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Evaluation of Avocado Germplasm Using Microsatellite Markers

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Abstract. Three horticultural races of avocado (*Persea americana* Mill.) are known: Guatemalan, Mexican, and West Indian. Each race has unique characteristics and current commercial varieties have been selected from within the races or from interracial hybrids. Using 14 microsatellite loci we investigated the genetic variation among 224 accessions (394 plants) maintained at the National Germplasm Repository (NGR) in Miami, Fla., and a set of 34 clones from the University of California South Coast Field Station (SCFS) located in Irvine, Calif. The 14 microsatellite loci had an average of 18.8 alleles per locus and average unbiased genetic diversity was 0.83. The total propagation error in the collection, i.e., plants that had been incorrectly labeled or grafted, was estimated to be 7.0%. Although many unique alleles did exist, no useful race-specific markers were found. A general concordance between the horticultural race and the clusters obtained from molecular data was observed. Principal Coordinate Analysis (PCA) grouped the Guatemalan and Mexican races into two distinct clusters. The West Indian also grouped into a unique major cluster but with an outlying group. Using the PCA a change in the racial designation or interracial hybrid status for 50 accessions (19.7%) is proposed. The unbiased gene diversity estimate was highest in the Mexican and Guatemalan races and lower in the West Indian group. This demonstrates the need to collect more of the West Indian germplasm to broaden the genetic diversity and to emphasize the identification of individuals conferring resistance to Phytophthora Root Rot (PRR).

The avocado (*Persea americana* Mill.) is an evergreen subtropical tree that is native from Mexico to northern South America and produces a fruit that is unique and nutritious. This fruit was known by the Aztecs as ahuacacuauhitl, which was later shortened by the Spaniards to aguacate. In the United States avocado was introduced into Florida in 1833, California in 1848 and to Hawaii by 1855 (Nakasone and Paull, 1998). Major commercial production of avocado in the United States is limited to California and Florida. In 2000, global production exceeded 2.4 MMT and the major producers were Mexico, Indonesia, South Africa and the United States (Anonymous, 2001).

P. americana has been subdivided into three horticultural groups: Mexican [*P. americana* var. *drymifolia* (Schect. & Cham.) Blake], Guatemalan (*P. americana* var. *guatemalensis* Wms.) and West Indian (*P. americana* var. *americana* Mill.) races. The West Indian race is known to be from the lowland areas of the Pacific coast of Central America and not the West Indies, while the Guatemalan and Mexican races are native to specific highland areas in each country (Scora and Bergh, 1992). The collection at the NGR-Miami contains 224 accessions with all three races represented, as well as hybrids between them. The earliest introductions were collected by Wilson Popenoe in Guatemala in the 1920s, and the newest introductions were collected by Avraham Ben-Ya'acov throughout Central and South America during the 1990s (Ben-Ya'acov, 1995; Popenoe, 1920). The University of California collection at the South Coast Field Station (SCFS) in Irvine contains a large number of accessions and breeding lines mostly of the Mexican and Guatemalan races and mixed interracial hybrids. These two collections contain a comprehensive representation of the genetic diversity currently in avocado germplasm collections.

The three racial groups can be distinguished by the percentage oil content in the fruit with the West Indian cultivars ranging from 2.5% to 8.0%, Guatemalan accessions from 10% to 13%, and the Mexican accessions ranging from 15% to 20% (Knight, 2002). The racial classes also vary phenotypically for characters such as fruit size and shape, skin thickness, skin color, seed size, and fruit ripening (Lahav and Lavi, 2002). Avocado is a diploid with 2n = 24 (Garcia, 1975) and sterility barriers do not exist between or among the three racial types (Lahav and Lavi, 2002). Avocado has a distinct flowering habit known as protogynous, diurnally synchronous dichogamy (Bergh, 1969). This type of reproductive behavior promotes outcrossing; however, significant amounts of self-pollination are known to occur in commercial plantings (Davenport et al., 1994). Named cultivars often originate from open-pollinated seedlings. The pollen parent is unknown but has often been estimated based on the flower types of available donor trees. Many of the cultivars grown in Florida are interracial hybrids between Guatemalan and West Indian types while those grown in California are hybrids between Mexican and Guatemalan types (R. Knight, personal communication). Morphological characters have been used to infer parentage, although these characters are influenced by environmental factors and may not unambiguously distinguish closely related genotypes or interracial hybrids.

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Table 1. Listing of the 254 Avocado accessions evaluated with microsatellite markers by racial lineages, interracial hybrid groups, and changes in lineages based on the microsatellite analysis.

Cultivar	Race	MIA	Source
Akbal	Guatemalan	654	SHRS
Anaheim	Guatemalan		SCFS
Brooks Late	Guatemalan	22618	SHRS
Carlsbad	Guatemalan		SCFS
Ereguayquin #7	Guatemalan	34962	SHRS
Gordo	Guatemalan		SCFS
Green Gold	Guatemalan		SCFS
HX73	Guatemalan		SCFS
Ishim	Guatemalan	657	SHRS
J241	Guatemalan		SCFS
JJ12	Guatemalan		SCFS
Julia	Guatemalan		SCFS
Kayab	Guatemalan	660	SHRS
L/28	Guatemalan		SCFS
L137	Guatemalan		SCFS
Lamat	Guatemalan	646	SHRS
MS9	Guatemalan		SCFS
NA251	Guatemalan		SCFS
Nabal	Guatemalan	656	SHRS
Nabal Seedling 1	Guatemalan	20713	SHRS
Nabal Seedling 2	Guatemalan		SHRS
Nimlioh a	Guatemalan	32160	SHRS
Nimlioh b	Guatemalan		SCFS
NN10	Guatemalan		SCFS
P.I.C. 9615	Guatemalan	35008	SHRS
PT37	Guatemalan		SCFS
Queen 8	Guatemalan	20722	SHRS
Reed	Guatemalan		SCFS
Taylor	Guatemalan	18262	SHRS
Williams	Guatemalan		SCFS
XX3	Guatemalan		SCFS
3/1/1	Mexican		SCFS
(Arue X Kanan)	Mexican	21110	SHRS
Atlisco No. 1	Mexican	4043	SHRS
Bacon	Mexican	20709	SHRS/SCFS
Bacon Seedling	Mexican	24427	SHRS
Brooksville Seedling 1	Mexican	23239	SHRS
Capac	Mexican	641	SHRS
Carchi	Mexican	635	SHRS
Celaya c-25	Mexican	28875	SHRS
Chota	Mexican	639	SHRS
Del Oro #2	Mexican	18106	SHRS
Duke	Mexican	17461	SHRS
Duke Seedling	Mexican	17468	SHRS
Egas	Mexican	640	SHRS
Esparta I	Mexican	34966	SHRS
Gainesville	Mexican	18431	SHRS
Gainesville Seedling	Mexican	17459	SHRS
Guayabamba	Mexican	24544	SHRS
Heyns	Mexican	32208	SHRS
Irving	Mexican		SCFS
Itzamna seedling 2	Mexican		SHRS
Jim	Mexican		SCFS
J. P. Young	Mexican	32157	SHRS
Mayo	Mexican		SCFS
Mexicola	Mexican	32159	SHRS/SCFS
Miramar de Monte de Oro	Mexican	34852	SHRS
Morocco No. 42	Mexican	19327	SHRS
Morocco No. 43	Mexican	19328	SHRS
Naranjo No. 1	Mexican	28867	SHRS
Naranjo No. 2	Mexican	28868	SHRS
Pancho	Mexican	29664	SHRS
Proteco	Mexican	24533	SHRS
Puebla	Mexican		SCFS
Reina Victoria	Mexican	24535	SHRS
Romain No. 1	Mexican	19329	SHRS
San Rafael	Mexican	34898	SHRS

