Genetic resources, breeding and avocado crop technologies (*Persea americana* Mill.) in Cuba

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Abstract

This paper puts forward the results of 40 years of research in avocado at the Outreach Station of Alquízar that belongs to the Tropical Fruit Crops Research Institute. The agromorphological characterization of the avocado collection of this institution is presented as well as a detailed illustrated catalogue of the most important 19 accessions through 101 descriptors. Information on the race, dicogamic group, origin and distinctive features of the cultivars and a practical handbook to manage this crop under our conditions is also provided: multiplication, nursery care, features that rootstocks should meet, climate, edaphic and phytosanitary requirements for the crop: season and spacing, fertilization, irrigation, weed control, pruning, harvest and postharvest principles. Comparison is done on the discrimination and informative power shown by morphoagronomic characters, izoenzymatic markers and DNA: AFLP, ISTR and SSR. The adaptation of parameter D (discrimination power) to morphoagronomic variables allowed the preparation of keys with phenotipic characters to distinguish the studied genotypes. The co-dominant molecular markers were the most effective ones to estimate diversity. The concept of biological or ecological diversity was done to evaluate the diversity of specie at a certain site through established classes according to the variations of phenotipic characters. The efficiency in the use of *in vitro* culture techniques and mutation induction was determined for preservation and breeding purposes.

Key words: Genetic resources, breeding, technologies

Recursos genéticos, mejoramiento y tecnologías del cultivo del aguacatero (*Persea americana* Mill.) en Cuba

Resumen

Se ofrecen los resultados de las investigaciones realizadas durante 40 años en la Unidad Científica Tecnológica de Base de Alquízar del Instituto perteneciente al Instituto de Investigaciones en Fruticultura Tropical en el cultivo del aguacate. Se brinda la caracterización agromorfológica de la colección de aguacateros de esta Institución y de forma exhaustiva, como catálogo ilustrado, de 19 accesiones económicamente más importantes mediante 101 descriptores. Se informa sobre la raza, grupo dicogámico, origen y características distintivas de los cultivares. Se brinda un manual practico, para el manejo del cultivo de este frutal en nuestras condiciones: multiplicación, atención a los viveros, características que deben reunir los patrones, requerimientos climáticos, edáficos y fitotécnicos para el cultivo: época y distancia de plantación, fertilización, riego, control de malezas, podas, atención fitosanitaria, así como los principios de cosecha y poscosecha. Se compara el poder de discriminación y de informatividad que presentan los caracteres morfoagronómicos, los marcadores izoenzimáticos y ADN: AFLP, ISTR y SSR. La adaptación del parámetro D (poder de discriminación) a variables morfoagronómicas, permitió la elaboración de claves con caracteres fenotípicos para la distinción de los genotipos estudiados. Los marcadores moleculares codominantes resultaron los más efectivos para estimar la diversidad. Se realizó la adaptación del concepto de diversidad biológica o ecológica para evaluar la diversidad de una especie en un sitio determinado, a través de clases establecidas en correspondencia con las variaciones de los caracteres fenotípicos. Se determinó la eficiencia del uso de técnicas de cultivos *in Vitro* y la inducción de mutaciones con fines de conservación y de mejoramiento genético.

Palabras claves: Recursos genéticos, mejoramiento, tecnologías

Introduction

Avocado (*Persea americana* Mill.) was introduced in Cuba more than 400 years ago. Nevertheless, with de foundation of the Experimental Station of Santiago de las Vegas in 1904, it began to claimed the attention as economical crop to farmers from the Havana province, and in 1958, Cuba became as the first avocado exporter country of the world. Later, in 1965, the Germplams Bank of Tropical and Subtropical Fruits was created and the greatest collection of this specie was established (Rodríguez *et al*., 1999).

Taking in account that avocado constitutes an important fruit in national and international scales, present paper assemble main results related to genetic resources, breeding and technologies applied in Cuba. For this, aspects of plant prospecting, characterization of material collected, uses of the genetic resources, technologic management, the efficiency of morphologic and molecular markers for discriminating and diversity estimation, as well as the utility of tissue culture and mutation induction for conservation and genetic breeding have been considered.

