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COMPARATIVE STUDY OF POLYMORPHISM LEVEL, DISCRIMINATION CAPACITY AND INFORMATIVENESS OF AFLP, ISTR, SSR AND ISOENZYMES MARKERS AND AGRO-MORPHOLOGICAL TRAITS IN AVOCADO

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AFLP, ISTR, SSR, isoenzyme markers and agro-morphological traits were compared in terms of their polymorphism level, discriminating capability and informativeness among 17 genotypes assembled in the Cuban avocado germoplasm, maintained at Alquízar under the auspices of the IIFT (Playa, Havana City, Cuba). *D* parameter adopted for agro-morphological traits was useful for genotype identification. Only four variables: fruit shape, fruit skin color, harvest season and fruit skin thickness were necessary for distinguishing all the individuals analyzed SSR, AFLP and ISTR were powerful techniques for avocado discriminating and varietal certification, but the high level of polymorphic loci detected by dominant markers highlights the discriminating capacity of these molecular 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 those 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 as *D*, *I*, I_u and *P* for all molecular markers. Then, both indexes probably reflect on the discriminating capability of avocado.

ESTUDIO COMPARATIVO DE LOS NIVELES DE POLIMORFISMO, CAPACIDAD DE DISCRIMINACIÓN E INFORMATIVIDAD DE CARACTERES MORFOAGRONÓMICOS Y DE LOS MARCADORES AFLP, ISTR, SSR E ISOENZIMAS EN AGUACATERO

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Se compararon los niveles de polimorfismo, capacidad de discriminación e informatividad de caracteres morfoagronómicos y marcadores AFLP, ISTR, SSR e isoenzimas, empleando 17 genotipos de la colección de germoplasma del IIFT. El poder de discriminación *D* utilizado para caracteres morfoagronómicos fue útil para la identificación de genotipos. Cuatro variables fueron suficientes 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 combinación de cebadores AFLP o ISTR se identificaron todos los individuos. A su vez, las isoenzimas resultaron técnicas de bajo costo útiles para este propósito en el germoplasma evaluado. Los niveles más altos de heterocigosidad esperada se detectaron con marcadores codominantes, pero los microsatélites superaron 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 cuando se utilizaron variables de alta 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. El índice de eficiencia del ensayo (A_i) y el índice 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 reflejan sobre la capacidad de discriminación en el aguacatero.

1. Introduction

The preservation of the genetic resources of avocado (*Persea americana* Mill.) began at the beginning if the last century in Cuba, but at the foundation of the Cuban Fruit Tropical and Subtropical genebank in 1965 (nowadays Institute on Tropical Fruit Research), the biggest collection of this species was established in the country.

In avocado, morphological data have traditionally used for germoplasm characterization and variety identification, but also have been supplemented by isoenzymes and DNA markers (Rodríguez *et al*., 2003). Avocado descriptors published by the International Plant Genetic Resource Institute (IPGRI, 1995) suggested the use of morphological traits and molecular markers to establish fingerprint of individual accessions. 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 (Donini *et al*,. 2000).

The objective of this paper is to compare the polymorphism levels, discriminating capacity and informativeness of agro-morphological traits, isoenzymes and DNA markers including Amplified Fragment Length Polymorphism (AFLP; Vos *et al*., 1995), Inverse Sequence-Tagged Repeat (ISTR; Rohde, 1996) and Simple Sequence Repeat (SSR; Litt and Luty, 1989; Tautz, 1989; Weber and May, 1989), for varietal identification and variability estimating of avocado.

2. Materials and methods

2.1. Plant materials

A total of 17 avocado accessions were used (Table 1). These varieties were evaluated for 14 qualitative traits recommended by IPGRI (1995) as highly discriminating descriptors: 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.

2.2. Isoenzyme analysis

Fully expanded, young leaves were harvested, wiped clean and stored at 2-3 ºC until using. A system of vertical electrophoresis and discontinuous buffers (González and González, 1981) were employed using polyacrylamide gel (8,5%) and 0,04 M tris-glycine buffer at pH = 8,3. 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 González *et al*. (2002).

2.3. Isolation and purification of genomic DNA

DNA isolation and purification were made according to the suggestions of Ramírez *et al*. (2004).

2.4. PCR amplification of genomic DNA

The following DNA marker techniques were used under standard reaction conditions with ^{33}P-labeled PCR primers: Amplified Fragment Length Polymorphism (AFLP) (Vos *et al*. 1995), Inverse Sequence-Tagged Repeat (ISTR) (Rohde, 1996) and Simple Sequence Repeat (SSR) (Litt and Luty, 1989; Tautz, 1989; Weber and May, 1989).