Cultivar	Race	MIA	Source
Seedless Mexican	Mexican	2684	SHRS
Stewart	Mexican		SCFS
Tamayo	Mexican	638	SHRS
Toltec	Mexican	14768	SHRS
Topa Topa	Mexican		SCFS
Young No.1	Mexican	20728	SHRS
Young No.2	Mexican	20729	SHRS
Young Slipskin	Mexican	21330	SHRS
Young Special	Mexican	21331	SHRS
Zutano	Mexican		SCFS
Antigua	West Indian	34858	SHRS
Arguello	West Indian	21895	SHRS
Argui 3	West Indian	34894	SHRS
Arue Seedling 1	West Indian	25422	SHRS
Avocatosa 3	West Indian	34868	SHRS
Aycock Red No. 3	West Indian	20022	SHRS
Aycock Red No. 19	West Indian	20021	SHRS
Belize	West Indian	20023	SHRS
Bernecker	West Indian	24244	SHRS
Biscayne Seedling 1	West Indian	3335	SHRS
Biscayne Seedling 2	West Indian		SHRS
Biscayne Seedling 3	West Indian		SHRS
Biscayne Seedling 4	West Indian		SHRS
Biscayne Seedling 5	West Indian		SHRS
Biscayne Seedling 6	West Indian		SHRS
Biscayne Seedling 7	West Indian		SHRS
Biscayne Seedling 8	West Indian		SHRS
Biscayne Seedling 9	West Indian		SHRS
Catalina	West Indian	17248	SHRS
Cellon's Hawaii Seedling	West Indian	6896	SHRS
Dade	West Indian	673	SHRS
Donaldson	West Indian	20024	SHRS
Dora	West Indian	20025	SHRS
Dr Dupuis No. 2	West Indian	25071	SHRS
Family	West Indian	3895	SHRS
Fuchs	West Indian	31946	SHRS
Galvan	West Indian	34867	SHRS
General Bureau	West Indian	19331	SHRS
Honalindo 1	West Indian	34872	SHRS
Jose Antonio	West Indian	17252	SHRS
Kilo 4 Ceiba	West Indian	20026	SHRS
Maxima	West Indian	23271	SHRS
Orizaba 4	West Indian	34856	SHRS
Pollock	West Indian	19846	SHRS
Progreso 1	West Indian	34854	SHRS
Raul Arango	West Indian	20029	SHRS
Ruehle	West Indian	19848	SHRS
Seedless Avocado	West Indian	8485	SHRS
Simmonds	West Indian	670	SHRS
Simmonds Seedling	West Indian	3900	SHRS
Tantima 2	West Indian	34870	SHRS
Tela	West Indian	34853	SHRS
Tito Perla	West Indian	20031	SHRS
Tower 2	West Indian	27195	SHRS
Trapp	West Indian	19849	SHRS
MIA 35729	West Indian	35729	SHRS
MIA 35734	West Indian	35734	SHRS
MIA 35732	West Indian	35732	SHRS
Waldin	West Indian	19850	SHRS
Wester	West Indian	675	SHRS
Wilson Popenoe	West Indian	3757	SHRS
6836a	GxM	20708	SHRS
6836c	GxM		SHRS
Antigua Market	GxM	11939	SHRS
Canaries	GxM	17872	SHRS
Ein vered	GxM	23693	SHRS
Ettinger	GxM	14304	SHRS/SCFS

Cultivar	Race	MIA	Source
Fuerte Popenoe	GxM	24542	SHRS
Horshim	GxM	23692	SHRS
Netaim	GxM	24088	SHRS
Nordshtein	GxM	24083	SHRS
Pinkerton	GxM	31113	SHRS
Sharwil	GxM	23332	SHRS
Tenerife	GxM	17690	SHRS
Winter mexican	GxM	18260	SHRS
Agnes	GxWI	23578	SHRS
Borrego	GxWI	17250	SHRS
Choquette	GxWI	22683	SHRS
Collinred Seedling B1	GxWI	6689	SHRS
Collinred Seedling B2	GxWI		SHRS
Collinred Seedling B3	GxWI		SHRS
Collinson	GxWI	400	SHRS
Dunedin	GxWI	3896	SHRS
Gripina 12a	GxWI	24223	SHRS
Gripina 12b	GxWI		SHRS
Hiawassee	GxWI	26063	SHRS
Lula	GxWI	7828	SHRS
Melendez	GxWI	19770	SHRS
Melendez 2	GxWI	34405	SHRS
Monroe	GxWI	19852	SHRS
Semil 34	GxWI	24224	SHRS
Semil 43	GxWI	24225	SHRS
Simon	GxWI	19853	SHRS
Suardia	GxWI	17253	SHRS
Tonnage	GxWI	19847	SHRS
Utuado	GxWI	19768	SHRS
Winslowson	GxWI	3889	SHRS
Winslowson Seedling	GxWI	3888	SHRS
Yon	GxWI	18261	SHRS
Courtright	WIxM	32154	SHRS
Brooksville Seedling 4	Complex hybrid	25464	SHRS
Brooksville Seedling 5	Complex hybrid	25865	SHRS
Brooksville Seedling 6	Complex hybrid	25867	SHRS
Brooksville Seedling 7	Complex hybrid	25868	SHRS
Brooksville Seedling 8	Complex hybrid	25870	SHRS
Brooksville Seedling 9	Complex hybrid	26924	SHRS
Brooksville Seedling 10	Complex hybrid	30506	SHRS
Brooksville Seedling 11	Complex hybrid	30507	SHRS
Brooksville Seedling 12	Complex hybrid	30508	SHRS
Brooksville Seedling 13	Complex hybrid	30509	SHRS
Brooksville Seedling 14	Complex hybrid		SHRS
Brooksville Seedling 15	Complex hybrid		SHRS
Brooksville Seedling 16	Complex hybrid		SHRS
Brooksville Seedling 17	Complex hybrid		SHRS
Irwing 120	Complex hybrid	20718	SHRS
Vero Beach (G.R.D.)	Complex hybrid	20536	SHRS
Vero Beach No. 01	Complex hybrid	18435	SHRS
Vero Beach No. 15	Complex hybrid	20538	SHRS
Vero Beach S.E.-2	Complex hybrid	20535	SHRS
P. nubigena		34880	SHRS
P. nubigena 2		34881	SHRS
P. schiedeana		34875	SHRS