Prospections, genetic resources characterization and cultivar selection:

At the foundation in 1965 of the Tropical and Subtropical Genebank of Fruit in Cuba, the new avocado collection was constituted by materials of two other collections, belonging to the Experimental Station of Santiago de las Vegas and the Soledad Botanical Garden in Cienfuegos; as well as by plants collected by prospections in all the country and a little quantity of foreign materials. This collection, with 406 accessions, was constituted mainly by plants of the West Indies race and in a least proportion by Guatemalan race and hybrids from Guatemalan x West Indies races (Rodríguez *et al*., 1999).

Trees, leaves, flowers and fruits of the avocado collection were highly variables (Rodríguez *et al*., 2011). There were both floral types: 45% corresponding to the type A and 55% to the type B. Specifically, fruit characterization of 322 cultivars using descriptors designed by IPGRI and other authors is offered (IPGRI, 1995; Rodríguez *et al*., 2007). Quantitative traits in fruits generally showed great variability. Characters with higher coefficient of variation were fruit weight, seed weight, length of seed cavity, pulp weight, skin weight and fruit skin thickness (Table 1). The oil content in the pulp, evaluated in 21% of total fruits, showed also variability. Mean value was 8.82%, with maximum value for Hass cultivar (17%) and minimum for Jardín cultivar (1.47%) (Rodríguez *et al*., 2011).

Table1. Characterization of the avocado (*Persea americana* Mill.) fruits according to quantitative characters.

	Fruit weight (g)	**Seed weight (g)**	**Seed weight / Fruit weight ratio**	**Fruit length (cm)**	**Fruit diameter (cm)**	**Fruit length / Fruit diameter ratio**	**Length of seed cavity (cm)**
Mean	703.89	114.54	0.17	15.71	9.78	1.63	8.28
Maximum	1840.00	235.00	0.33	36.90	13.58	4.29	24.70
Minimum	76.00	22.60	0.05	7.60	4.50	0.90	4.31
C. V. (%)	29.39	31.95	31.83	23.01	10.80	25.34	30.52
S. D.	206.85	36.60	0.05	3.62	1.06	0.41	2.53

	Diameter of seed cavity (cm)	**length / Diameter of seed cavity ratio**	**Pulp weight (g)**	**Pulp weight /Fruit ratio**	**Skin weight (g)**	**Skin weight /Fruit ratio**	**Skin thickness (mm)**
Mean	5.89	1.44	538.13	0.76	53.04	0.08	1.15
Maximum	7.90	4.41	1619.20	0.89	124.80	0.27	3.20
Minimum	3.10	0.79	58.50	0.54	1.30	0.02	0.10
C. V. (%)	13.19	35.39	33.08	7.63	32.69	26.29	28.57
S. D.	0.78	0.51	177.99	0.06	17.34	0.02	0.33

S. D. Standard deviation; C. V. Coefficient of variation

Fruit shape oblate, spheroid, high spheroid, ellipsoid, narrowly obovate, obovate, pyriform and clavate were observed (Figure 1A). Moreover, fruit skin color (Figure 1B), flesh color (Figure 1C) and flesh texture (Figure 1D) were highly variables.

Studies developed have permitted the selection of different cultivars with good yields during a large period of the year (Rodríguez *et al*., 2003).