2.4.1. Amplified Fragment Length Polymorphism (AFLP).

For the selective amplification a total of ten primer combinations (E32XM32: E-AAC x M-AAC, E32XM36: E-AAC x M-ACC, E32XM38: E-AAC x M-ACT, E32XM39: E-AAC x M-AGA, E32XM44: E-AAC x M-ATC, E32XM45: E-AAC x M-ATG, E32XM46: E-AAC x M-ATT, E32XM47: E-AAC x M-CAA, E33XM36E-AAG x M-ACC, E33XM42: E-AAG x M-AGT), were used. The amplification steps were the following: 94^{o}C, 30 s; 65^{o}C (-0.7^{o}C/cycle), 30 s, and 72^{o}C, 60 s during 12 cycles, until reaching the optimal annealing temperature of 56^{o}C. At this temperature, 24 more cycles were performed to complete the amplification.

2.4.2. Inverse Sequence-Tagged Repeat (ISTR)

Primers for forward F_3 and backward reaction B_2B previously designed (Rohde *et al*., 1996) were used in PCR amplification. PCR reactions were performed according to standard protocols (Rohde *et al*., 1995) and the amplification program consisted of the following steps: step 1, 95ºC/3min; step 2, 95ºC/ 30sec; step 3, 45ºC/30sec; step 4, 72ºC/2min; step 5, 72ºC/10min, with 40 cycles of steps 2 to 4.

2.4.3. Simple Sequence Repeat (SSR)

Fifteen SSR primer pairs: AM1, AM2, AM3, AM5, AM6, AM8, AM9, AM10, AM11, AM13, AM14, AM15, AM16, AM17 and AM18 (Ramírez *et al*. (2005) were used. The reaction was processed at 94^{o}C for 30 s, followed by 32 cycles consisting of 94^{o}C for 15 s, 45^{o}C to 50^{o}C for 25 s (depending on the primers) and 68^{o}C for 25 s, and a final extension step of 68^{o}C for 2 min.

2.5. Gel electrophoresis analysis

After the reactions, the amplified AFLP, ISTR or 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 1 x TBE buffer, pH 8.9 at 40 W. After the run, 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 days.

2.6. 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 independently of 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.

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) different indicators were estimated according to Belaj *et al.* (2003) and Ramírez *et al*. (2005).

Also, for morphological traits, the morphological 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 (Simpson, 1949) was determined using the formula: $D_s = \Sigma p_i^2$, 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. A lineal correlation was determined between morphological (D_m) and Simpson (D_s) diversity indexes.

3. Results

3.1. Polymorphism levels and discriminating capacity of agro-morphological traits and molecular markers

The analysis with agro-morphological traits 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.

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 variables analyzed. This phenotypic polymorphism

generated from 2 to 7 distinctive patterns with a maximum of only two unique patterns.

In accordance with these results, four reproductive variables: fruit shape, fruit skin color, fruit skin thickness and harvest season were necessary to take in account for discriminating of all individuals (Table 1).

A comparison of the levels of polymorphism and discriminating capacity of the morphological traits and the four molecular markers is summarized in Table 2. All molecular markers proved to be highly polymorphic and were effective for discriminating the avocado accessions studied, although best results were observed with ISTR and AFLP techniques. For instance, the highest values of the number of polymorphic bands and the average of number of polymorphic bands per assay unit were detected with these molecular markers. Similar results were obtained with the average of the number of total and unique patterns per assay unit. In contrast, the values of these indexes generated by the 47 polymorphic stages of the morphological traits were the lowest, respectively. Intermediate values were found with isoenzymes and SSR. None of these indexes for each marker type did correlate to the total number of bands (or stages) scored.

As a consequence of the low average of confusion probability for the four genetic markers, high values of discriminating power were determined, especially for ISTR and AFLP. As expected, the lowest discriminating capacity was found for qualitative morphological 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, 12, 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 morphological traits only up to 2 individuals can be discriminated.

3.2. Comparison of informativeness obtained with morphological traits, isoenzymes and DNA markers

A total of eight indexes were calculated to assess the informativesness levels of the four molecular markers used. Moreover, to compare these results with phenotypic characters, diversity indexes were calculated on the basis of the total number of stages, the presence (1) or absence (0) of the stages of each variable and by the frequency of them derived, independently of the number of loci and the inheritance type involved in the morphological traits.
The morphological diversity index ranged from 0.43 for pedicel shape and cotyledon surface to only 0.11 for trunk surface, surface of young twig, petal pubescence and sepal pubescence. 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 lineal correlation ($r = -0.70^{**}$) with the Simpson diversity index was found.