Cultivar	Race	MIA	Source
Previously labeled as MxW.			
Gottfried Seedling 1	West Indian		SHRS
Gottfried Seedling 2	West Indian		SHRS
Gottfried Seedling 3	West Indian		SHRS
Gottfried Seedling 4	West Indian		SHRS
Kosel	West Indian	19247	SHRS
Previously labeled as GxM			
6836b	West Indian		SHRS
Tensen	West Indian	17114	SHRS
Previously labeled as Mexican.			
Itzamna Seedling 1	GxWI		SHRS
Brooksville Seedling 2	Complex hybrid	24305	SHRS
Brooksville Seedling 3	Complex hybrid	25856	SHRS
Gottfried	Complex hybrid	632	SHRS
Miramar de Monte de Oro a	Complex hybrid		SHRS
Previously labeled as West Indian.			
Avacatosa	Guatemalan	34861	SHRS
Orizaba 3	Guatemalan	34871	SHRS
Gen. Francisco Robles	Mexican	24534	SHRS
Orizaba 6	Mexican	34864	SHRS
Biscayne	GxM	26512	SHRS
Novillero	GxWI	34860	SHRS
Previously labeled as Guatemalan.			
Apakia 1	Mexican	35007	SHRS
Key Largo	MxWI	22121	SHRS
La Piscina	Mexican		SHRS
Tertoh	West Indian	650	SHRS
Dickinson	GxWI	2374	SHRS
Lima Late	GxWI	20034	SHRS
Collins	MxWI	21135	SHRS
Collins Seedling	MxWI	2897	SHRS
Queen	MxWI	20028	SHRS
MIA 35730a	MxWI	35730a	SHRS
MIA 35730b	MxWI	35730b	SHRS
MIA 35730c	MxWI	35730c	SHRS
Previously labeled as GxWI.			
Sitio del Nino #5	Guatemalan	34961	SHRS
D.W.I. Bank	West Indian	19767	SHRS
Girrardin	West Indian	23343	SHRS
Gripina	West Indian	19769	SHRS
Previously labeled as Complex hybrid.			
Marsheline	West Indian	20712	SHRS
Trappson	West Indian	1276	SHRS
CRC 195-36	GxM	25874	SHRS
Hass	GxM		SCFS
Iriet	GxM		SCFS
Irwing 59	GxM	20715	SHRS
Irwing 65	GxM	20720	SHRS
Irwing 78	GxM	20716	SHRS
Irwing 96	GxM	20717	SHRS
Irwing 134	GxM	20712	SHRS
Lamb Hass	GxM		SCFS
Teague	GxM	20714	SHRS
Yama 003	GxM	20724	SHRS
Yama 381	GxM	20726	SHRS
Yama 423	GxM	20727	SHRS
Hawaii	GxWI	3893	SHRS
Previously labeled as unknown.			
"636"	Guatemalan	636	SHRS
MIA 35731	Mexican	35731	SHRS
MIA 35733	West Indian	35733	SHRS
"8486"	GxM	8486	SHRS
San Miguel	GxM	31961	SHRS
Alicia Cordero	GxWI	34965	SHRS
Pope	GxWI	32161	SHRS
Semil 38	GxWI	34419	SHRS
Argui 1	Complex hybrid	34891	SHRS
Brogden	Complex hybrid	31303	SHRS
Egami	Complex hybrid	34892	SHRS
El Venado	Complex hybrid	34863	SHRS
Leyad Aro a	Complex hybrid	34893	SHRS
Leyad Aro b	Complex hybrid		SHRS
San Jeirer 1	Complex hybrid	34887	SHRS

Molecular markers are often used to clarify genetic relationships between individuals. In avocado germplasm evaluated using minisatellite markers, racial pattern differences were observed and fingerprinting of 26 cultivars was accomplished (Lavi et al., 1991). Mhameed et al. (1997) used variable number of tandem repeats (VNTR) markers to evaluate genetic relationships among 24 avocado cultivars that had been classified using morphological traits. The VNTR data supported the racial classification for most of the accessions. In another study, RAPD markers were used to evaluate 16 accessions representing the three races. Race-specific markers were identified and the similarity between races ranged from 53% to 58% (Fiedler et al., 1998). Similarly, Davis et al. (1998) used Restriction Fragment Length Polymorphism (RFLP) to evaluate a set of 36 cultivars from the University of California SCFS and their results were in agreement with the previous studies.

Microsatellite markers have been developed for avocado and utilized to produce a crude linkage map (Sharon et al., 1997). These markers have distinct advantages over the other types of molecular markers used to classify avocado germplasm. Their advantages include their abundance in most genomes, uniform distribution, hypervariability, codominance, and PCR-based protocols. Even though the genetic diversity analysis from the aforementioned studies sampled a small number of accessions, they have adequately demonstrated the usefulness of molecular markers in the classification of avocado germplasm.

Our objectives were to estimate genetic diversity within the large collection maintained at the USDA, NGR in Miami and a subset of the collection maintained at the SCFS by the University of California using microsatellite markers. Genetic diversity within and between populations of avocado was of interest, particularly within the West Indian race that is known to have genes involved with tolerance to phytophthora root rot (PRR). Another objective was to clarify the anecdotal information on parentage of mixed race hybrids and seedling selections from commercial cultivars. Since all plants (clones) of a given accession were genotyped, we also investigated the fidelity of germplasm propagation within accessions, as clonal collections are known to contain identical accessions with different names and mixtures of genotypes with the same name (Schnell et al., 1999).

Materials and Methods

Plant materials. Leaf material was sampled from the *Persea* germplasm collection at the NGR in Miami, Fla., and from the SCFS, University of California, Irvine, Calif**.** The number of plants genotyped for this study was 428. This included 254 accessions, 224 from the NGR-Miami and 34 from the University of California SCFS at Irvine with four cultivars common to both collections. Of the 224 accessions at the NGR, 104 accessions were represented by multiple plants. These 274 plants include 54 accessions with two, 37 accessions with three, 10 accessions with four, and three accessions with five plants.

In total, individuals of the following backgrounds were studied: 51 Mexican, 35 Guatemalan, and 65 West Indian, 100 interracial hybrids, and three related species. Hybrids were designated as follows: Complex (CH), Guatemalan x West Indian (G x W), Guatemalan x Mexican (G x M), and Mexican x West Indian (M x W). The out-group consisted of two accessions of *P. nubigena* and one accession of *P. schiedeana* (Table 1).

Table 2. Microsatellite loci and primers used in the analysis of the avocado germplasm collections developed by Sharon et al. (1997).

