Proceedings VI World Avocado Congress (Actas VI Congreso Mundial del Aguacate) 2007.
Viña Del Mar, Chile. 12 – 16 Nov. 2007. ISBN No 978-956-17-0413-8.

GENETIC RELATIONSHIP ESTIMATION OF TAIWAN AVOCADO CULTIVARS BY VOLATILE CONSTITUENTS OF LEAVES

H. Wu[1], C. Chou[1,2], T-L. Chang[1] and I-Z. Chen[1]

[1] Department of Horticulture, National Taiwan University. Taipei, Taiwan, R.O.C.

email: chenyo@ntu.edu.tw, fax: 886-2-23620760

[2] Sinetics International Co., Ltd. Taipei, Taiwan, R.O.C.

Volatile constituents of leave samples were analyzed to distinguish the genetic relationship among 46 Avocado (*Persea americana* Mill.) cultivars (28 from Taiwan and 18 from El Salvador). A solid phase microextraction (SPME) device was used to extract the volatile constituents from avocado leaves in heated headspace vial, and injected into GC-MS directly. The intensity of volatile aroma of avocado leaf in Mexican races was higher than Guatemalan and West-Indian races. Estragole and β-caryophyllene are the most abundant compounds in Mexican and Guatemalan races respectively, and can be used as indicators in races classification. The qualitative and quantitative results of GC-MS analysis were analyzed by principle component analysis (PCA) and cluster analysis to estimate the variation of individual cultivar races of avocado.

Key words: Headspace solid phase microextraction (HS-SPME), GC-MS, Chemotaxonomy

ESTIMACIÓN DE LA RELACIÓN GENÉTICA DE CULTIVARES DE AGUACATE DE TAIWÁN POR LOS COMPONENTES VOLÁTILES DE LAS HOJAS

H. Wu1, C. Chou1,2, T-L. Chang1 y I-Z. Chen1

1 Department of Horticulture, National Taiwan University. Taipei, Taiwan, R.O.C.

email: chenyo@ntu.edu.tw, fax: 886-2-23620760

2 Sinetics International Co., Ltd. Taipei, Taiwan, R.O.C.

Los componentes volátiles de las muestras de hojas fueron analizados para distinguir la relación genética entre 46 cultivares (28 de Taiwán y 18 de El Salvador) de aguacate (Persea americana Mill.). Un dispositivo de microextracción en fase sólida (MEFS o SPME en inglés) fue utilizado para extraer los componentes volátiles de las hojas de aguacate en viales con headspace calentados y directamente inyectado en el GC-MS. La intensidad del aroma volátil de hojas del aguacate fue más alto en las razas mexicanas que en las razas guatemaltecas y antillanas. Estragol y β-cariofileno son los componentes más abundantes en las razas mexicanas y guatemaltecas respectivamente, y pueden ser indicadores en la clasificación de raza. Los resultados cualitativos y cuantitativos del análisis de GC-MS fueron analizados mediante el Análisis del Componente Principal (ACP o PCA en inglés) y análisis de conglomerados para estimar la variación de las razas de cultivares individuales de aguacate.

Palabras claves: Headspace micro extracción en fase sólida (MEFS o SPME en inglés), GC-MS, Quimitaxonomía

1. Introduction

Avocado (*Persea americana* Mill.) is in the genus *Persea* of the Lauraceae. Three horticultural or ecological races of avocado have conventionally been identified, including Mexican, Guatemalan, and West Indian races. The origin of avocado was in Latin America, and three races found in different altitude. West Indian race thrived from sea level to about 1000 m, Guatemalan race from about 1000 to 2000 m, and Mexican race from about 1500 to 3000m (Popenoe, 1952). The different climate adaptation and ecological conditions caused these races could be distinguished by morphological, physiological, and horticultural traits (Bergh, 1992). However, it is seemed to be no sterility barrier among races, and probably presented a natural distribution of races overlap (Bergh, 1969).

Rhodes *et al*. (1971) used 67 morphological characters, such as fruit skin and texture, seed integument, and leaf scent etc., as the indicator of a numerical taxonomy of avocado. Root characters were different between Guatemalan and West Indian races (Borys *et al*.,1985). Avocado leaves of Mexican race had apparent anise-like scent compared with other two races (Bergh, 1969).