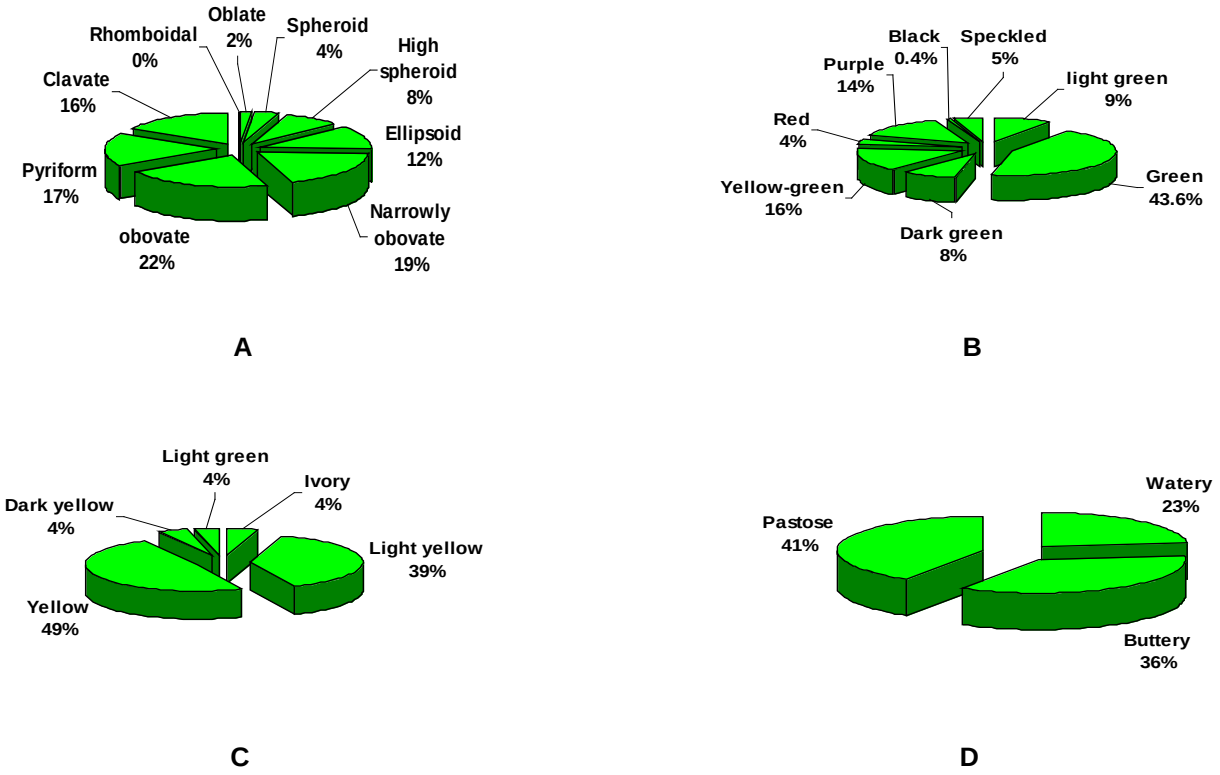

Figure 1. Characterization of the avocado (*Persea americana* Mill.) fruits according to qualitative characters.

Catalogue of avocado cultivars

An exhaustive characterization of 19 important avocado cultivars was made. The information was processed in form of catalogue containing photos of fruits and young twigs, as well as the evaluation of 101 descriptors: 65 qualitative and 36 quantitative (Rodríguez *et al*., 2003a; 2004). Also, in additional notes, information about origin, flowering type and some other distinctive characteristics of cultivars are offered. Cultivars considered were the following: 'Amado Gómez # 1', 'California', 'Casimiro Soledad', 'Catalina', 'Centro América # 3', CH 1 # 3', 'Chavao # 3', 'Choquette', 'Duke–7', 'Hass', 'Itzamná', 'Jaruco # 1', 'José Antonio', 'Los Moros', 'Lula La Pepilla', 'Miguel García', 'Sicilia # 6', 'Suardía Estación' and 'Wilson Popenoe'. Cultivars mentioned have different floral types and belong to diverse ecological races. Cultivars with good yields during a large period of the year have been selected (Figure 2).

Handbook for avocado cultivation

The document refers the technology and management used in avocado, including seedbed, nursery, plantation and harvest. Horticultural races, flowering type, as well as botanical and agronomic characteristics of principal cultivars and rootstocks are offered. Also climatic requirement for avocado cultivation are referred (Jiménez *et al*., 2005).

The multiplication process includes two phases: the sexual in seedbeds and the asexual in nurseries. There are three principal grafting methods utilized for avocado propagation in Cuba: lateral graft, lateral graft with stock pruned on the top (Figure 3a) and cleft grafting (Figure 3b). Also, the

former has been successfully employed for canopy change in commercial plantation (Jiménez *et al*., 2005).