Locus	Repeat	Primers (5'-3')	Annealing temp (°C)	Alleles (no.)	Size range (bp)	Diversity $[\hat{H} \pm V(\hat{H})]$
AVAG05	$(AG)_{10}$	GGATCTTGATGTGTGGGGGAG				
		CCTGTCGGAAAAGACTATGCG	50	19	83–125	0.7906 (0.0394)
AVAG03	$(TC)_{17}$	GCACTTCCTAAACTTGCAGGT				
		CTGAACATCCAATGACAAACATCC	45	14	92–122	0.8414 (0.0551)
AVAG25	$(TC)_{14}$	ATGGTTTTTTCCTGCCCTTT				
		AACAAGCCCCCTAAAAGAA	50	20	96–140	0.8640 (0.0596)
AVAG13	$(CT)_{18}$	CTGCGATAACAACTGGAC				
		AACTAGGACCTGAAACCG	50	29	96–160	0.9150 (0.0912)
AVAG11	$(AG)_{20}$	AGCGATGAACATTACCA				
		ATTTCTTCAACCCATCTGTC	50	15	105–161	0.7553 (0.0388)
AVMIX03	$(TG)_{16}$,$(AG)_{20}$	GATATTCCTGTTGTCACTGC				
		GATATTCCTGTTGTCACTGC	50	23	139–196	0.8912 (0.0718)
AVAG21	$(CT)_{22}$	TGTAAGTTTTAACCCCACAA				
		AATCACTATTAGAGTTTTCAGTCG	50	30	153–219	0.8911 (0.0729)
AVAG07	$(TC)_{15}$	ATCCAAAATGCACAAGGTGAGG				
		TGTCGCTATGTCCAAAATGTGG	50	8	98–114	0.6524 (0.2801)
AVMIX04	$(AG)_{12}$,$(CAA)_5$, $(ACAG)_{10}$	CCGTTTGCTTCCTGTATC				
		GTTATCCCTTCCACTTTC	50	19	158–194	0.8922 (0.0750)
AVAC01	$(TG)_{15}$	CTGGTTGCTCTCTTGTCTACATAATA				
		CGGTTTTGTAAGTTGATAG	40	16	95–185	0.8627 (0.0597)
AVMIX02	$(TC)_6$,$(TCC)_4$	GAGTCACGCTCGTAGGCT				
		TATAAATTCAAATGACAC	40	10	147–135	0.7335 (0.0363)
AVAG06	$(CT)_{18}$	CGACCTCTTCTTATACTC				
		GTACCTCTGATAATGAGCAT	40	15	59–89	0.8625 (0.0629)
AVAG10	$(CT)_{22}$	GAATTACAAAGCACTAGAG				
		GTAGAAAGTGGGCACACAT	45	30	149–234	0.8695 (0.0607)
AVAG22	$(GA)_{15}$	GATCATCAAGTCCTCCTTGG				
		GATCTCATAGTCCAAATAATGC	55	16	96–130	0.8206 (0.0597)

DNA extraction was performed on 200 mg samples of leaf tissue using the Fast DNA kit (BIO 101, Inc.; Carlsbad, Calif.) and a cell disrupter (FastPrep FP 120; Savant Instruments, Inc.; Holbrook, N.Y.). The kit protocol for plant tissue was followed including the optional SPIN protocol. Tissue was homogenized using the Garnet Matrix and two ¼-inch spheres as the Lysing Matrix combination, at speed 5 for 30 s, repeated three times. DNA was quantified on a spectrophotometer (DynaQuant 200; Amersham Pharmacia; Piscataway, Calif.)

Microsatellite markers and PCR amplification. The microsatellite markers used in this study were reported by Sharon et al. (1997). Initially, 39 primer pairs were tested of which 14 were selected based on amplification consistency and level of polymorphism. Microsatellite locus name and primer sequence are listed in Table 2. PCR amplification reactions were carried out in a total volume of 10 μL, or 20 μL for multiplex reactions, containing 0.25 ng·μL^{-1} genomic DNA. All PCR reactions contained 0.025 U/μL Amplitaq (Applied Biosystems, Inc.; Foster City, Calif.), 0.2 mM dNTPs, 0.25 μM each forward and reverse primers, 1× GeneAmp PCR buffer (1.5 mM $MgCl_2$, 10 mM Tris-HCl pH8.3, 50 mM KCl, 0.001% (w/v) gelatin). Thermal cycling profile consisted of the following: 4 min denaturation at 94 °C; followed by 33 cycles of denaturation at 94 °C for 30 s, 1 min at appropriate annealing temperature for each primer (Table 2), 1 min extension at 72 °C ; with a final 7 min 72 °C extension. PCR was carried out on a DNA Engine tetrad thermal cycler (MJ Research, Inc.; Watertown, Mass.). The following PCR multiplex reaction combinations were used: AVAG11 and AVMIX03, AVAG21 and AVAG07, and AVMIX02 and AVAG06, all other primer pairs were run individually.

Electrophoresis. Capillary electrophoresis (CE) was performed on an genetic analyzer (ABI Prism 3100; Applied Biosystems, Inc.) using Performance Optimized Polymer 4 (POP 4, Applied Biosystems, Inc.). Samples were prepared immediately before electrophoresis by adding 1 μL of PCR product to 12 μL of deionized formamide and 0.1 μL of GeneScan 500 ROX size standard (Applied Biosystems, Inc.), then denatured at 95 °C for 5 min, and chilled on ice. PCR products and size standards were doubled for preparation of multiplex PCR amplifications. Samples were injected electrokinetically at 3 kV for 10 s and were run at 15 kV and 60 °C for 25 min. Resulting data were analyzed with GeneScan 3.7 (Applied Biosystems, Inc.) for internal standard and fragment size determination. Allelic designations were ascertained using Genotyper 3.7 (Applied Biosystems, Inc.).

Data analysis. Gene diversity values for each locus and averages across all loci for the three races and interracial groups were calculated using Nei's (1987) unbiased estimate $\hat{H} = n(1 - \sum p^2_i/n - 1$, where n = number of individuals sampled, p_i is the frequency of the ith allele. The variance of this statistic was calculated as $V(\hat{H}) = 2/[(n-2)[\sum p^3_i - (\sum p^2_i)^2] + \sum p^2_i - (\sum p^2_i)^2]$.

Unbiased gene diversity (H_{nb}) and observed heterozygosity (H_{obs}) were estimated from the allele frequencies of the Guatemalan, Mexican, and West Indian populations as well as CH, G x M, G x W, M x W, and the *Persea* spp. group, at each locus using GENETIX ver. 4.0 (Montpellier, France).

The principal coordinate analyses (PCA; Sokal and Rohlf, 1998) were performed on subsets of the data using the SAS System for Windows ver. 8.0 (SAS Institute; Cary, N.C.) with modified Rogers' distance (Wright, 1978). Due to the number of taxa and resulting difficulty in visualizing individuals in the 3-D PCA, the Guatemalan, Mexican, and West Indian populations were analyzed together first. This was followed by the analysis of the other interracial hybrid groups, one at a time, to determine where they clustered in relation to the three primary populations.

The relationships between the populations were also represented using a phenetic tree constructed from allele frequencies averaged over populations, using the Cavalli-Sforza and Edwards (1967) chord distance and the neighbor joining method (NJ; Saitou and Nei, 1987). Statistical analysis was accomplished by testing for similarities in allele frequencies using genotypic counts between groups as suggested by Weir (1996). Chi-square analysis of the contingency tables was generated using the Proc. Freq procedure with Monte Carlo simulation for estimates of exact P values and 1000 iterations. The Monte Carlo option was used due to the size of the data set and the computational resources required by the analysis (SAS, 1999).

