare the levels of polymorphism, discriminating capacity and informativeness of agro-morphological traits and the four molecular markers employed (isoenzyme, SSR, AFLP and ISTR), for each assay unit (*U*: qualitative trait, enzymatic system, or the product of PCR amplification obtained with one set of primers) the following were estimated:

1) Number of polymorphic bands (or stages) (n_p);
2) Number of non polymorphic bands (or stages) (n_{np});
3) Total number of bands (or stages) $n = n_p + n_{np}$;
4) Average number of polymorphic bands (or stages) per assay unit (n_p/U);
5) Number of loci (*L*): in the case of AFLP and ISTR markers the theoretical maximum number of loci is equal to total number of bands ($n_p + n_{np}$) obtained for each marker type.[8] In isoenzymes were determined by inspection visual according to bands intensity and position;[12]
6) Number of loci per assay unit: $n_u = L/U$;
7) Number of banding (or stage) patterns (T_p);
8) Number of unique banding (or stage) patterns (T_{up});
9) Average number of banding (or stage) patterns/assay unit (*I*);
10) Average number of unique banding (or stage) patterns/assay unit (I_u);
11) Confusion probability (C_j) of the *j*th assay unit:[7]

$$C_j = \sum_{i=1}^{I} Pi \frac{(Np_i - 1)}{N - 1}$$

where p_i is the frequency of the *i*th pattern; *N*, sample size; *I*, total number of pattern generated by the *j*th assay unit;

12) Discriminating power (D_j) of the *j*th assay unit:[7] $D_j = 1 - C_j$;
13) Limit of *Dj* as *N* tends toward infinity:

$$D_L = \lim\left(D_j\right) = 1 - \sum_{i=1}^{I} p_i^2$$

14) Effective number of patterns per assay unit:[8]

$$P = \frac{1}{1 - D_L}$$

15) Average number of alleles per locus (n_{av}). For microsatellite the average number of alleles per locus according to the formula: $n_{av} = n_p/L$.[8] For AFLP and ISTR two alleles per assay were considered ($n_{av} = 2$).[8] In isoenzymes were determined according to the following expression:[12] $n_{av} = n/L$;

16) Expected heterozygosity (H_{ep}) of the polymorphic loci: $H_e = 1 - \Sigma p_i^2$ where p_i is the allele frequency of the *i*th allele and the arithmetic mean of the expected heterozygosity of the polymorphic loci:[8]

$$H_{ep} = \frac{\sum H_{np}}{n_p}$$

where n_p is the number of markers analyzed. For morphologic traits, the morphologic diversity index was defined as: $D_m = 1 - \Sigma p_i^2$ where p_i is the stage frequency of the *i*th stage of the polymorphic trait and the arithmetic mean of the index:

$$D_M = \frac{\sum D_m}{n}$$

where *n* is the number of variables analyzed. In addition, the Simpson diversity index was determined using the formula: $D_s = \Sigma p_i^2$,[30] where p_i is the morph-type frequency of the of the *i*th morph-type of the polymorphic trait and the arithmetic mean were determined using the expression:

$$D_S = \frac{\sum D_s}{n}$$

where *n* is the number of variables analyzed. In all cases, the values were corrected by the sample size as:

$$H_{ep} = \frac{2nH_e}{2n-1}, \qquad D_M = \frac{2nD_m}{2n-1} \quad \text{or} \quad D_S = \frac{2nD_s}{2n-1}$$

for the four genetic markers and agro-morphologic traits, respectively;

17) Fraction of polymorphic loci (β):[31]

$$\beta = \frac{n_p}{n_p + n_{np}}$$

18) Expected heterozygosity (H_e):[31] $H_e = \beta H_{ep}$;

19) Effective number alleles per locus (n_e):[32]

$$n_e = \frac{1}{\sum p_i^2}$$

where p_i is the frequency of the *i*th allele;

20) Total number of effective alleles (N_e):[33]

$$N_e = \sum n_e$$

21) Assay efficiency index (A_i):[33] $A_i = \frac{N_e}{U}$

22) Effective multiplex ratio:[31] $E = n_u \beta$

23) Marker index (MI):[31] $MI = EH_{ep}$

A linear correlation between morphologic (D_m) and Simpson (D_s) diversity indexes was also determined.

RESULTS

Polymorphism levels and discriminating capacity of agro-morphologic traits and molecular markers

The analysis of these characters was based on the polymorphism detected by the presence (1) or absence (0) of the different stages (classes) of each variable and the frequencies of patterns of them derived. A comparison of the efficiency of these variables according to the polymorphism levels and discriminating capacity is offered (Table 1).

