The comparison of persimmon genotypes (*Diospyros kaki* Thunb.) by using RAPD and FAME data

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Abstract

In the current study, FAME (Fatty acid methyl esters) and RAPD profiles were used to examine biochemical and genetic relationships between 31 selected persimmon genotypes sampled from Persimmon Repository, Black Sea Agricultural Research Institute, Samsun, Turkey. Fatty acid composition of persimmon leaves was determined by using gas chromatography. The dendrogram realized from the RAPD markers grouped the 31 genotypes into three major clusters. Cluster 1 included 8, cluster 2 included 14 and cluster 3 included 9 genotypes. The fatty acid results showed that there were differences among persimmon genotypes both percent and presence of fatty acids in leaves. Some relationships between RAPD and FAME data were found. The linolenic acid was detected only in genotypes in cluster 3. Behenic acid was detected only in genotypes within cluster 1. According to these results, it can be concluded that the use of RAPD and FAME data could be useful indicator for characterization and grouping of persimmon genotypes.

Keywords: Biochemical Markers, Fatty Acids, Persimmon, RAPD

Introduction

The persimmon fruit is of Japanese origin and commonly grows in the warm regions of the world. There are four species of Diospyros, namely, *Diospyros kaki*, *Diospyros virginiana*, *Diospyros oleifera* and *Diospyros lotus* out of which *Diospyros kaki* (Japanese persimmon) is the most important species from commercial point of view [1]. Persimmon (*Diospyros kaki*) is an important fruit in Japan, China and Korea and is also gaining popularity in the Mediterranean countries including Turkey. It is normally marketed as fresh fruit, but processing of this fruit is of great interest due to its important biologically active compounds [2].

The demand to persimmon fruits in the last decade in Turkey increased and resulting production increase in the country [3]. It has been becoming an alternative fruit for fruit growers in Turkey and the country have important persimmon genetic resources mostly from *Diospyros kaki* and *Diospyros lotus*. However, it has yet to be registered any persimmon cultivars in Turkey [4].

Characterization of germplasm is essential to identify individual genotypes and the also extent of variability existing among the accessions. Characterization is also a process in which characters are subjected to systematic data recording and analyses which finally helps in elucidating the genetic and biochemical diversity among the accessions. The comprehensive information obtained from such an exercise would help the breeder, geneticist and conservationist to effective utilize the valuable genetic resources [5].

Recent advances in the field of plant science are creating exciting possibilities for the rapid and accurate determination of biochemical and genetic variations between plant species and cultivars. Fatty acid methyl esters (FAME) analysis and nucleic acid based techniques, such as RAPD (Random Amplified Polymorphic DNA), have been utilized for determination of relationship within and among plant species in addition to morphological characters since 1990s [6,7]. Earlier classifications and evaluations of the persimmon were done primarily based on phenotypic expressions of the plants such as growth form, leaf morphology and fruit properties [4,8,9]. But information from these environmentally influenced morphological characteristics is not sufficient to identify persimmon genotypes because the differences between them are often subtle and misleading. Hence, robust and environmentally little influenced genotypic traits are to be used for proper identification and estimation of genetic diversity among these genotypes.

There has been no basic information on the genome and also no chemotaxonomic evaluation of persimmon genotypes present in Turkey is available and FAME and RAPD technique may provide a rapid means of assessment diversity among closely related genotypes [10], we have selected the same in our study to assess the diversity among the 31 selected persimmon genotypes. This data provides a scientific basis for future selection and management of germplasm.

Materials and methods

Sample collection

The leaves sampled from 31 selected persimmon genotypes (*Diospyrus kaki* L.) found Persimmon Repository, Black Sea Agricultural Research Institute, Samsun, Turkey. The selected genotypes were named as BSP 1 to 31 (BSP: Black Sea Persimmon) and maintained under similar cultural practices at Black Sea Agricultural Research Institute. Twenty leaves from the top of 90 day old primary branches of five clonally propagated plants per genotypes were collected separately and stored immediately at –80°C for DNA extraction.

Determination of fatty acids in leaves

Fatty acid composition was analyzed according to a previous method and fatty acids were separated by gas chromatography (HP6890, Hewlett Packard, Palo Alto, CA) with a fused-silica capillary column (25 m by 0.2 mm) with cross-linked 5% phenyl methyl silicone [11].

DNA extraction

Genomic DNA was extracted from powdered plant materials using frozen nitrogen described by Lin et al. [12].

RAPDs

Samples were screened for RAPD variation using standard 10-base primers supplied by Operon. Thirty µl of reaction cocktail was prepared as follows: 10x Buffer 3.0 µl, dNTPs (10mM) 1.2 µl, magnesium chloride (25mM) 1.2 µl, primer (5µM) 2.0 µl, *Taq* polymerase (5unit) 0.4 µl, water 19.2 µl sample DNA 3.0 µl (100ng/ µl). Total 60 RAPD primers were tested in this study and among them 9 primers producing reproducible banding patterns were selected. Therefore the results are based on 9 primers. The thermalcycler (Eppendorf Company) was programmed as 2 min at 95°C; 2cycles of 30 sec at 95°C, 1 minute at 37°C, 2 minute at 72°C; 2 cycles of 30 sec at 95°C, 1 minute at 35°C, 2 minutes at 72°C; 41 cycles of 30 sec at 94°C, 1 minute at 35°C, 2 minute at 72°C; followed by a final 5 minute extension at 72°C then brought down to 4°C.