Several different DNA markers had been applied to estimate genetic relationship. Restriction fragment length polymorphism (RFLP) was used to evaluate genetic relationship among subgenus *Persea* (Furnier *et al.,* 1990). Randomly amplified polymorphic DNA (RAPD) was used to estimate the rate of outcrossing and diversity of germplasm (Fiedler *et al.,* 1998; Kobayashi *et al.,* 2000). Minisatellite (Lavi *et al.,* 1991), and microsatellite (Ashworth and Clegg, 2003; Ashworth *et al.,* 2004; Mhameed *et al.,* 1996; Schnell *et al.,* 2003) were also used for the same purposes above.

Headspace-solid phase microextraction (HS-SPME) GC-MS has been widely used for flavor and fragrance analysis(Li *et al.,* 2003), the lack of availability of commercial fragrance standards and the absorption based equilibrium nature made HS-SPME GC-MS reproducibility and quantization difficult. The stable isotope dilution GC-MS using deuterium internal standard has been widely used in US EPA method for semi-volatile organic compounds (SVOC) quantitation (US EPA method 8270C). These stable isotope labeled compounds can be used as the internal standards for calibrated quantitation fragrance analysis in Avocado cultivars leaves (Wu, 2007)

Avocado cultivars were introduced into Taiwan since 1918. The climate of Taiwan was not suitable for the introduced cultivars. Some local cultivars were selected from hybrids among introduced cultivars, but there were little records about the breeding history of Taiwan local cultivars. Avocado leaves have known to have strong fragrance such as anise-like scent. In this study, volatile constituent of avocado leaves were analyzed by a stable isotopes internal standard based headspace-solid phase microextraction (HS-SPME) coupled to GC-MS, and the analytical data of GC-MS were used for numerical taxonomy of avocado samples.

2. Materials and Methods

Regent and Standards*:* HPLC grade methanol was purchased from Merck (Germany). Internal standard, 1,4-dichlorobenzene-d_4 and naphthalene-d_8 in EPA Method 8270 Internal standards mixture (AccuStandard, USA), were used for relative quantitation. Estragole (Riedel-de Haën, Germany), trans-anethole (Fluka, Germany), β-pinene, methyl eugenol, β-caryophyllene, and phenylacetaldehyde (Sigma-Aldrich, Germany) were used as standards for compound identification.

Samples*:* Mature avocado leaves were collected from El Salvador and Taiwan. Twenty-six Taiwan avocado leaf samples, freezed at - 20 ^{o}C before analysis, were collected from Chiayi Agricultural Experimental Station (CAES) and Shen's orchard. Fourteen silica-gel dehydrated leaf samples were supplied by

Germplasm Reserves of Institute of Agricultural Experiments of El Salvador. The summary of cultivar name and source of avocado leaves for volatile constituents analysis in this study is listed in Table one.

Equipment: Agilent 5890 GC-5972 MSD (Agilent, USA) was operated in electron-impact ionization mode at 70 eV. The column was HP-5-MS (30 m × 0.25 mm internal diameter, 1 μm film thickness, Aglient, USA). Solid phase microextraction (SPME) fiber was 50/30 μm DVB/PDMS (Supelco, USA).

Sample preparation: Avocado leaf discs were placed into headspace vial (25 mL) with the injection of 1μL internal standard solution (400 μg/mL). Vial was heated at 75 ^{o}C for 20 min, and volatile compounds of leaves were trapped on SPME fiber for 5 min. SPME fiber was then direct thermal desorption in GC injection port.

Chromatographic condition: Oven temperature program was started at 35 ^{o}C for 5 min, 5 ^{o}C/min to 160 ^{o}C, maintain 2 min, 40 ^{o}C/min to 280 ^{o}C, maintain 1 min. Splitless injection mode was selected. Injection port temperature was set at 280 ^{o}C, and transfer line was set at 280 ^{o}C. Helium (99.9995 %) was the carrier gas with a flow rate of 1 mL/min.

Data analysis: The data spectrum were identified by Wiley275 (Wiley, USA) and NIST05 (NIST, USA) standard mass spectrum libraries. Calibrated abundance was calculated from the abundance divided by the abundance of 'Duke 7'. Similarity matrix was calculated by Euclidean distance with the converted base-10 logarithm values of calibrated abundance, and calculated by using R 2.4.1 (Statistics dept. U. Auckland), and clustered by unweighted pair-group method using arithmetic average (UPGMA).