Results

Level of polymorphism. The 14 microsatellite loci were highly polymorphic. The number of alleles varied from eight (AVAG07) to 30 (AVAG21), with an average of 18.8 alleles per locus and the average gene diversity was 0.83 (0.65 to 0.91) (Table 2). Eleven of the 14 microsatellites were composed of simple dinucleotide repeat motifs. Seven of these gave amplification products differing by either two bases or multiples of two for each allele. An example of the allelic diversity is given in Fig. 1 for locus AVAG06 showing 8 of the 15 alleles detected at this locus in eight accessions including *P. nubigena*. AVAG13 had two of 29 alleles that were not multimers of the repeat unit, while AVAG21 contained nine of 30 alleles that differed by a single base. Additionally, AVAG10 and AVAG11 generated two of 29 and three of 15 alleles, respectively, that were not multimers of the repeat unit. Two of the three microsatellite

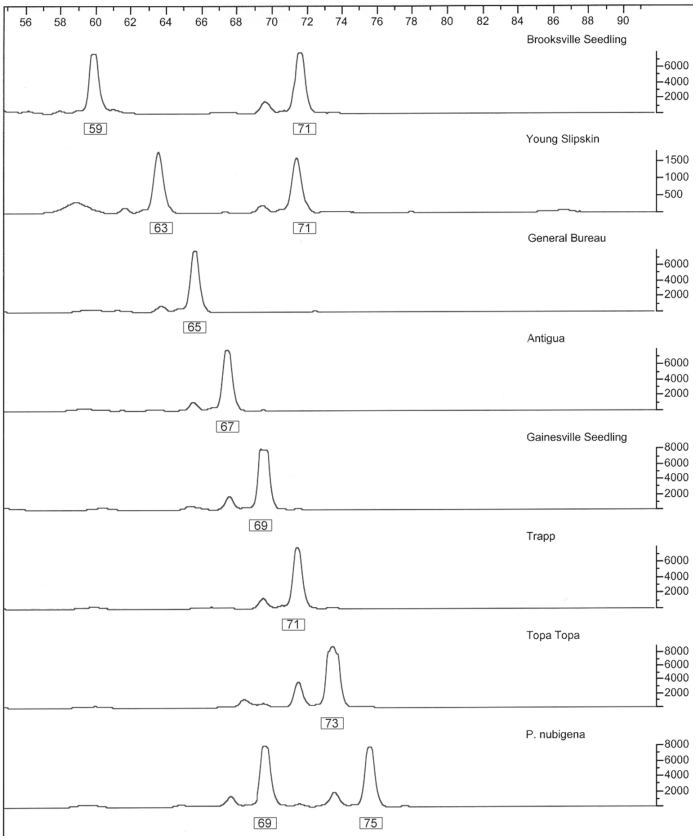

Fig. 1. Allele sizes in base pairs detected at the AVAG-06 microstatellite locus in eight *Persea* accessions illustrating eight alleles using the computer program Genotyper ver 4.0. The major peak in each electropherogram is the PCR amplified allele at this locus.

Table 3. Allele size for each locus and comparison of Ettinger and Pinkerton for allele sizes at the 14 microsatellite loci.

Allele	AVAG05	AVAG03	AVAG25	AVAG13	AVAG11	AVMIX03	AVAG21	AVAG07	AVMIX04	AVACO1	AVMIX02	AVAG06	AVAG10	AVAG22
1	83	92	96	96	105	139	153	---	158	95	147	59	149	96
2	85	94	98	98	107	141	167	98	160	99	149	63	174	98
3	87	96	100	100	109	143	171	100	162	103	153	65	176	100
4	89	98	102	102	113	145	173	102	164	105	155	67	178	102
5	91	100	104	104	115	147	175	104	166	107	157	69	180	104
6	93	102	106	106	116	151	177	106	168	109	159	71	182	106
7	95	104	108	108	118	152	179	108	170	111	163	73	184	108
8	97	106	110	110	125	154	180	112	172	113	165	75	185	110
9	99	108	112	112	129	156	181	114	174	115	167	77	186	112
10	101	110	114	114	131	158	183		176	117	185	79	187	114
11	103	112	118	116	132	160	186		178	119		81	188	116
12	105	114	120	118	133	162	187		180	121		83	190	118
13	107	116	122	120	137	164	188		182	123		85	192	120
14	109	122	124	122	147	166	189		184	127		87	194	122
15	111		128	126	161	168	190		186	131		89	196	124
16	113		130	128		170	191		188	135			298	130
17	119		134	130		172	193		190				200	
18	121		136	132		174	194		192				202	
19	125		138	134		178	195		194				204	
20			140	136		182	196						206	
21				138		190	198						208	
22				141		192	199						210	
23				143		196	201						216	
24				146			202						218	
25				148			203						220	
26				152			204						222	
27				154			207						224	
28				158			209						228	
29				160			215						230	
30							219						234	
Allele size														
Ettinger(Akko Expt. Station)														
	95/99	96	100	134/140	109/115	146/147	172/184	108/120	164/170	113/117	153/171	81/87	153	103/117
Ettinger (NGR-Miami)														
	87/95	94	102	122/128	109/161	143/172	167/190	100/112	162/170	109/113	157/167	77/83	190	100/114
Pinkerton(Akko Exp. Station)														
	95/99	98/106	106/110	130	107	146/170	192/200	110/113	180	117	153/171	77/85	149/153	117
Pinkerton (NGR-Miami)														
	89/91	96/102	104/106	118	107	143/166	181/198	102/106	180	NA[z]	149/167	69/81	149/186	114

[z]NA = no amplification.

loci that contained mixed repeat motifs gave amplification products differing by two bases or multiples of two for each allele.

Accession identification. Of the 104 accessions with duplicate plants, 85 (82%) had identical allelic configurations over all loci. Nonidentical allelic configurations usually resulted when one of three or four trees was not the same genotype. Many of these off-type plants had been previously detected based on phenotypic differences and the molecular data confirmed the misidentification. The 104 duplicated accessions are represented by 274 plants, only 19 of which were genotypically different from their sibling clones. The rate of error associated with propagation of the avocado collection is estimated to be 7.0%. Of the four cultivars common to both the NGR and SCFS, three were identical between collections, 'Bacon', 'Ettinger', and 'Mexicola'. 'Nimlioh' differed at 12 of the 14 loci. The cultivars 'Ettinger' and 'Pinkerton' were used in the study by Sharon et al. (1997) where the microsatellite markers were developed. These two cultivars were also included in our study. The comparison of allele sizes for 'Pinkerton' produced very similar fragment sizes, ours being on average 4.5 bp smaller, with the exception of alleles at two loci. For locus AVAG10 Sharon et al. (1997) reported allele sizes of 149 and 153 bp, whereas our alleles are 149 and 190 bp. Another difference occurred at locus AVACO1 where we obtained no amplification products, whereas Sharon et al. (1997) reported one amplification product at 117 bp. The comparison of allele sizes for 'Ettinger' also produced very similar fragment sizes, ours being on average 4.8 bp smaller, with the exception of alleles at six loci that were on average 20 bp larger. For example, the largest difference occurred at locus AVAG11 where Sharon et al. (1997) reported allele sizes of 109 bp and 115 bp, whereas our amplification products were 109 bp and 161 bp (Table 3).