The total number of stages varied from 2 for pedicel shape, trunk surface, young twig surface, petal pubescence and sepal pubescence to 7 for fruit shape. All of them were polymorphic in the accessions analyzed. This phenotypic polymorphism generated from 2 to 7 distinctive patterns. However, a maximum of only two unique patterns were detected.

As a consequence of the low values of confusion probability, the highest discrimination capacity with fruit and leaf shapes were detected. Nevertheless, the latest one was not very useful for distinguishing different genotypes since more than one type of leaf shapes were observed in twenty leaves randomly collected in the same tree. For instance, oblong, oval and roundish leaves in 'Suardía Estación' cultivar were found (Table 2).

The lowest values of discriminating power were obtained for trunk surface, young twig surface, petal pubescence and sepal pubescence. Moreover, the last three variables jointly with the pubescence of leave surface gave the same information since high positive correlation between them was detected when 30 avocado accessions were analyzed.

The effective number of patterns indicates the size of an ideal population in which, given the frequencies of the patterns obtained with a primer or a marker system, all of the individuals can be distinguished. Therefore, more than six genotypes can be distinguished with fruit shape trait when the population size tends to infinity, while only more than two did for pedicel shape, trunk surface, young twig surface, petal pubescence and sepal pubescence.

In accordance with these results, four reproductive variables were necessary to take in to account for discriminating all individuals (Table 3). Not all of them were distinguished when only vegetative traits for overall tree were considered.

Also, the results with the four molecular markers are offered (Table 1). The analysis with isoenzymes indicated that in PX, PPO and AO a total of 10, 8 and 7 bands were found, respectively, grouped in 5 or 4 loci. All loci were polymorphic in PX and AO systems, while 3 from 4 (75 %) showed polymorphism in PPO.

The total number of banding patterns ranged from 9 for AO to 17 for PX, and the unique patterns from 5 to 17, respectively, with intermediate values for PPO. Low confusion probability was obtained for the three enzymatic systems assayed, especially for PX and PPO. The discriminating capacity showed the higher values for PX (1.00) and PPO (0.98), while AO reached the lowest value (0.87). These results revealed that PX system could discriminate all the accessions studied.

The effective number of patterns indicates that with PX system up to 17 accessions can be distinguished when the population size tends to infinity. However, in PPO and AO, up to 13 and 5 accessions can be discriminated, respectively.

In SSR, all bands were polymorphic in each primer combination assayed, with the total number of bands ranging from 4 for AM9 to 12 for AM2 and AM6. The highest value of banding patterns was 14 for AM6 and AM12, while only 5 were detected for AM9. Unique banding patterns ranged from 2 to 12, indicating that more than one primer combination could be used to distinguish all the accessions studied. These results are in correspondence with the values of confusion probability and discriminating power calculated. The effective number of patterns ranged from 3.18 for AM9 to 11.56 for AM1, AM3, AM6, AM12 and AM14.

The total number of bands in AFLP ranged from 23 for AA8 to 51 for AA1 and AA7 and the number of polymorphic bands from 4 for AA8 to a maximum of 22 for AA3. The total number of banding patterns varied from 6 to 17 while the unique patterns from 1 to 17. These results showed that AFLP primer combinations AA1, AA2, AA4 and AA10 were effective for the identification of all accessions. This result agrees with the values of confusion probability (0) and discriminating power (1) obtained in these assay units. The lowest values of effective number of patterns were observed in two of the 10 primer combinations used: AA6 (5.90) and AA8 (4.90). The rest of assay units ranged from 9.32 to 17 in accordance with D_L values estimated.

In ISTR, the single primer combination assayed scored a total of 157 bands, all of them polymorphic. The analysis detected 17 unique banding patterns that permitted to distinguish the genotypes studied. This is in correspondence to the values of confusion probability (0) and discriminating capacity (1) and effective number of patterns per assay unit (17) determined.

Informativeness levels of agro-morphologic traits and molecular markers

Table 4 offers the results obtained with 14 phenotypic variables and also with dominant and codominant molecular markers.

The morphological diversity index ranged from 0.11 for trunk surface, young twig surface, petal pubescence and sepal pubescence to 0.43 for pedicel shape and cotyledon surface. Moreover, relative high values of this index were detected for fruit skin thickness (0.41), flesh texture (0.41) and harvest season (0.38). An intermediate values were observed in the rest of variables analyzed. A significant negative linear correlation ($r = -0.70^{**}$) with the Simpson diversity index was found.

The average of the number of alleles per locus in isoenzymes ranged from 1.8 for AO to 2 for PX and PPO. Similarly, the effective number of alleles per locus was higher in the same enzymatic markers, with respective values of 2.09 and 1.88 and slightly lower in AO (1.50). This was reflected in the expected heterozygosity of

Table 1. Levels of polymorphism and discriminating capacity of agro-morphologic traits, isoenzymes and DNA markers in avocado (*Persea americana* Mill.).