3. <u>Results and Discussion</u>

'Duke7' is commonly used as the rootstock with strong anise-like scent of leaves. Calibrated abundance was calculated from the abundance divided by the abundance of 'Duke7'.

$$\text{Calibrated abundance} = \text{abundance} \times \frac{A_{IS}}{A_{IS'Duke7'}} \times \frac{S}{S_{'Duke7'}}$$

A_{IS} – internal standard abundance

$A_{IS'Duke7'}$ – internal standard abundance of 'Duke7'

S – leaf disc area in headspace vial

$S_{'Duke7'}$ –leaf disc area of ‘Duke7’

Not all the target compounds could be recognized clearly by library search results. Sesquiterpene I (retention time = 23.55), sesquiterpene II (R.T. = 24.97), sesquiterpene III (R.T. = 26.26), sesquiterpene IV (R.T. = 27.44) and sesquiterpene V (R.T. = 28.18) could be only recognized as sesquiterpenes ($C_{15}H_{24}$, MW = 204) with their molecular ion. The forms of these sesquiterpenoids could be α-cubebene, tetradecane, δ-cadinene and ledene. The standard of the compounds list above are not available commercially. In this study, these compounds were named sesquiterpene I, II, III, IV, and V, respectively.

The volatile constituent patterns of the cultivar within the same ecological races were similar (Figure 1, 2, and 3). Two major characteristics of fragrance among the cultivars of three ecological races could be recognized, which are race-specific compounds and abundance-varied characteristic compounds. Twelve race-specific compounds and ten characteristic compounds (Table 2) were found among all testing data of pure race samples. Race-specific compounds could only be found in one or two ecological races. For instance, hexanal and phenylacetaldehyde could found only in West Indian race, chavicol and methyl eugenol could found only in Mexian race, and sesquiterpene I and caryophyllene could found only in Guatemalan race.

Abundance-varied characteristic compounds can be found in all three ecological races, but their calibrated abundance varied. The abundance of estragole in Mexican race samples were 10^3 to 10^4 times greater then other two race samples. All the other abundance-varied characteristic compounds had similar behavior as estragole in Mexican race samples. Without proper conversion of calibrated abundance, the difference between Guatemalan and West Indian race samples could be neglected in comparison with Mexican race samples. Base-10 logarithm was chosen as the numerical conversion method of calibrated abundance for further analysis. Similarity matrix was calculated by Euclidean distance with the converted base-10 logarithm values of calibrated abundance, and clustered by UPGMA and generated dendrogram by R 2.4.1 software. The dendrogram of all testing sample data generated by UPGMA showed in Figure 4.

Avocado samples were separated into three major groups in according with three ecological races on the dendrogram of volatile constituents test results. Most of the Taiwan cultivars and pure West Indian race samples from Taiwan were clustered in the group A. Samples from El Salvador and pure Guatemalan race from Taiwan were clustered in the group B, and Samples pure Mexian race from Taiwan were clustered in the group C.

With the benefits of easy operation, fast analysis, good repeatability and reproducibility, volatile constituent analysis of leaves could be used as a morphological taxonomy method for avocado. Calibrated abundance by internal standards of testing data of GC-MS could be merged into database, and generated a new dendrogram with the all data in the database. Experimental results proved that this is a feasible method for genetic relationship identification.

Acknowledgement

The authors would like to appreciate the assistance on sampling of Mr. T-M. Jong, Mr. C-D. Shan in Taiwan, and Mr. K-L. Huang in El Salvador.

4. References

Ashworth, V. E. T. M., Clegg, M. T. 2003. Microsatellite markers in avocado (*Persea americana* Mill.): genealogical relationships among cultivated avocado genotypes. J. Hered. 94: 407-415.

Ashworth, V. E. T. M., Kobyashi, M. C., de la Cruz, M., Clegg, M. T. 2004. Microsatellite markers in avocado (*Persea americana* Mill.): development of dinucleotide and trinucleotide markers. Sci. Hortic. 101: 255-267.

Bergh, B. 1992. The Origin, nature, and genetic improvement of the avocado. California Avocado Society Yearbook 76: 61-75.

Bergh, B. O. 1969. Avocado (*Persea americana* Miller). pp. 23-51 in F. P. Ferwerda and F. Wit [ed.], Outlines of perennial crop breeding in the tropics. Landbouwhogeschool, Wageningen, Netherlands, Misc. Paper 4.

Borys, M. W., Barrera, Guerra, J. L., Luna-Lopez, J. 1985. Some root characteristics of avocado (*Persea americana* Mill.) seedlings of the West Indian and Guatemalan races. California Avocado Society Yearbook 69: 111-122.