Genetic differentiation between horticultural races and relatedness of cultivars. The PCA supports the grouping for the West Indian, Guatemalan, and Mexican races as illustrated in Fig. 2A where only the individuals within each race were analyzed. The PCA was able to summarize 29.7% of the total variability onto the three axes shown in this plot (18.3%, 6.4%, and 5.0%, respectively, for Prin1, Prin2, and Prin3). The PCA based on gene frequency suggested that the race of some accessions was incorrectly assigned. Six of the West Indian accessions did not group with the West Indian cluster. Two of these six, 'General Francisco Robles' and 'Orizaba 6' are clearly Mexican; both contained estragole in their leaves. Two others, 'Avocatosa' and 'Orizaba 3' clustered with the Guatemalan group while 'Biscayne' is most likely a M x W hybrid and 'Novillero' is most likely a G x M hybrid. Based on the PCA, 12 of the Guatemalan accessions clustered in problematic areas. 'Tehtoh' is clearly a West Indian accession, 'La Piscina' is Mexican and nine of the other accessions are mixed racial hybrids, 'Collins Seedling 2', 'Collins', 'MIA35730a', 'MIA35730b', 'MIA35730c', and 'Key Largo' all are M x W hybrids while 'Dickinson' and 'Lima Late' are G x W hybrids. 'PIC9651' clustered in the Mexican accessions; however it was listed as a Guatemalan accession (Ben-Ya'acov, 1995) and does not contain estragole in the leaves. Based on the PCA, five of the Mexican accessions clustered in problematic areas. 'Brooksville Seedling 2' and 'Brooksville Seedling 3' seem to be West Indian or West Indians hybrids while 'Gottfried' and 'Miramar de Monte de Oro' seem to be CH. 'Itzamna Seedling 1' clustered as a G x W. These changes are illustrated in Fig. 2A where the original racial designations are indicated with different symbols and the putatively misidentified accessions are labeled. In

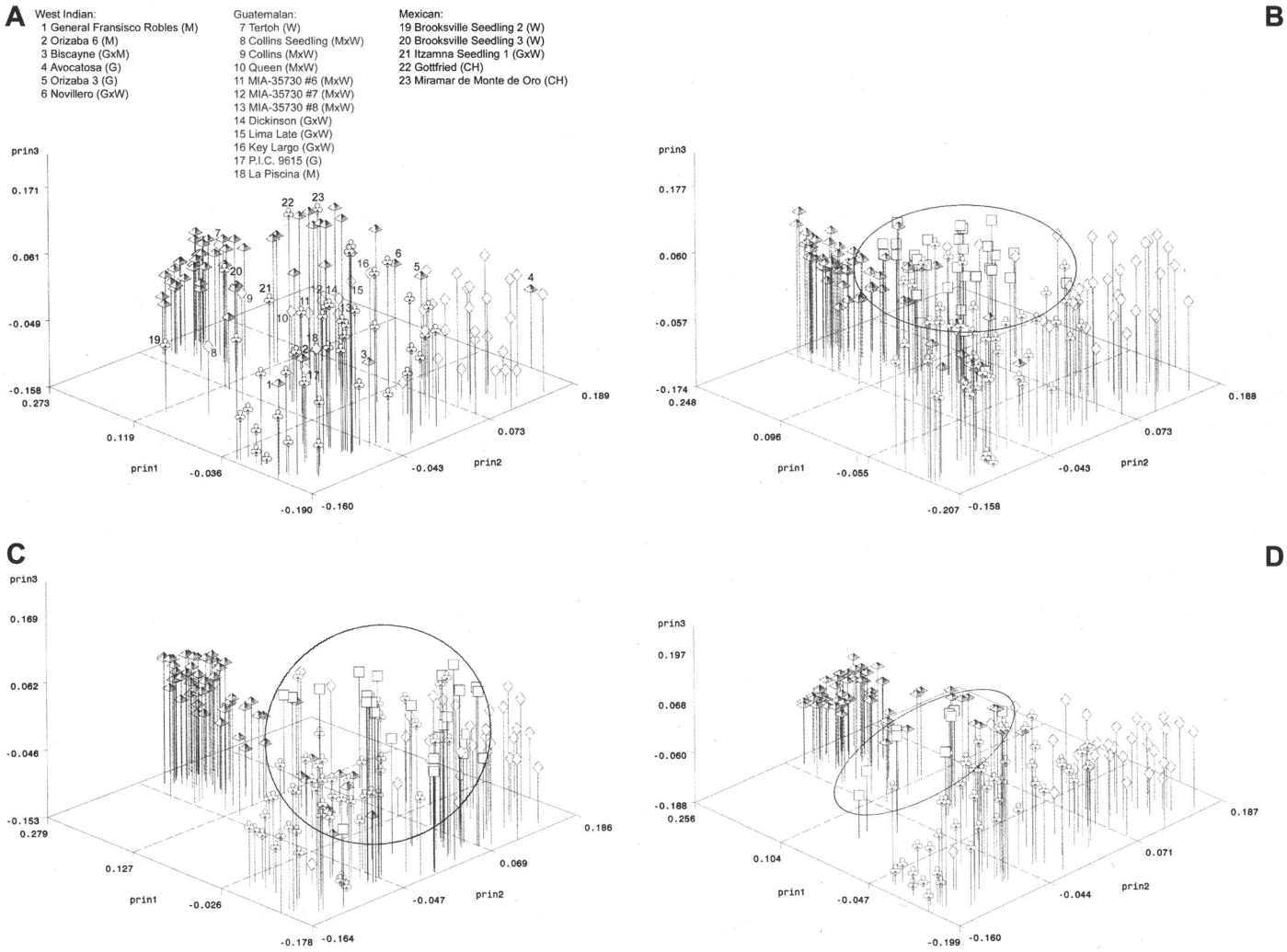

Fig. 2. Principal coordinate analysis (PCA) for the microsatellite evaluation of the avocado germplasm. ♦ Guatemalan accessions, ♣ Mexican accessions, Δ West Indian accessions. (**A**) PCA for the Guatemalan, Mexican, and West Indian avocado accessions based on 14 microsatellite loci and illustrating incorrect population classification within each racial group, correct classification in parenthesis. This plot contains 29.7% of the total variability, 18.3% for the first principal coordinate axis, 6.4% for the second, and 5.0% for the third. Symbols (♦, ♣, Δ) indicate original classification. (**B**) PCA for the Guatemalan, Mexican, West Indian races, and the G x W hybrids. Plot contains 27.9% of the total variability, 17.4% for the first principal coordinate axis, 5.9% for the second, and 4.6% for the third; ❑ = G x W. (**C**) PCA for the Guatemalan, Mexican, West Indian races, and the GxM hybrids. Plot contains 29.5% of the total variability, 20.1% for the first principal coordinate axis, 5.1% for the second, and 3.9% for the third; ❑ = G x M. (**D**) PCA for the Guatemalan, Mexican, West Indian races, and the MxW hybrids. Plot contains 29.9% of the total variability, 20.4% for the first principal coordinate axis, 5.0 % for the second, and 4.0% for the third; ❑ = M x W.

a similar manner, the other interracial hybrid groups were analyzed and changes made based on the PCA. All of the Unknown accessions (15) could be placed in racial or hybrid groups so they were no longer considered as a group for analysis purposes. A total of 50 accessions (19.7%) were changed based on the PCA and these changes are listed in Table 1. All further analysis, including PCA and phylogenetic, was done using this corrected dataset.