	Indexes abbreviations									
	n_p	n_{np}	n	L	T_p	T_{up}	C_j	D_j	D_L	P
Assay units	Morphologic traits									
Leaf shape	4	0	4	-	7	2	0.14	0.86	0.81	5.25
Trunk surface	2	0	2	-	2	1	0.88	0.12	0.11	1.12
Color of young twig	4	0	4	-	4	1	0.38	0.62	0.58	2.39
Surface of young twig	2	0	2	-	2	1	0.88	0.12	0.11	1.12
Petal pubescence	2	0	2	-	2	1	0.88	0.12	0.11	1.12
Sepal pubescence	2	0	2	-	2	1	0.88	0.12	0.11	1.12
Fruit shape	7	0	7	-	7	2	0.13	0.87	0.82	5.67
Fruit skin color	4	0	4	-	4	2	0.40	0.69	0.56	2.28
Pedicel shape	2	0	2	-	2	0	0.56	0.44	0.42	1.71
Fruit skin thickness	3	0	3	-	3	1	0.37	0.63	0.60	2.47
Flesh texture	3	0	3	-	3	0	0.37	0.63	0.60	2.47
Seed shape	5	0	5	-	5	2	0.25	0.75	0.71	3.40
Cotyledon surface	3	0	3	-	3	0	0.34	0.66	0.62	2.65
Harvest season	5	0	5	-	5	2	0.24	0.76	0.71	3.48
	Enzymatic systems									
Peroxidases	10	0	10	5	17	17	0.00	1.00	0.94	16.99
Ascorbate oxidases	7	0	7	4	9	5	0.13	0.87	0.82	5.45
Polyphenol oxidases	7	1	8	4	14	11	0.02	0.98	0.92	12.56
	Simple Sequence Repeat (microsatellites)									
AM1	10	0	10	1	13	9	0.03	0.97	0.91	11.56
AM2	12	0	12	1	10	7	0.10	0.90	0.84	6.42
AM3	7	0	7	1	13	9	0.03	0.97	0.91	11.56
AM4	8	0	8	1	10	6	0.08	0.92	0.87	7.41
AM5	10	0	10	1	13	11	0.05	0.95	0.89	9.32
AM6	12	0	12	1	14	12	0.03	0.97	0.91	11.56
AM7	6	0	6	1	11	7	0.06	0.94	0.89	8.76
AM8	6	0	6	1	10	6	0.10	0.90	0.85	6.72
AM9	4	0	4	1	5	2	0.27	0.73	0.69	3.18
AM10	7	0	7	1	12	8	0.04	0.96	0.90	9.97
AM11	8	0	8	1	11	6	0.05	0.95	0.89	0.32
AM12	9	0	9	1	14	12	0.03	0.97	0.91	11.56
AM13	8	0	8	1	10	7	0.08	0.92	0.87	7.41
AM14	10	0	10	1	13	9	0.03	0.97	0.91	11.56
AM15	7	0	7	1	9	5	0.13	0.87	0.82	5.45
	Amplified Fragment Length Polymorphism (AFLP)									
AA1	21	30	51	51	17	17	0.00	1.00	0.94	16.99
AA2	11	29	40	40	17	17	0.00	1.00	0.94	16.99
AA3	22	28	50	50	16	15	0.01	0.99	0.93	15.21
AA4	13	26	39	39	17	17	0.00	1.00	0.94	16.99
AA5	15	29	44	44	16	15	0.01	0.99	0.93	15.21
AA6	10	22	32	32	10	8	0.12	0.88	0.83	5.90
AA7	16	35	51	51	15	14	0.02	0.98	0.92	12.57
AA8	4	19	23	23	6	1	0.15	0.85	0.80	4.90
AA9	10	35	45	45	13	11	0.05	0.95	0.89	9.32
AA10	10	22	32	32	17	17	0.00	1.00	0.94	16.99
	Inverse Sequence-Tagged Repeat (ISTR)									
F_3/B_2B	157	0	157	157	17	17	0.00	1.00	0.94	16.99

n_p Number of polymorphic bands (or stages).
n_{np} Number of monomorphic bands (or stages).
n Total number of bands (or stages).
L Number of loci.
T_p Number of banding (or stage) patterns.
T_{up} Number of unique banding (or stage) patterns.
C_j Average confusion probability.
D_j Average discriminating power.
D_L Average limit of discriminating power.
P Effective number of patterns per assay unit.

Table 2. Characterization of avocado (*Persea americana* Mill.) using highly discriminating descriptors.