Cultivar Los Moros.
Harvest season: March - Jun

Cultivar Casimiro Soledad.
Harvest season: July - August

Cultivar Catalina.
Harvest season: September - October

Cultivar Miguel.
Harvest season: December – February

Figure 2. Harvest season of different avocado (*Persea americana*. Mill.) cultivars.

Figure 3. Operational sequences of the principal grafting methods utilized in Cuban avocado nurseries. A: lateral graft with stock pruned on the top; B: cleft grafting.

Although the traditional nursery (Cañizares, 1973) has been employed in Cuba, nursery developed in plastic bags with adequate substrate is the way more used at the moment (Farrés, 2002; Jiménez *et al*., 2005).

Orchards include pre-productive and productive stages. Selection and soil preparation, plantation season, as well as type and plantation distances are described. In this way, distances of 16 x 16 m, 12 x 12 m and 10 x 10 m have been employed (Cañizares, 1973); however, nowadays shorter distances are preferred: 10 x 5 m, 8 x 8 m and 7 x 7 m (Jiménez *et al*., 2005).

Cultivation labors applied on nurseries and orchards are also described: organic and inorganic fertilization; chemical, mechanic and combined methods for overgrowths control; chemical, biological and shared methods for plague control; as well as types of watering and prune, respectively. In orchards, there are different types of prune used for suckers elimination, canopy formation, branches clearing, tree rehabilitation, canopy changing and for reduction the size of the tree through lateral and topping prunes (Jiménez *et al*., 2005).

Because of the economical repercussion, intercropping has been another task included in this handbook. Principal crop has been associated with fruits of low size, like pineapple, papaya and dwarf guavas, and also by other crops of short life cycles: beans, sweet potato, corn, etc. All this have permitted the rent-ability of perennial crops (Jiménez *et al*., 2005).

Important consideration related with harvest and post-harvest have been also considered in the handbook (Jiménez *et al*., 2005).

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

Agro-morphologic traits and molecular markers were compared (Table 2) in terms of their discriminating power and informativeness among 17 genotypes assembled in the Cuban avocado germplasm (Rodríguez *et al*., 2009).

D parameter (Tessier *et al*., 1999) adopted for agro-morphologic traits (Rodríguez *et al*., 2009) was useful for genotype identification. Only four morphologic traits were necessary for distinguishing all the individuals analyzed: fruit shape, fruit skin color in mature fruits, harvest season and fruit skin thickness (Rodríguez *et al*., 2007a; 2009). DNA extraction and purification techniques were successfully standardized (Ramírez *et al*., 2004). SSR, AFLP and ISTR markers were powerful techniques for avocado discriminating and cultivar 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 (Rodríguez *et al*., 2009). The higher values of expected heterozygosity were detected with codominant markers, but the value for microsatellites doubled or more the ones obtained with isoenzymes and dominant markers (Rodríguez *et al*., 2007; 2009). The morphologic diversity index designed by Rodríguez *et al*. (2009) 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 (Rodríguez *et al*., 2007; 2009).

Characterization of the genetic diversity of a collection from the avocado Cuban germplasm by means of morph-agronomic and molecular markers

An analysis of the diversity among avocado cultivar was undertaken based on agro-morphological traits, isoenzymes and DNA markers.

The high phenotypic variability of the avocado fruit was proven through the evaluation of 14 characters in two populations of West Indies race selected. Values of coefficients of variation over 25% in different quantitative traits were determined (Rodríguez *et al*., 2000). Moreover, the principal components analysis grouped 39 of the 41 cultivars in their three presumable races with only five variables: harvest season, percentage of the skin weight as compared to the fruit, fruit surface texture, fruit skin thickness and leaf anise smell, (Rodríguez *et al*., 2000). Also, another set of experiments showed that only three variables (peduncle length, fruit surface texture and the adherence of skin to flesh) the cluster analysis permitted to assemble cultivars in two main groups. The former was constituted by West Indies race and hybrids of West Indies x Guatemalan races, and the other by Guatemalan race (Lima *et al*., 1988; Rodríguez *et al*., 2000).