Fiedler, J., Bufler, G., Bangerth, F. 1998. Genetic relationships of avocado (*Persea americana* Mill.) using RAPD markers. Euphytica 101: 249-255.

Furnier, G. R., Cummings, M. P., Clegg, M. T. 1990. Evolution of the avocados as revealed by DNA restriction fragment variation. J. Hered. 81: 183-188.

Lavi, U., Hillel, J., Vainstein, A., Lahav, E., Sharon, D. 1991. Application of DNA fingerprints for identification and genetic analysis of avocado. J. Amer. Soc. Hort. Sci. 116(6): 1078-1081.

Li, H. P., Li, G. C. and Jen, J. F. 2003. Determination of organochlorine pesticides in water using microwave-assisted headspace solid-phase microextraction and gas chromatography. J. Chroma. A. 1012: 129–137.

Mhameed, S., Sharon, D., Hillel, J., Lahav, E., Kaufman, D., Lavi, U. 1996. Level of heterozygosity and mode of inheritance of variable number of tandem repeat loci in avocado. J. Amer. Soc. Hort. Sci. 121(5): 778-782.

Popenoe, W. 1952. Central American fruit culture. Ceiba 1: 225-227.

Rhodes, A. M., Malo, S. E., Campbell, C. W., Carmer, S. G. 1971. A numerical taxonomic study of the avocado (*Persea americana* Mill.). J. Amer. Soc. Hort. Sci. 96: 391-395.

Schnell, R. J., Brown, J. S., Olano, C. T., Power, E. J., Krol, C. A., Kuhn, D. N. , Motamayor, J. C. 2003. Evaluation of avocado germplasm using microsatellite markers. J. Amer. Soc. Hort. Sci. 128: 881-889.

USEPA, 1995. SW-846, Method 8270C, Semi-volatile organic compounds (SVOC) by gas chromatography-mass spectrometry (GC-MS): capillary column technique.

Wu, H., Chou, C., Chen, K., Chang, T-L., Chen, I-Z. 2007. Avocado (*Persea americana* Mill.) genetic relationship identification by using leaf volatile constituents as indicators. J. Taiwan Soc. Hort. Sci. (in press)

Table 1. Cultivar name and source of avocado leaves for volatile constituents analysis in this study.

Source	Cultivar name
CAES, Taiwan (17 samples)	‘76-s-6’, ‘79-6-5-3’, ‘Bacon’, ‘CAES1’, ‘CAES2’, ‘CAES3’, ‘CAES4’, ‘Duke 7’, ‘Esther’, ‘Hong-ci-chao’(C), ‘Hong-sin-yuan’(C), ‘Horshim’, ‘Jhang-an’(C), ‘Stewart’, ‘Toro Canyon’, ‘WS1Q5’, ‘Zutano’
Shen’s, Taiwan (8 samples)	‘Chang-jhong’, ‘Hong-ci-chao’(S), ‘Hong-sin-yuan’(S), ‘Jhang-an’(S), ‘O24D’, ‘Pollock’-1, ‘Pollock’-2, ‘Pollock’-3
El Salvador (14 samples)	‘Ahuachapán’, ‘Béneke’, ‘Cordero 2’, ‘Ereguayquín 1’, ‘Ereguayquín 3’, ‘Guirola’, ‘Juguete’, ‘Letona Morado’, ‘Lima’ , ‘Lorenzana’, ‘Mercedes’, ‘Nejapa’, ‘San Benito’, ‘Sitio del Niño 3’

Table 2. Average and standard deviation of calibrated abundance of race-specific compounds in three avocado races.

Race-specific compound	Mexican race	Guatemalan race	West Indian race
hexanal	ND	ND	34424 ± 15470
β-myrcene	ND	11504 ± 9574	12199 ± 7494
phenylacetaldehyde	ND	ND	5434 ± 2397
γ-terpinene	ND	6973 ± 7489	4343 ± 3283
nonyl aldehyde	ND	8444 ± 5708	4304 ± 1018
chavicol	324875*	ND	ND
trans-anethole	1092508 ± 551347	ND	1288*
sesquiterpene I	ND	4198 ± 3049	3303*
methyl eugenol	4812841*	ND	ND
caryophyllene isomer	ND	ND	7113 ± 5200
sesquiterpene III	ND	5209 ± 6001	3171*
sesquiterpene IV	191043 ± 15453	14796 ± 23858	ND

ND = not detected

* compound found only in one or two samples

Table 3. Average and standard deviation of calibrated abundance of characteristic compounds in three avocado races.