No	Varieties	Morph-agronomic traits													
		Ls	Ts	Ct	St	Pp	Sp	Fs	Fc	Ps	Fst	Ft	Ss	Cs	Hs
1	Amado Gómez	OB,OV,O	R	G	G	S	S	HS	YG	CY	M	B	BO	I	M
2	California	OV,R	R	R	P	D	D	O	G	CO	G	P	BR	R	L
3	Casimiro Soledad	OB,OV	R	C	P	D	D	C	G	CY	M	B	BO	R	M
4	Catalina	OV	R	Y	P	D	D	OB	YG	CO	M	P	BO	I	L
5	Centro America No. 3	OV,R	R	C	P	D	D	NO	G	CO	G	W	BC	R	VL
6	CH-1 No. 3	OB,R	R	C	P	D	D	HS	G	CO	M	B	BR	R	L
7	Choquette	OV	R	R	P	D	D	HS	G	CO	G	W	BR	I	L
8	Duke-7	OB,OV,R	S	G	P	D	D	NO	G	CY	T	P	O	I	E
9	Hass	OB,OV,R	R	C	P	D	D	OB	B	CY	G	P	BO	S	VL
10	Itzamná	OB,OV	R	R	P	D	D	P	G	CO	G	B	BC	S	VL
11	Jaruco No. 1	OB	R	C	P	D	D	OB	G	CO	G	P	BO	I	L
12	Jose Antonio	OB,OV	R	C	P	D	D	C	YG	CO	M	P	BO	I	M
13	Los Moros	OV	R	C	P	D	D	NO	P	CY	M	B	BO	I	E,M
14	Lula	OV	R	C	P	D	D	NO	G	CO	G	P	BC	R	L
15	Monroe Estación	OV,R	R	C	P	D	D	HS	YG	CO	M	B	BR	S	L
16	Sicilia No. 6	OV	R	C	P	D	D	P	YG	CO	M	B	C	R	M
17	Suardia Estación	OB,OV,R	R	R	P	D	D	S	G	CO	G	P	BR	I	VL

Ls Leaf shape: oblong (OB); oval (OV); roundish (R); ovate(O)
Ts Trunk surface: smooth (S); rough (R).
Ct Color of young twig: yellow (Y); green (G); coopery (C); reddish (R).
St Surface of young twig: glabrous (G); pubescent (P).
Pp Petal pubescence: sparse (S); dense (D).
Sp Sepal pubescence: sparse (S); dense (D).
Fs Fruit shape: obovate (OB); narrowly obovate (NO); high spheroid (HS); pyriform (P); clavate (C); oblate (O); spheroid (S).
Fc Fruit skin color: yellow-green (YG); green (G);purple (P); black (B).
Ps Pedicel shape: cylindrical (CY); conical (CO).
Fst Fruit skin thickness: thin (T), medium (M); gross (G).
Ft Flesh texture: watery (W); buttery (B); pastose (P).
Ss Seed shape: ovate (O); broadly ovate (BO); cordiform (C); base flattened, apex rounded (BR); base flattened, apex conical (BC).
Cs Cotyledon surface: smooth (S); intermediate (I); rough (R).
Hs Harvest season. early (E); medium (M); late (L); very late (VL).

these assay units. The values were higher for PX (0.51) and PPO (0.43) than for AO (0.29). Also, the total number of effective alleles in PX duplicated the values obtained for the other enzymatic systems assayed. The marker index ranged from 1.16 for AO to 2.55 for PX with intermediate value for PPO (1.48). The distinctive value in PX was a consequence of the effective multiplex ratio and the expected heterozygosity values detected.

The average of the number alleles *per locus* in SSR varied from 4 for AM9 to12 for AM2 and AM6, and the effective number of alleles *per locus* from 3.28 for AM15 to 6.53 for AM1. High expected heterozygosity but low values of marker index, as a consequence of the effective multiplex ratio were found in overall assay units. Both, H_{ep} and *MI* ranged from 0.60 for AM9 to 0.87 for AM1, AM6 and AM11.

In AFLP, the effective number of alleles *per locus* ranged from 1.31 for AA6 to 1.73 for AA8, but high values of the total number of effective alleles and effective multiple ratio were usually found due to the total number of bands (loci) scored with this molecular marker. For that reason, high marker index in each assay unit (from 1.65 for AA8 to 6.20 for AA3) were detected, in spite of the relative low values of expected heterozygosity. The values of H_{ep} ranged from 0.22 for AA6 to 0.41 for AA8. Similar result were obtained in ISTR marker, but the distinctive values of the total number of effective alleles, effective multiple ratio and marker index were a consequence of the total of 157 loci detected with the single primer combination employed.

Comparison of polymorphism levels, discriminating capacity and informativeness of agro-morphologic traits and molecular markers

A comparison of the levels of polymorphism, discriminating capacity and informativeness of the agro-morphologic traits and the four molecular markers was carried out (Table 5).