There were use three enzymatic systems by means of discriminating capacity and diversity estimating (Rodríguez *et al*., 2003).The cluster analysis offered an specific band pattern that permit the identification of all cultivars. Moreover, moderate level of genetic diversity was detected. The five

group formed do not clustered cultivars accurately according to the presumable horticultural races (Rodríguez *et al*., 2000; 2003). Probably more enzymatic systems are necessary to reach this objective.

Table 2. 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*		Morph-agronomic traits	Isoenzymes	SSR	AFLP	ISTR
Number of assay unit	U	14	3	15	10	1
Number of polymorphic bands (or stages)	n_p	47	24	124	132	157
Number of monomorphic bands (or stages)	n_{np}	0	1	0	275	0
Total number of bands (or stages)	n	47	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

* From Belaj *et al*. (2003) and Rodríguez *et al*. (2009).

Later, in 2005, 22 agro-morphological traits were processed by a Principal Component and Classification Analysis in order to determine phenotypic diversity of a group of cultivars belongs to the Cuban avocado collection, as well as, the phenotypic traits contributing to race classification. In addition to other determined, two another phenotypic traits (leaf dimension and period length from flowering to fruit maturity) see as useful to this purpose (Ramírez *et al*., 2005).

Two molecular markers for diversity estimating were also used: AFLP and SSR. Bands scored from a total of 12 amplified restriction length polymorphism (AFLP) primer combinations and 16 simple sequence repeat (SSR, microsatellite) were used to determine distance estimates. Cluster analyses were performed based on genetic distance matrices using the unweighted pair group mean arithmetic analysis (UPGMA). Cultivars cluster within racial groups confirming the ecological classification. Thus,

horticultural races from the West Indies and Guatemalan as well as their hybrids were positioned in different groups as expected (Ramírez *et al*., 2005).

Correlation efficiencies R between phenotypic and genetic distance estimates were statistically significant. This analysis demonstrated that genetic dissimilarities based on AFLP and SSR were properly correlated (r = 0.59). Phenotypic distance estimate was also moderately correlated with distances estimated based on AFLP (0.58) and microsatellite (0.54) data (Ramírez *et al*., 2005).

Application of biotechnologies to improve avocado breeding in Cuba

In vitro zygotic embryo culture (Rodríguez *et al*., 1997; 1999a; Fuentes *et al*., 2004) and micropropagation in avocado (Rodríguez *et al*., 1999a; Capote *et al*., 2001) have been techniques established in Cuba for propagation and conservation alternatives. Also, mutation induction and biotechnological techniques are approaches used in avocado breeding (Fuentes *et al*., 2003; 2004a; 2009).

In vitro germination and rooting of zygotic embryos, multiplication rate and plantlets *ex vitro* acclimatization were studied. Percentage of germinated entire embryos were higher using mature than immature embryos. Near of 80 % of entire plantlets obtained by embryo culture were adapted to greenhouse conditions. Based on inhibition of entire sprout fraction, radiosensibility curves for 'Duke-7' and 'Hass' cultivars were developed. Inhibition of entire sprout fraction was described by a second order polynomial equation, according to the equation $Ln(x) = a + b_1x + b_2x^2$, where Ln (x) is the logarithm of the fraction of germinated entire embryo, x is the radiation dose; and a, b_1 and b_2 are the equation parameters (Table 3). Fit of experimental data and theoretical model was equal to 0.96 and 0.95 for 'Hass' and Duke-7' radiosensibility curves, respectively. LD_{50} values defined as the dose, at which the 50 % of entire sprout fraction are inhibited, were determined in 27 and 28 Gy for 'Hass' and 'Duke-7' cultivars, respectively. Gamma-rays mutagenic doses for zygotic embryos of both cultivars were also established between 19 and 25 Gy. Applied mutagenic dose did not affect significantly plantlets development. However, leaf and root anomalies, atrophied and chlorophyll-deficient shoots and albinism were observed at doses higher than LD_{50} values (Fuentes *et al*., 2003; 2004a; 2009).