Characteristic compound	Mexican race			Guatemalan race			West Indian race		
trans-2-hexenal	788772	±	665659	27446*			98962	±	28551
α-pinene	259050	±	182858	21191	±	16465	12912	±	6341
β-pinene	593495	±	322313	46360	±	55905	63490	±	37232
limonene + eucalyptol	2415054	±	1361622	72576	±	32246	253615	±	98102
estragole	30420012	±	27968424	18428	±	25136	4426	±	3876
α-copaene	489744	±	343566	13553	±	26790	17970	±	16470
sesquiterpene II	280289	±	130629	9081	±	4744	5339	±	3539
caryophyllene	3402598	±	2976023	116097	±	165022	77369	±	74865
α-humulene	359975	±	281218	15317	±	18766	9451	±	11934
sesquiterpene V	399511	±	273064	22672	±	26049	14453	±	14796

* compound found only in one or two samples

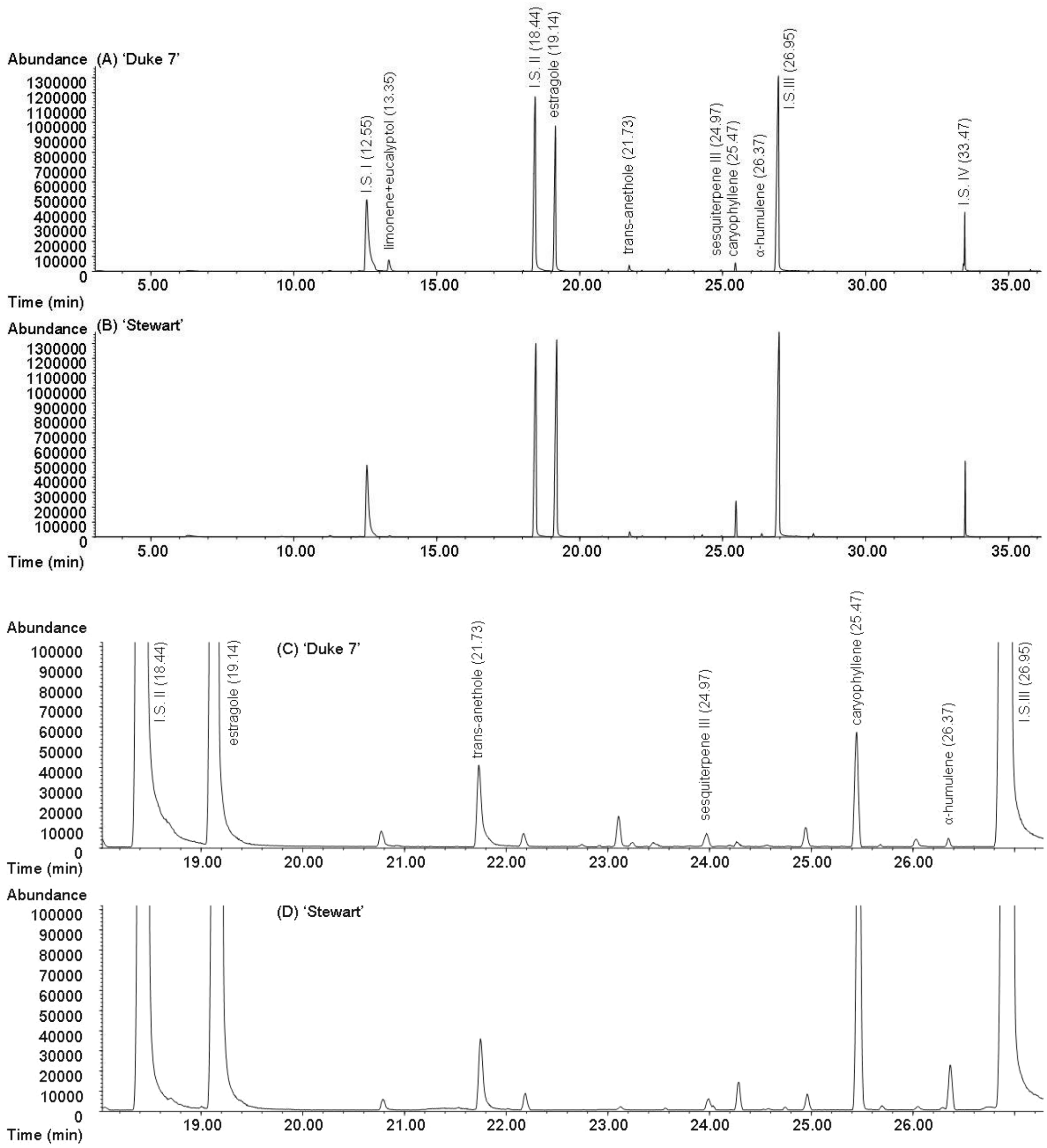

Figure 1. Total ion chromatogram and indicator compounds of avocado Mexican race samples. (A) 'Duke 7', (B) 'Stewart'; enlarged chromatograms (from 18 min to 27 min) of (A) and (B) showed in (C) and (D).

I.S. = internal standard, only I and II were used as internal standard. I - 1,4-dichlorobenzene-d_4, II - naphthalene-d_8, III - acenaphthene-d_{10}, IV - phenanthrene-d_{10}