DNA markers proved to be highly polymorphic. SSR, AFLP and ISTR scored a total of 124, 407 and 157 bands, respectively. All of them were polymorphic in SSR and ISTR while the percentage of polymorphism in AFLP was 32.4 %. However, the average number of polymorphic bands per assay unit was higher in AFLP and ISTR than in SSR. Also isoenzymes showed high polymorphism level, since 24 of the 25 bands scored were polymorphic (96 %). In agro-morphologic traits, all 47 stages were polymorphic, but in contrast, low average number of polymorphic stages per assay unit (3.36) was found. None of these indexes for each marker type did correlate to the total number of bands (or stages) scored.

Table 3. Key made with four reproductive agro-morphologic traits for identification avocado (*Persea americana* Mill.) genotypes.

Variables				
Fruit shape	Fruit skin color[1]	Harvest season	Fruit skin thickness	Genotypes
Oblate				California
Spheroid				Suardía Estación
Clavate	Green			Casimiro Soledad
	Yelow-green			José Antonio
Obovate	Green			Jaruco No. 1
	Yelow-green			Catalina
	Black			Hass
Piriform	Green			Itzamná
	Yelow-green			Sicilia No. 6
Narrowly obovate	Purple			Los Moros
	Green	Precocious (March-May)		Duke 7
		Late (September-November)		Lula
		Very late (December-February)		Centro América No. 3
High spheroid	Yellow-green	Medium (June-August)		Amado Gómez
		Late (September-November)		Monroe Estación
	Green	Late (September-November)	Medium (1.40 ± 0.23)	CH 1 No. 3
			Gross (2.05 ± 0.14)	Choquette

1. In mature fruits.

As a consequence of the low average of confusion probability for the four molecular markers, high values of discriminating power were determined. These ranged from 0.93 for SSR to 1.00 for ISTR, with intermediate values for isoenzymes (0.95) and AFLP (0.96). As expected, the lowest discriminating capacity (0.52) was found for qualitative morphologic traits. D_L values, estimated for all the markers were close to the actual discriminating power of each of them calculated, respectively.

The effective number of patterns per assay unit indicated that more than 17, 11, 10 and 8 accessions for ISTR, AFLP, isoenzymes and SSR, respectively, can be distinguished with a primer combination (or enzymatic system) when the population size tends to infinity. Using morphologic traits only up to two individuals can be discriminated.

An average of 8.27 alleles per locus was detected in SSR. For the same marker, the effective number of alleles per locus was 4.65, while for ISTR and AFLP were lower, with values of 1.39 and 1.42, respectively. In isoenzymes, the value was slightly higher (1.82) with respect to both dominant genetic markers analyzed. This was clearly reflected in lower values of expected heterozygosity for both AFLP and ISTR markers.

Also, morphological diversity index, calculated with 14 agro-morphologic variables was lower than the values of expected heterozygosity for SSR and isoenzymes and very similar with those observed for AFLP and ISTR. The highest assay efficiency index and marker index were found in dominant markers (224.65 and 45.53 for ISTR and 19.55 and 4.04 for AFLP, respectively). The distinctive values for these indexes in ISTR were a consequence of the level of simultaneous detection of several polymorphic markers in the single primer combination assayed, that influenced directly to the total number of effective alleles and the effective multiple ratio. However, the lowest values for assay efficiency index and marker index were observed in SSR (5.05 and 0.81, respectively) and slight higher in isoenzymes (7.35 and 1.58).

DISCUSSION

This paper describes the levels of polymorphism of different marker types and their effectiveness for identification purposes and variability estimate of avocado, a tropical fruit tree of commercial importance in Latin America.

Polymorphism by means of morphological traits has been traditionally used for avocado cultivars classification and identification.[1,3,6,9,10] In this way, a key to identify different variants of 'Hass' cultivar successfully using descriptors of overall trees, leaves and fruits has been offered. However, many observations are necessary for the selection of these variables, representing a time-consuming task.

Some authors defined the *D* parameter for varieties identification.[7] Discriminating power (*D)* can be used to compare different types of markers even if only the allele frequencies are known.[7,8] The extensiveness of this concept for agro-morphological data, using the frequencies of the stages (classes) of each variable, permitted the selection of variables with high discriminating capacity to identify a set of genotypes.

For avocado characterization, using minimal highly discriminating descriptors have been suggested.[17] Nevertheless, this study demonstrated that a distinctive value of discriminating capacity was detected in each of the 14 variables analyzed. Probably these differences not only

Table 4. Informativeness levels detected with agro-morphologic traits, isoenzymes and DNA markers in avocado (*Persea americana* Mill.).