Table 3. Fit and equation parameters obtained from radio sensibility curves for the 'Duke-7' and 'Hass' cultivars

		Equation parameters		
Cultivars	R-Square	a	b_1	b_2
Duke-7	0.95 *	-0.3943	0.0166	0.0009
Hass	0.96 *	-0.4711	0.0775	0.0004

(*) Significant for $p < 0.0001$

Also, experiments combining saline stress and *in vitro* embryo culture were developed (Fuentes *et al*., 2009). Inhibition of the fraction of germinated entire embryos depended on the saline concentration, according to the equation $Ln(x) = a + b\ x$, where Ln(x) is the logarithm of fraction of germinated entire embryos, x is the NaCl concentration (mM); and a = -0.0865 and b = -0.0108 being the intercept in Y and scope, respectively. The fit of .experimental data and theoretical model was equal to 0.96 ($p < 0.003$). Based on the equation parameters, lethal media dose (LD_{50}) values were calculated as 56 mM of Nacl for Duke-7. Doses values higher than LD_{50} were clearly toxic for Duke-7 zygotic embryos. A concentration of 157 mM NaCl corresponding to LD_{20} value was chosen as a selective saline dose for breeding because this value was highly toxic for 'Duke-7' embryos.

In the other hand, a collection of *Phytopohthora* spp. and *Phytium* spp. strains isolated from commercial avocado plantation was constructed. Studies have been done for the identification and characterization of those isolates based on morphological, physiological and molecular (ITS primers) markers. Amplification using ITS primers permitted to differentiate *Phytopohthora* spp. and *Phytium* spp. strains. Then, the usefulness of this combined approach for the identification fungal isolates is confirmed. Conductimetric bioassays have been done trying to find more effective strains of *P. cinnamomi* for the isolation of fungal toxic filtrates to be used during *in vitro* selection of zygotic embryos of 'Duke 7' rootstock (Coto *et al*., 2007).

These *in vitro* methodologies appear as an useful alternatives to traditional breeding methods, particularly for improving agronomic characteristics as salt tolerance and rot-root resistance in avocado.

Concluding remarks

Several conclusions were derived from these studies: *i)* characterization of the Cuban avocado collection has showed great variability in quantitative and qualitative traits measured in trees,

leaves, flowers and fruits, *ii)* both floral types in cultivars with similar harvest season have been identified, giving the possibility to select adequate pollinators for each case, *iii)* near 90% of cultivars produce fruits with more than 500g, constituting probably a limitation for foreign markets, *iv)* according to morph-agronomic traits, the collection was mainly constituted by West Indies race, but also by Guatemalan race, Mexican race and hybrids, *v)* a group of cultivars producing fruits during all the year have been selected, *vi)* a catalogue of main cultivars and a handbook for avocado cultivation was already done, *vii) D* (discriminating capacity) parameter designed by molecular markers comparison could be adopted successfully for distinguishing avocado cultivars by the use of morphologic traits, *viii)* DNA markers (ISTR, AFLP and SSR) were useful for discriminating avocado cultivars and for varietal certification; moreover, isoenzymes was a low cost technique functional for this purpose in the germplasm, *ix)* a high degree of heterozygosity was detected in avocado using microsatellite (SSR) markers, *x)* in addition to the classical traits used to race classification, six phenotypic characters (percentage of the skin weight as compared to the fruit, fruit surface texture, leaf dimension, peduncle length, harvest season, and period length from flowering to fruit maturity) see as useful for this purpose, *xi)* cultivars cluster within racial groups confirming the ecological classification, *xii)* phenotypic variation properly reflects the genetic distance among avocado cultivars, but AFLP and microsatellite markers were useful for providing a more precise estimate of genetic distance among avocado breeding material, *xiii)* correlations between phenotypic and AFLP and microsatellite data suggesting the convenience of use genetic distances estimates based on these marker systems as an indicative of phenotypic diversity in avocado cultivars, *xiv)* the combination of molecular marker, zygotic embryo culture and mutation induction technologies can be an excellent alternative for the improvement of important characteristics in breeding programs, and *xv*) putative mutant lines have been obtained and were planted for future analysis.

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