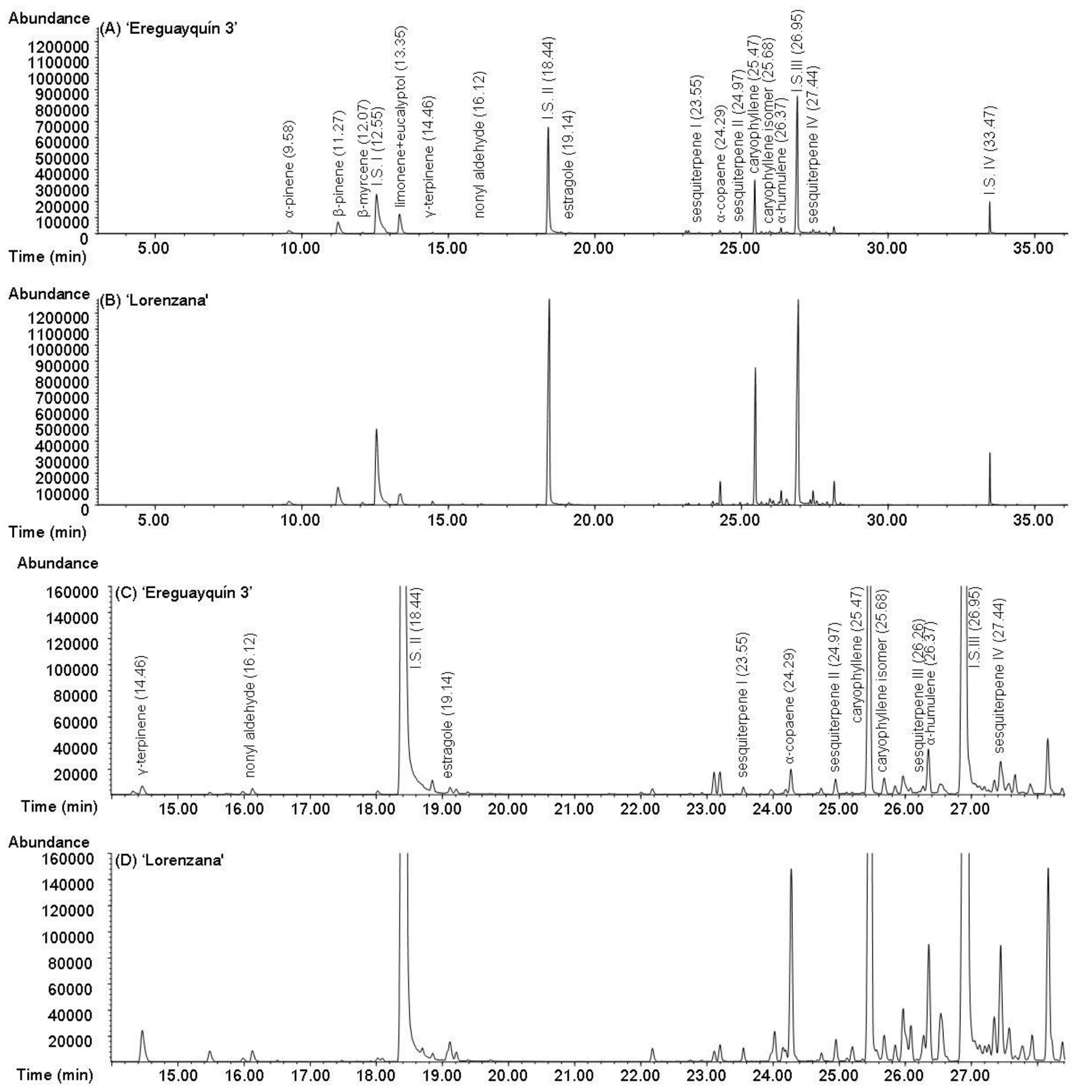

Figure 2. Total ion chromatogram and indicator compounds of avocado Guatemalan race samples. (A) 'Ereguayquín 3' ; (B) 'Lorenzana'; enlarged chromatograms (from 14 min to 28 min) of (A) and (B) showed in (C) and (D). I.S. = internal standard, only I and II were used as internal standard. I - 