	Indexes abbreviations								
	n_{av}	D_M/H_{ep}	D_S	β	H_e	n_e	N_e	E	MI
Assay units	Morphologic traits								
Leaf shape	-	0.33	0.20	-	-	-	-	-	-
Trunk surface	-	0.11	0.92	-	-	-	-	-	-
Color of young twig	-	0.30	0.43	-	-	-	-	-	-
Surface of young twig	-	0.11	0.92	-	-	-	-	-	-
Petal pubescence	-	0.11	0.92	-	-	-	-	-	-
Sepal pubescence	-	0.11	0.92	-	-	-	-	-	-
Fruit shape	-	0.24	0.19	-	-	-	-	-	-
Fruit skin color	-	0.29	0.48	-	-	-	-	-	-
Pedicel shape	-	0.43	0.61	-	-	-	-	-	-
Fruit skin thickness	-	0.41	0.45	-	-	-	-	-	-
Flesh texture	-	0.41	0.45	-	-	-	-	-	-
Seed shape	-	0.29	0.31	-	-	-	-	-	-
Cotyledon surface	-	0.43	0.38	-	-	-	-	-	-
Harvest season	-	0.38	0.31	-	-	-	-	-	-
	Enzymatic systems								
Peroxidases	2.00	0.51	-	1.00	0.51	2.09	10.43	5.00	2.55
Ascorbate oxidases	1.80	0.29	-	1.00	0.29	1.50	5.99	4.00	1.16
Polyphenol oxidases	2.00	0.43	-	0.86	0.37	1.86	5.63	3.44	1.48
	Simple Sequence Repeat (microsatellites)								
AM1	10	0.87	-	1.00	0.87	6.53	6.53	1.00	0.87
AM2	12	0.86	-	1.00	0.86	5.95	5.96	1.00	0.86
AM3	7	0.82	-	1.00	0.82	4.93	4.93	1.00	0.82
AM4	8	0.76	-	1.00	0.76	3.88	3.88	1.00	0.76
AM5	10	0.85	-	1.00	0.85	5.63	5.63	1.00	0.85
AM6	12	0.87	-	1.00	0.87	6.52	6.52	1.00	0.87
AM7	6	0.82	-	1.00	0.82	4.83	4.83	1.00	0.82
AM8	6	0.70	-	1.00	0.70	4.12	4.12	1.00	0.70
AM9	4	0.60	-	1.00	0.60	2.39	2.39	1.00	0.60
AM10	7	0.85	-	1.00	0.85	5.57	5.57	1.00	0.85
AM11	8	0.87	-	1.00	0.87	6.43	6.43	1.00	0.87
AM12	9	0.86	-	1.00	0.86	6.02	6.02	1.00	0.86
AM13	8	0.76	-	1.00	0.76	3.81	3.81	1.00	0.76
AM14	10	0.86	-	1.00	0.86	5.90	5.90	1.00	0.86
AM15	7	0.72	-	1.00	0.72	3.28	3.28	1.00	0.72
	Amplified Fragment Length Polymorphism (AFLP)								
AA1	2.00	0.29	-	0.41	0.12	1.44	30.35	21.00	6.07
AA2	2.00	0.38	-	0.28	0.10	1.66	18.27	11.00	4.10
AA3	2.00	0.28	-	0.44	0.12	1.43	31.49	22.00	6.20
AA4	2.00	0.34	-	0.33	0.11	1.53	19.95	13.00	4.43
AA5	2.00	0.31	-	0.34	0.11	1.47	22.08	15.00	4.66
AA6	2.00	0.22	-	0.31	0.08	1.31	13.12	10.00	2.17
AA7	2.00	0.27	-	0.31	0.09	1.40	22.45	16.00	4.35
AA8	2.00	0.41	-	0.17	0.07	1.73	6.90	4.00	1.65
AA9	2.00	0.28	-	0.22	0.06	1.43	14.28	10.00	2.81
AA10	2.00	0.38	-	0.31	0.12	1.66	16.58	10.00	3.82
	Inverse Sequence-Tagged Repeat (ISTR)								
F_3/B_2B	2.00	0.29	-	1.00	0.29	1.39	224.65	157.00	45.13

n_{av} Average number of alleles per locus.
H_e Expected heterozygosity.
D_M Morphologic diversity index.
n_e Effective number alleles per locus.
H_{ep} Expected heterozygosity of the polymorphic loci.
N_e Total number of effective alleles.
D_S Simpson diversity index.
β Fraction of the polymorphic loci.
E Effective multiple ratio.
MI Marker index.

Table 5. Comparison of polymorphism levels, discriminating capacity and informativeness of agro-morphologic traits, isoenzymes, SSR, AFLP and ISTR markers in avocado (*Persea americana* Mill.).