PCA for the Guatemalan, Mexican, and West Indian populations and the three interracial groups are illustrated in Fig. 2B–D. The PCA that includes the G x W hybrid grouping accounted for 28% of the total variation and the G x W accessions clustered between the two source populations (Fig. 2B). Likewise, Fig. 2C illustrates the G x M accessions clustering between the two source populations and the PCA accounts for 29.5% of the total variation. Fig. 2D illustrates the M x W accessions again clustering between the two source populations with the PCA accounting for 29.9% of the variation. The PCA for the CH group is not illustrated but these cluster together in a different area that overlaps with the other interracial hybrids.

Significant differences were found for each racial comparison, Guatemalan vs. West Indian, Guatemalan vs. Mexican, and Mexican vs. West Indian for 13 of the 14 loci from the Chi-square analysis. Significant frequency differences were also detected for locus AVAG22 between the Guatemalan vs. West Indian and

Table 4. Chi-square values for tests of similarity of allele frequency by racial background.[z]

Locus	AVAG05	AVAG03	AVAG25	AVAG13	AVAG11	AVMIX03	AVAG21	AVAG07	AVMIX04	AVACO1	AVMIX02	AVAG06	AVAG10	AVAG22
G vs. W	135.83**	84.05**	83.14**	141.18**	97.45**	114.82**	98.33**	76.20**	94.09**	105.42**	151.85**	100.66**	123.13**	20.75*
G vs. M	70.17**	29.12**	32.71**	68.74**	50.34**	68.83**	68.28**	44.15**	81.69**	44.98**	68.24**	45.36**	67.34**	14.42
M vs. W	133.65**	112.53**	120.11**	156.22**	150.15**	144.40**	149.90**	108.77**	155.91**	78.72**	183.45**	144.69**	140.98**	21.47*

[z]G = Guatemalan, M = Mexican, and W = West Indian races.
*,**Signifiant at $P < 0.05$ or 0.01, respectively.

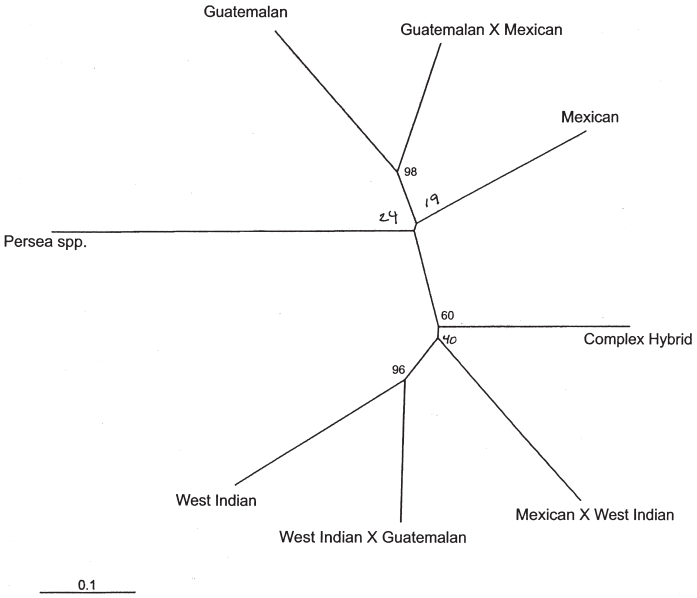

Fig. 3. Neighbor-joining tree of Guatemalan, Mexican, and West Indian races and interracial hybrids based on the Cavalli-Sforza and Edwards chord distance calculated from microsatellite data. Bootstrap percentages were computed using 1000 replications. Bootstrap values below 50% are not shown.

Mexican vs. West Indian but not for the Guatemalan vs. Mexican comparison (Table 4).

Phylogenetic analysis of the microsatellite data for the three populations and the inter-population hybrids was in agreement with the previously reported genetic relationships that separated the Guatemalan, Mexican and West Indian races (Davis et al., 1998; Fiedler et al., 1998; Mhameed et al., 1997). The NJ tree based on Cavalli-Sforza and Edwards (1967) chord distance grouped the three races into distinct clusters. The *Persea* spp. were distinct from any other group. The Guatemalan race and G x M hybrids clustered together with a high bootstrap value and the West Indian and W x G clustered together again with a high bootstrap value, all other groups had little bootstrap support (Fig. 3).

All of the groups had a high average allele number, with the exception of the M x W group that only contained eight individuals (3.57 to 13.35, Table 5). All loci were polymorphic when considered over all groups. H_{nb} was high in the Mexican (0.83) and Guatemalan populations (0.81) and lower for the West Indian group (0.61). The other groups had a narrow range 0.79 to 0.75. H_{obs} did not follow the same pattern being higher in the Guatemalan population (0.75), lower in the Mexican (0.66) and lower still in the West Indian (0.50). The M x W, G x W hybrids and CH had a high H_{obs} ,0.87, 0.79 and 0.78 respectively, while the *Persea* spp. and G x M were similar, 0.70 (Table 5).

With the exception of the M x W hybrids, all other racial groupings contained unique alleles, 27 among the Mexican accessions, 16 in the West Indian group, seven among the Guatemalan accessions, and from one to four among the other groups (data not shown). Of these unique alleles, only three were at a frequency greater than 0.05. Two of these were found in both of the *Persea* spp. accessions (0.33 and 0 .75 due to the existence of a null allele in *P. schiedeana*) and the other in the CH group (0.13). Only one allele in the Guatemalan population had a frequency greater than 0.50, allele 9 for locus AVMIX02 with a frequency of 0.71. Both the Mexican and West Indian populations contained this allele but at low frequencies of 0.17 and 0.04, respectively. The highest frequency in the Mexican population was 0.44 at locus AVAG11 for allele 2 and 0.46 at locus AVMIX02 for allele 2. The West Indian population had alleles with frequencies greater than 0.50 for eight of the loci, AVAG05 allele 6 (0.75), AVAG03 allele 6 (0.53), AVAG11 allele 2 (0.72), AVAG07 allele 3 (0.92) AVACO1 allele 5 (0.58), AVMIX02 allele 3 (0.90), AVAG06 allele 3 (0.51) and AVAG10 allele 1 (0.62). Allele 3 at AVAG07 was common to most of the West Indian accessions, 58 accessions were homozygous for this allele, six were heterozygous leaving only one accession not having this allele. This allele also occurred in the Guatemalan and Mexican populations but at lower frequencies, 0.34 and 0.28, respectively. Allele 3 at AVMIX02 also occurred in a high frequency with 36 accessions homozygous, 18 heterozygous and 11 not containing this allele. The frequency of this allele was low in the Guatemalan population (0.07) and moderate in the Mexican (0.20).

Discussion

Attributes that make microsatellites desirable as molecular markers include their hypervariablity, abundance and automated experimental procedures for detection. By using an automated high throughput CE system, we were able to analyze a large number of individuals for 14 microsatellite loci. The pattern of the PCR products produced by amplification of the genomic DNA was usually simple. It was possible to distinguish the full size amplification products containing the microsatellite from the stutter products and from the +A product. High levels of microsatellite polymorphism have been attributed to two molecular mechanisms, replication slippage and unequal crossing over (Johnson et al., 1992; Messier et al., 1996). Eleven of the 14 microsatellite loci had 15 or more alleles in this study and loci AVAG21, AVAG13, and AVAG10 could be considered hypervariable with 30, 29, and 30 alleles

Table 5. Genetic variation within the three horticultural races and among hybrid populations across 14 microsatellite loci; No.= sample size; $P_{0.95}$ = proportion of polymorphic loci when most frequent allele does not exceed 95%; A = mean number of alleles per locus; H_{nb} = unbiased gene diversity (Nei, 1978); H_{obs} = observed heterozygosity. Standard deviations are indicated in parentheses.