1,4-dichlorobenzene-d_4, II - naphthalene-d_8, III - acenaphthene-d_{10}, IV - phenanthrene-d_{10}

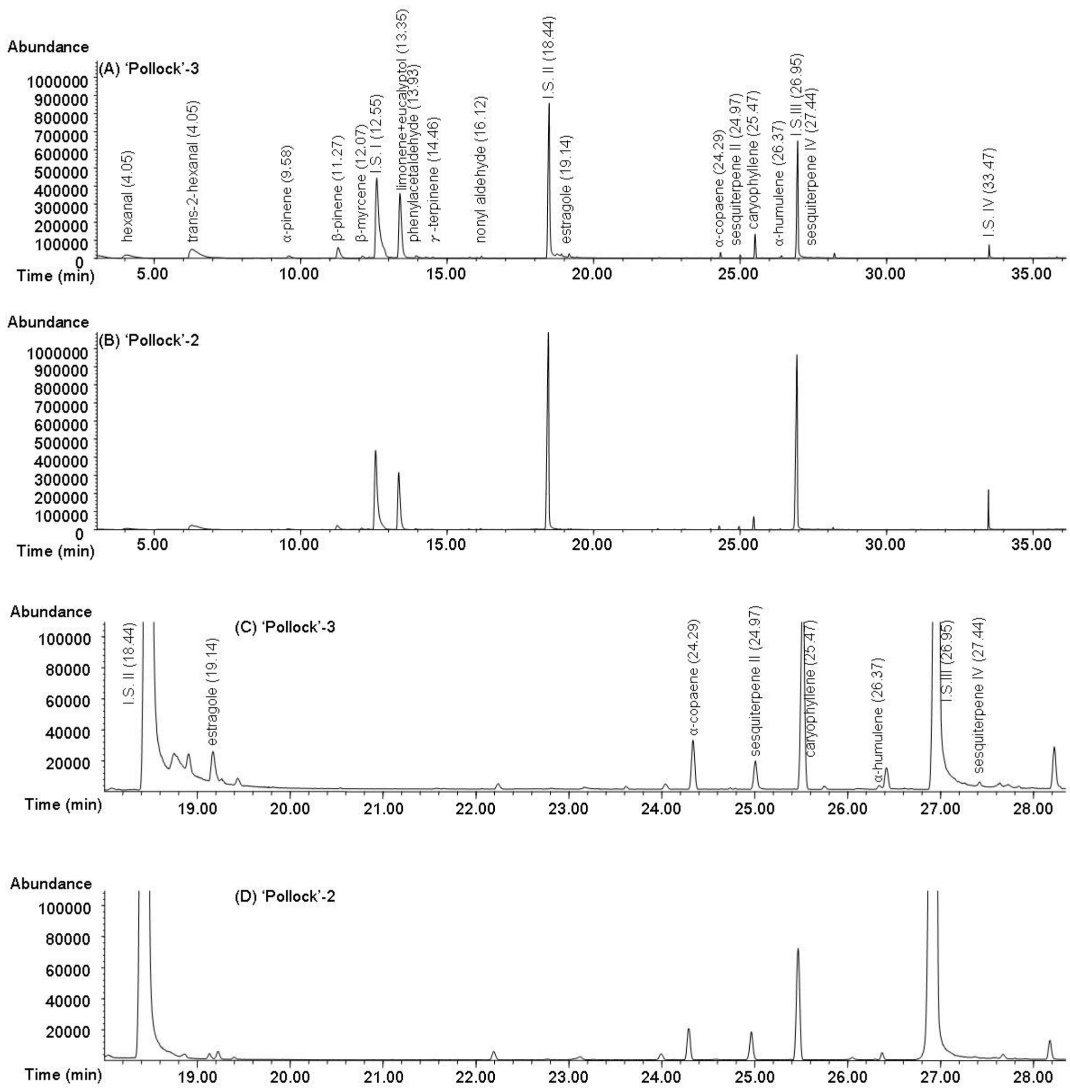

Figure 3. Total ion chromatogram and indicator compounds of avocado Mexican race samples. (A) 'Pollock'-3 ; (B) 'Pollock'-2; enlarged chromatograms (from 18 min to 29 min) of (A) and (B) showed in (C) and (D).