Indexes with their abbreviations		Morphagronomic traits	Isoenzymes	SSR	AFLP	ISTR
Number of assay unit	U	14	3	15	10	1
Number of polymorphic bands (or stages)	n_p	48	24	124	132	157
Number of monomorphic bands (or stages)	n_{np}	0	1	0	275	0
Total number of bands (or stages)	n	48	25	124	407	157
Average number of polymorphic bands (or stages) per assay unit	n_p/U	3.36	8	8.27	13.20	157
Number of loci	L	-	13	15	407	157
Average number of loci per assay unit	n_u	-	4.03	1	40.7	157
Number of banding (or stage) patterns	T_p	51	40	168	144	17
Number of unique banding (or stage) patterns	T_{up}	15	33	116	132	17
Average number of banding (or stage) patterns per assay unit	I	3.64	13.30	11.20	14.40	17.00
Average number of unique banding (or stage) patterns per assay unit	I_u	1.07	11.00	7.73	13.20	17.00
Average confusion probability	C_j	0.47	0.05	0.07	0.04	0.00
Average discriminating power	D_j	0.52	0.95	0.93	0.96	1.00
Average limit of discriminating power	D_L	0.49	0.89	0.87	0.91	0.94
Effective number of patterns per assay unit	P	1.96	9.32	7.75	10.78	17.00
Average number of alleles per locus	n_{av}	-	1.92	8.27	2.00	2.00
Expected heterozygosity of the polymorphic loci. Morphological diversity index/Simpson diversity index	H_{ep}. D_M/D_S	0.29/0.52	0.41	0.81	0.31	0.29
Fraction of the polymorphic loci/ Fraction of the polymorphic trait	β	-	0.96	1.00	0.32	1.00
Expected heterozygosity	H_e	-	0.39	0.81	0.10	0.29
Effective number alleles per locus	n_e	-	1.82	4.65	1.42	1.39
Total number of effective alleles	N_e	-	22.05	75.78	195.50	224.65
Assay efficiency index	A_i	-	7.35	5.05	19.55	224.65
Effective multiple ratio	E	-	3.87	1.00	13.02	157.00
Marker index	MI	-	1.58	0.81	4.04	45.53

depend on the variables selected, but also by the set of individuals sampled. Avocado has pronounced phenotypic variability and plasticity, and breeding programs has focused on creating hybrids from the three ecological races in tropical and subtropical areas,[34] thereby, the selection of morphological traits for identification purposes may be determined on each group of genotypes. In this study, only four of them were necessary for distinguishing all the individuals analyzed, but the low value of the effective number of pattern per assay unit obtained with morphologic traits and the possible influence of environmental conditions on phenotypic expression, suggest the use of additional markers for discriminating purpose when numerous cultivars need to be accurately characterized and identified.

Isoenzymes are variant molecular forms of enzymes that are readily separated and detected by standard starch or polyacrylamide gel electrophoresis. Proteins and isoenzymes have been used in many crops, including trees, for cultivar identification, but for many species insufficient polymorphism is a problem.[35] However, isoenzyme profiles used in this study, demonstrated high capacity for discriminating avocado genotypes, since only peroxidases system could differentiate of all individuals analyzed. These results agreed with the fact that peroxidases is one of the most polymorphic isoenzymes in plants,[36] and confirmed those obtained by authors in relation to their utility for avocado identification.[11-13]

Additionally, some proteins can exhibit spatial and temporal variation as well as variation due to environments influence.[37,38] In this sense, different peroxidases profiles were observed with samples taken of distinct fruit tissues of avocado cultivar 'Banes',[39] and from leaves harvested in juvenile and adult stages in the same cultivar.[40] For this reason, peroxidases system was proposed as marker for distinguishing the juvenile and mature phases rather than clues to the mechanism underlying phase change in avocado and other species.[38] Then, their use for varietal identification is limited to local genotypes since isoenzyme profiles are not transferable.

At present, PCR-based marker systems have been preferred as molecular markers for varietals identification, but AFLP and microsallites dominate the scene of variety profiling and hence identification since the reproducibility of RADP across different laboratories is discussed,[19] and a low discriminating capacity has been determined in comparison with SSR in grape,[7] and with AFLP and SSR in olive.[8] Also, retrotransposon sequences (ISTR) have detected a remarkable degree of polymorphism in genetically highly related genotypes of barley (*Hordeum vulgare* L.) and tomato (*Lycopersicon esculentum* Mill.),[21] mango (*Mangifera indica* L.),[41] ornamental plants,[19] and avocado.[13,16] Moreover was demonstrated that just two ISTR primer combinations must be sufficient in the characterization a particular crop, one yielding a species-specific fingerprint and the other discriminating between the different varieties or parent for hybrid production.[21]