Table 2 comprises a comparison of the informativeness of the morphological traits and the four molecular markers. An average of 8.27 alleles per locus was detected in SSR. For the same markers, 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 the expected heterozygosity values for all molecular markers. The morphological diversity index, calculated with 14 morphoagronomic variables was 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 45.13 in ISTR and 19.55 and 4.04 in 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).

4. Discussion

This paper describes the levels of polymorphism of different marker types and their effectiveness for identification purposes and variability estimating of avocado.

The high level of polymorphism observed for morphoagronomic traits and for all four molecular markers is consistent with results from previous studies carried out on avocado accessions by means of morphological data (Rodríguez *et* al., 2003), isoenzymes (Lima *et al*., 1982; Sánchez *et al*., 1993; González *et al*., 2002) and different DNA markers (Ramírez *et al*., 2002; Chang *et al*.,2003; Rodríguez *et al*., 2003; Ramírez *et al*., 2005), thereby confirming the great diversity within the cultivated avocado germplasm (Ashworth and Clegg, 2003).
The higher values of expected heterocigosity were observed in codominant markers and reflect the level of informativeness of these genetic 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. Similar results were obtained in olive by Belaj *et al*. (2003). However, the value of morphological diversity index was very close to those obtained with ISTR and AFLP.

The concept of diversity of the species, according to ecologic or biologic biodiversity analysis, has been adopted recently for morphological data. For this purpose, the local accessions or varieties from a particular region are classified by defined classes based on of the phenotypic expression of the morphological traits (Chávez, 2003). Louette *et al*. (1997) and Aguirre *et al*. (2000) estimated the diversity successfully using the Shannon, Simpson and Margalef indexes in different maize samples.

The fact that morphological diversity and Simpson diversity indexes were correlated, suggest that the former also can be used for variability estimating in avocado germplasm. 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 (Chávez, 2003).

Tessier *et al*. (1999) defined the *D* parameter for varieties identification. *D* can be used to compare different type of markers even if only the allele frequencies are known. The extensiveness of this concept for morphological data, using the frequencies of the stages (classes) of each variable, permitted the selection of a set of morphological traits with high discriminating capacity to identify the genotypes of the germoplam collection, or a selection of them involved in such experiment or breeding program.

For avocado characterization, using minimal highly discriminating descriptors in the germplasm have been suggested (IPGRI, 1995). 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 depend on the variables selected, but also by the set of individuals sampled. The *D* parameter adopted here for morphological traits can be useful for this purpose. In this sense, only four of them were necessary for distinguishing all the accessions studied. In spite of these results, the low value of the effective number of pattern per assay unit obtained with morphological traits and the possible influence of environmental conditions on phenotypic expression, suggest the use of additional markers for discriminating purpose and for the management of germplasm banks when numerous cultivars need to be accurately characterized and identified.

Isozymes 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 identification purposes, but for many species insufficient polymorphism is a problem (Dettori and Palombi, 2000). Isozyme profiles developed in this study nevertheless, demonstrated high capacity for discriminating avocado accessions, since only peroxidase system could differentiate of all individuals analyzed. These results confirm those obtained by González *et al*. (2002) and the utility of isoenzyme analysis for avocado identification.

Additionally, some proteins can exhibit spatial and temporal variation as well as variation due to environments (Beckman and Soller, 1983; Li-Chun *et al*., 2003). Different peroxidase profiles were observed with samples taken of distinct fruit tissues of avocado 'Banes' (Lima *et al*., 1982), and from leaves harvested in the same accession in juvenile and adult stages (Sánchez-Romero *et al*., 1993). For this reason, their use for identification purposes is limited to a local germoplasm since isoenzyme profiles are not transferable.

With the advent of PCR-based marker system, RADP, AFLP and microsatellites (SSR) techniques have been preferred as molecular markers for varietal

identification in fruit trees (Tessier *et al*., 1999; Dettori and Palombi, 2000; Aranzana *et al*., 2001; Balaj *et al*., 2003), but at present, AFLP and microsallites dominate the scene of variety profiling and hence identification since the reproducibility of RADP across different laboratories is discussed (Donini *et al*., 2000). Also, retrotransposon sequences (inverse sequence-tagged repeat, ISTR) have detected a remarkable degree of polymorphism in genetically highly related genotypes of barley (*Hordeum vulgare*), tomato (*Lycopersicon esculentum*) (Rohde, 1996) and ornamental plants (Donini *et al*., 2000).