Population	No.	$P_{0.95}$	A	H_{nb}	H_{obs}
Guatemalan	35	1.0	11.29	0.806 (0.110)	0.746 (0.136)
Mexican	51	1.0	13.35	0.830 (0.065)	0.664 (0.163)
West Indian	65	1.0	11.14	0.607 (0.226)	0.496 (0.242)
G x W[z]	32	1.0	9.93	0.770 (0.099)	0.792 (0.102)
G x M[y]	30	1.0	9.86	0.792 (0.062)	0.702 (0.174)
M x W[x]	08	1.0	6.00	0.788 (0.090)	0.866 (0.115)
Complex hybrids	30	1.0	8.79	0.752 (0.109)	0.779 (0.144)
Persea spp.	03	1.0	3.57	0.793 (0.134)	0.702 (0.294)

[z]Listed in germplasm records at Guatemalan x West Indian Hybrids.
[y]Listed as Guatemalan x Mexican hybrids.
[x]Listed as Mexican x West Indian hybrids.

detected, respectively (Table 2). When the entire 264 accessions were considered as a single population only 41 of the 256 alleles had a frequency >0.10. Only one allele had a frequency >0.50 and this was at locus AVAG07. High levels of polymorphism have been reported in other plant species. Saghai Maroof et al. (1994) detected 28 and 37 alleles at loci HVM3 and HVM4, respectively, in barley (*Hordeum vulgare*) and Dow et al. (1995) found high levels of polymorphism with the number of alleles at each locus ranging from 11 to 20 in wild populations of bur oak (*Quercus macrocarpa*).

The initial classification of the accessions by racial or hybrid group was based on the USDA records maintained at the NGR-Miami and on the national germplasm database system, GRIN. Six of the West Indian accessions, 12 of the Guatemalan, and five of the Mexican accessions were of questionable characterization based on the molecular data. Estragole is a compound known to be produced in the Mexican race and in interracial hybrids involving Mexican parents. The compound imparts an anise-like scent in the leaves and the skin of the fruit (King and Knight, 1987). As a verification of the molecular data, the four accessions, two listed as West Indian and two listed as Guatemalan, that clustered with the Mexican group were found to contain estragole in the leaves. The accession 'Key Largo' was classified as Guatemalan but clustered at the interface of the Mexican and West Indian accessions and also contained estragole in its leaves. In contrast, the accession 'P.I.C. 9615' clustered within the Mexican accessions, yet the leaves do not contain estragole. It was classified as being of the Guatemalan race when collected and this classification was not changed (Ben-Ya'acov, 1995)

Most of the differences between fragment sizes found in the comparison of 'Ettinger' and 'Pinkerton' in this study versus that of Sharon et al. (1997) can be attributed to the increased sensitivity of the CE procedure as opposed to the use of polyacrylamide gels. Using CE we can detect differences of a single base pair in length for microsatellite fragments and the size calling is based on an internal standard placed into each sample, again giving increased confidence in accuracy of the size determination. The inability to generate amplification products for 'Pinkerton' with primers for locus AVAC01 indicates the existence of a null allele for this accession, suggesting that it is genetically different from the 'Pinkerton' accession studied by Sharon et al. (1997). We consistently detected much larger amplification products at AVAG10 (149 to 234) than were detected by Sharon et al. (1997). We are currently sequencing the alleles from AVAG10 to determine if we are amplifying the same dinucleotide repeat. Further differences between fragment sizes found in the comparison of 'Ettinger' and 'Pinkerton' in this study versus that of Sharon et al. (1997) are being investigated for other loci as well. Additionally under investigation is the basis of the existence of alleles that do not differ in size by either two bases or multiples of two bases for dinucleotide repeat microsatellites.

The Mexican, West Indian, and Guatemalan races did contain many unique alleles; however, they were all at low frequency and were not useful for predicting the horticultural race. This is in contrast to the RAPD analysis where race-specific markers were identified (Fiedler et al., 1998) and the VNTR analysis using DNA fingerprinting reported by Mhameed et al. (1997). In both of those studies a relatively small number of individuals were examined, 16 accessions in the RAPD study and 24 accessions in the VNTR study. We examined 254 accessions and did not find any useful race-specific markers and while many unique alleles did exist, the low frequency restricted their usefulness as race-specific markers. The West Indian race does contain alleles at two loci that have frequencies greater than 90%, AVAGG07 allele 3 and AVMIX02 allele 3. The absence of both of these alleles in an accession would indicate that the accession is not in the West Indian race.

Mhameed et al. (1996) found high levels of H_{obs}, 98%, among avocado cultivars using VNTR; however using microsatellite markers the H_{obs} was found to be 62%. In this study, the average H_{obs} for the Guatemalan, Mexican, and West Indian races was 0.64 or very similar to the observations of Mhameed et al. (1996). In their study overall H_{nb} was 0.56 which is lower than that found in this study where the average H_{nb} was 0.75 for the Guatemalan, Mexican, and West Indian races. H_{nb} was highest in Mexican and Guatemalan races and considerably lower in the West Indian race. The results indicate that avocado accessions of Mexican and Guatemalan races are highly heterozygous and heterogenous while those of the West Indian race are more homozygous and homogenous (Table 5). Davis et al. (1998) concluded that the West Indian and Guatemalan races were more similar to each other than either is to the Mexican race based on RFLP data. Our data suggest that each of the races is distinct and based on the Chi-square analysis significantly different from one another for all of the 14 loci surveyed. This is similar to the analysis by Fiedler et al. (1998) using RAPDs where the similarities delimited three groups of equal rank.

The selection of PRR-tolerant rootstocks has been reported and the tolerance has been identified in progeny of the West Indian race (Zilberstaine et al., 1995). Fourteen of the accessions evaluated in this study have been found to impart tolerance to PRR based on screening of maternal half-sib families (Ploetz et al., 2002). No obvious relationship became apparent within the PCA as they are scattered throughout the West Indian cluster and hence it is not likely that any of these microsatellites are associated with PRR resistance. H_{nb} was low among the West Indian population (0.61) indicating that this collection of accessions is less diverse than the Guatemalan or Mexican accessions. Since PRR is such an important problem in avocado production areas, an increased sampling effort among the West Indian types is warranted. We are currently developing a project with the Centro Agronómico Tropical de Investigación y Enseñanza (CATIE) in Costa Rica to collect new West Indian accessions in selected areas of Central America.

The results presented in this study demonstrate a basic agreement between the horticultural race and the phylogenetic clusters originating from the molecular analyses. The use of the molecular data has allowed a more informed classification of avocado germplasm and will allow genetic verification of newly propagated accessions when adding them to the germplasm collection. The accessions that have been designated as possible errors or difficult to classify racial types have been entered into the GRIN system with a notation indicating that the racial designations of these accessions may need modification. Further phenotypic evaluation of the avocado germplasm collection is needed for comparison with the molecular data. This analysis demonstrates the usefulness of molecular data in the management of plant genetic resources.

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