Some authors using minisatellites probes,[14] and RAPD markers,[15] identified a set of avocado genotypes. Also, the reproducibility of the three DNA markers used in this study,[21,42,43] and the very similar and high D_L values estimated, make them a powerful tool for avocado discriminating. These results reinforce the useful of AFLP,[8,41,44] ISTR,[13,16,41] and microsatellites,[7,8,45,46] for genotype identification in different fruit species. However, the high level of polymorphic loci detected in avocado by dominant markers highlights the discriminating capacity of these markers. With a single AFLP or ISTR primer combination all the individuals were identified with a specific banding patterns, while in microsatellites two primer combinations were necessary for this purpose.

Results reported here confirmed the high level of heterozygosity previously suggested in avocado.[14,34,47-49] The higher values of expected heterozygosity were observed in codominant markers and reflect the level of informativeness of these markers, but the value detected with microsatellites doubled or more the obtained with isoenzymes and dominant markers. These values followed the pattern SSR > Isoenzymes > AFLP > ISTR, as a consequence of the effective number of alleles *per locus* detected in each molecular marker. Also, SSR reached higher heterozygosity value than AFLP did in olive,[8] but in contrast, in rice (*Oriza sativa* L.) this value was lower for isoenzymes in comparison with AFLP.[36] This difference is probably due to the form of polymorphic bands were scored for isoenzymes: 1/0 in rice, and homozygotic and heterozygotic genotypes in this study and/or for distinct nature of both plant species.

The concept of diversity of the species, according to ecologic or biologic analysis, has been adopted recently for morphologic data. For this, local accessions or varieties from a particular location are classified by defined classes based on the phenotypic expression.[50] Some authors have estimated the diversity using the Shannon, Simpson and Margalef indexes in different maize (*Zea mays* L.) samples successfully.[51,52]

The fact that morphological diversity and Simpson diversity indexes were correlated, suggest that the index used here for estimating the diversity with morphological data can be used for variability estimating and comparable with the expected heterozygosity (also called diversity index) determined with isoenzymes and DNA markers, since they have the same mathematical base. As known, morphological traits are influenced by the environment conditions, but if the analysis contains variables with high repeatability, in other words, heritability ($\gamma > 1$), this problem is minimized.[50] In this sense, the value of morphological diversity index was lower than SSR and isoenzymes and much closed to those obtained with ISTR and AFLP.

The utility of a given genetic marker was defined by the balance between the level of polymorphism and its capacity to identify multiple polymorphisms.[31] The highest values of marker index (*MI*) of dominant markers (ISTR and AFLP) depended more of the number of polymorphic bands obtained in each profile than on the allelic heterozygosity found among accessions. However, lower values of this index were observed for SSR and isoenzymes in spite of the high level of heterozigosity determined with these last markers. These results agreed with those obtained in olive and in rice.[8,36] In contrast, in soybean was detected that SSR scored higher value of *MI* that did dominant markers (RAPD),[31] and reinforce the need for specific studies of marker comparisons for each plant species previously reported.[8]

Similarly to the *MI* values, the information measured as the assay efficiency index (A_i), which correlates with the number of effective alleles per assay unit was greater for ISTR and AFLP than for codominant markers. Also, higher values of A_i detected by AFLP, compared with SSR, were reported in olive and in maize inbreed lines.[8,33]

The lower *MI* values scored with microsatellites in comparison with isoenzymes and dominant markers used in this study contrast with the previous information. SSR are versatile genetic markers that combine

the useful properties of high variability, codominant inheritance and good reproducibility,[34,53] and their codominance makes them suitable for tracing paternity and tracking pollen movement.[54] This fact, and that *MI* and A_i had the same pattern that *D*, *I*, I_u and *P* (ISTR > AFLP > isoenzyme > SSR) suggest that marker index and assay efficiency index are probably indicators of discriminating capacity in avocado.

CONCLUSIONS

Several conclusions are derived from this study: *i) D* parameter can be adopted successfully for discriminating capacity with morphological traits, *ii)* morphological traits are less effective for varietal identification in comparison with all molecular markers analyzed, *iii)* all DNA markers were useful for discriminating avocado cultivars and for varietal certification *iv)*, isoenzymes is a low cost technique useful for discriminating avocado accessions in local germplasm, *v)* a high degree of heterozygosity was detected in avocado using microsatellite markers, *vi)* the morphologic diversity index is a good estimator of diversity among avocado genotypes when variables of high heritability are used and comparable with the expected heterozygosity scored with isoenzymes and DNA markers and *vii)* marker index (*MI*) and assay efficiency index (A_i) values are probably indicators of the discriminating capacity in avocado.

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