These three DNA markers used in this study have discriminated all genotypes very effectively, and their reproducibility have been demonstrated (Janssen *et al*.,1996; Rohde, 1996; Jones *et al*., 1997), but the high level of polymorphic loci detected in avocado accessions by dominant markers highlights their discriminating capacity. With a single AFLP or ISTR primer combination all the individuals were identified with a specific banding patterns, while best results obtained with AM8 and AM15 SSR primers involved confusions each one even including accessions from different ecological race. This result is in accordance with those obtained by Ramírez *et al*. (2002) using Inverse Sequence Tagged Repeat (ISTR) analysis in this species.

Powell *et al*. (1996) defined the utility of a given genetic marker, by the balance between the level of polymorphism and its capacity to identify multiple polymorphisms. 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, the lowest value of this index was observed in SSR in spite of the high level of heterozigosity determined with this marker. These results agree with those obtained by Belaj *et al*. (2003) in olive.

The very low *MI* scored with microsatellites in comparison with isoenzymes and dominant markers used in this study contrast with the information given by this former. SSR are versatile genetic markers that combine the useful properties of high variability, codominant inheritance and good reproducibility and their codominance makes them suitable for tracing paternity and tracking pollen movement (Ashworth and Clegg, 2003). This fact, and that *MI* and A_i had the same pattern that *D* (ISTR > AFLP > isoenzyme > SSR), suggest that marker index and assay efficiency index reflect more on the discriminating capacity than on the informativeness of these genetic markers.

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Table 1. Key made with four reproductive morphological traits for identification avocado (*Persea americana* Mill.) accessions .

Variables				Accessions
Fruit shape	**Fruit skin color[1]**	**Harvest season**	**Fruit skin thickness**	
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 (Mar-May)		Duke 7
		Late (Sep-Nov)		Lula La Pepilla
		Very late (Dec-Feb)		Centro América No. 3
High spheroid	Yellow-green	Medium (Jun-Aug)		Amado Gómez No. 1
		Late (Sep-Nov)		Monroe Estación
	Green	Late (Sep-Nov)	Medium Gross	CH 1 No. 3 Choquette

[1] In mature fruits

Table 2. Comparison of polymorphism, discriminating capacity and informativeness of molecular markers and morphological traits in avocado (*Persea americana* Mill.) accessions.

Indexes with their abbreviations		**Isoenzyme**	**SSR**	**AFLP**	**ISTR**	**Morphological traits**
Number of assay unit	U	3	15	10	1	14
Number of polymorphic bands (or stages)	n_p	24	124	132	157	47
Number of monomorphic bands (or stages)	n_{np}	1	0	275	0	0
Total number of bands (or stages)	n	25	124	407	157	47
Average number of polymorphic bands (or stages) per assay unit	n_p/U	8	8,27	13,20	157	3,36
Number of loci	L	13	15	407	157	Unknown
Average number of loci per assay unit	n_u	4,03	1	40.7	157	Unknown
Number of banding (or stage) patterns	T_p	40	168	144	17	51
Number of unique banding (or stage) patterns	T_{up}	33	116	132	17	15
Average number of banding (or stage) patterns per assay unit	I	13,30	11,20	14,40	17,00	3,64
Average number of unique banding (or stage) patterns per assay unit	I_u	11,00	7,73	13,20	17,00	1,07
Average confusion probability	C_j	0,05	0,07	0,04	0,00	0,47
Average discriminating power	D_j	0,95	0,93	0,96	1,00	0,52
Average limit of discriminating power	D_L	0,89	0,87	0,91	0,94	0,49
Effective number of patterns per assay unit	P	9,32	7,75	10,78	17,00	1,96
Average number of alleles per locus	n_{av}	1,93	8,27	2,00	2,00	-
Expected heterozygosity of the polymorphic loci[1]/ Morphological diversity index[2]/Simpson diversity index[3]	H_{ep}. D_M/ D_S	0,41[1]	0,81[1]	0,31[1]	0,29[1]	0,29[2]/0,52[3]
Fraction of the polymorphic loci	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	A_i	7,35	5,05	19,55	224,65	-
Assay efficiency index	E	4,15	1,00	13,20	157,00	-
Effective multiple ratio	MI	1,73	0,81	4,04	45,13	-