I.S. = internal standard, only I and II were used as internal standard. I - 1,4-dichlorobenzene-d_4, II - naphthalene-d_8, III - acenaphthene-d_{10}, IV - phenanthrene-d_{10}

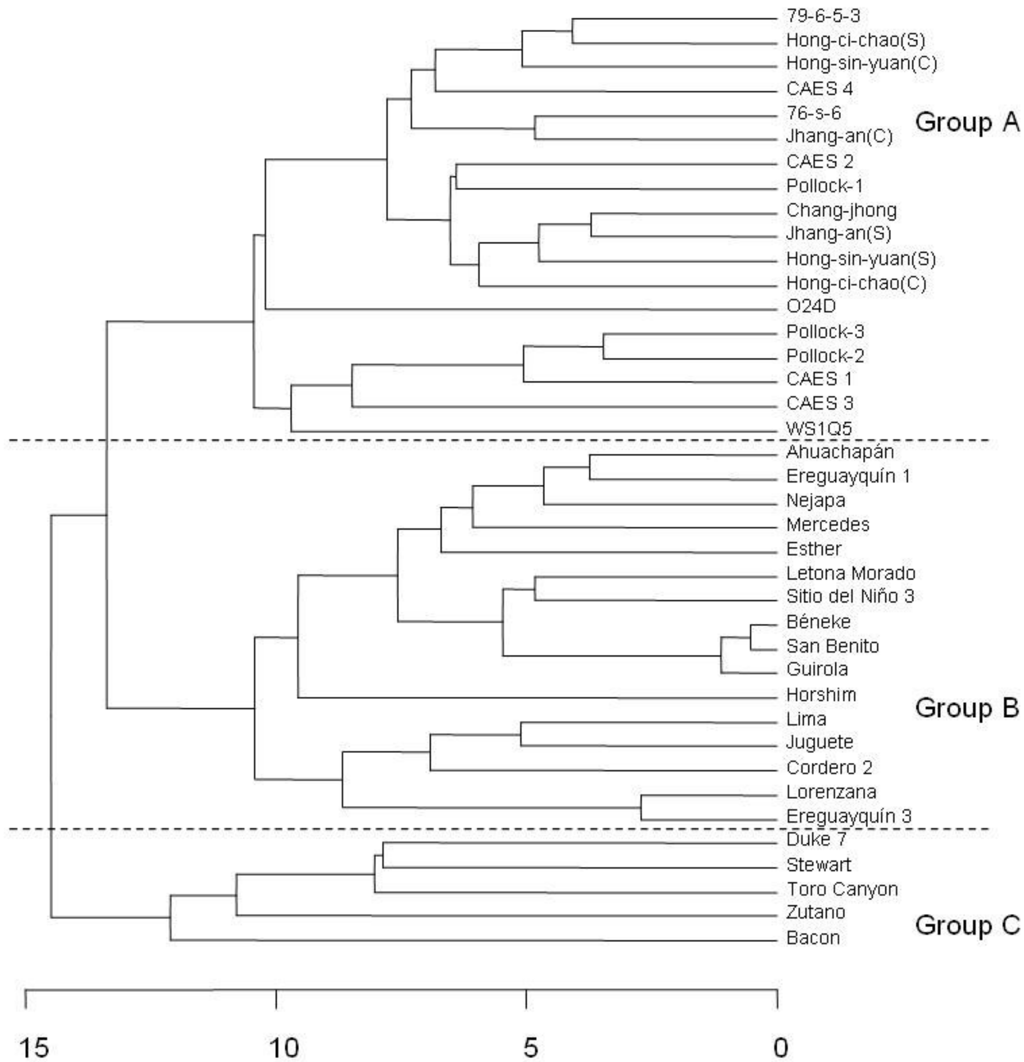

Figure 4. Dendrogram of 39 avocado samples (24 pure race samples and 15 Taiwan cultivar samples) derived from analysis using indicator compounds based on distances obtained from the similarity matrix (Euclidean distance) of calibrated abundance in logarithm and clustered by unweighted pair group method arithmetic average.