Electrophoresis

The PCR products (27 µl) were mixed with 6x gel loading buffer (3 µl) and loaded onto an agarose (1.5% w/v) gel electrophoresis in 0.5xTBE (Tris-Borate- EDTA) buffer at 70 V for 150 min. The gel was stained in ethidium bromide solution (2 µl Etbr/100ml 1xTBE buffer) for 40 min and visualized under UV in Bio Doc Image Analysis System with Uvisoft analysis package (Cambridge, UK).

Data Analysis

All the 60 primers were tested at least twice for reproducibility of banding pattern. Out of 60 decamer primers used for RAPD analysis, 9 primers, viz. OPA-01, OPH-14, OPH-18, OPH-19, OPW-13, OPW-20, OPY-01, OPY-08 and OPY-15 producing reproducible banding patterns were selected. All the bands in the range of resolution were scored. The gel pictures acquired through gel documentation system into the computer were processed and scored to obtain binary data. The presence/absence data (1, 0) matrix was analyzed using the standard procedure in NTSYS PC2 package. The genetic distance or similarity was determined by Jaccard similarity index [13]. The pair wise genetic distances of the samples were used to construct dendogram using Unweighted Pair-Group Method of Arithmetic Average (UPGMA).

Results and discussion

FAME

The fatty acid composition in leaves in 31 persimmon genotypes is summarized in Table 1. The great variation on presence and percentage was found among genotypes (Table 1). Eleven fatty acids were identified and quantified in persimmon leaves which were 14:0 (Myristic acid), 15:0 (Pentadecenoic acid), 16:0 (Palmitic acid), 18:0 (Stearic acid), 19:0 (Nonadecanoic acid), 22:0 (Behenic acid) and 24:0 (Lignoceric acid) as saturated and 16:1 (Palmitoleic acid), 18:1 (Oleic acid), 18:2 (Linoleic acid) and 18:3 (Linolenic acid) as unsaturated (Table 1).

Table 1. Fatty acid content (%) of leaves of 31 persimmon genotypes

Genotypes	Fatty Acids (%)										
	Behenic (14:0)	Penta decenoic (15:0)	Palmitic (16:0)	Palmit oleic (16:1)	Stearic (18:0)	Oleic (18:1)	Linoleic (18:2)	Linolenic (18:3)	Nona decenoic (19:0)	Behenic (22:0)	Lignoceric (24:0)
BSP1	-	-	22.22	18.58	2.07	30.83	5.11	18.11	-	0.87	-
BSP2	1.22	-	22.75	2.26	3.70	22.54	19.22	15.54	5.02	1.32	-
BSP3	-	-	12.11	2.90	5.14	24.86	12.07	20.17	15.19	1.18	-
BSP4	-	-	16.90	5.41	4.29	28.70	11.13	13.42	11.17	1.31	-
BSP5	-	-	20.66	9.25	1.97	33.86	9.77	10.06	7.97	1.17	-
BSP6	1.47	-	17.73	6.14	5.76	26.59	10.36	12.43	13.74	1.45	-
BSP7	-	-	8.43	5.07	2.14	24.73	4.61	26.13	17.37	0.91	-
BSP8	-	-	18.87	6.93	3.18	29.72	12.51	21.72	4.64	1.08	-
BSP9	1.27	-	19.19	2.31	4.57	26.08	11.35	16.30	8.19	1.16	-
BSP10	-	1.04	16.30	9.37	6.22	40.31	14.11	-	4.81	1.07	1.66
BSP11	1.01	1.34	15.23	10.98	6.42	25.19	9.44	-	12.12	1.10	0.50
BSP12	2.36	2.41	19.50	10.07	1.13	48.45	3.97	-	3.84	3.08	0.83
BSP13	1.14	-	19.19	4.08	4.13	27.79	24.68	-	9.14	0.78	0.87

BSP14	1.22	1.38	28.13	12.71	5.32	14.01	13.73	-	11.40	1.63	1.08
BSP15	-	-	20.99	8.58	2.15	42.63	9.70	-	4.06	2.29	0.91
BSP16	-	-	18.53	4.95	5.48	40.64	11.70	-	6.55	1.33	0.88
BSP17	1.42	1.13	16.76	4.10	1.55	40.31	9.51	-	12.04	1.99	1.94
BSP18	1.04	1.93	17.31	2.74	4.06	35.60	14.38	-	6.13	1.04	0.67
BSP19	-	-	21.99	5.63	1.61	41.92	12.15	-	6.92	1.25	1.05
BSP20	-	-	6.33	3.53	7.14	47.08	20.07	-	-	0.74	4.72
BSP21	-	-	4.43	3.17	9.54	44.18	20.41	-	-	0.87	0.76
BSP22	0.83	-	10.31	1.49	5.03	35.50	33.26	-	7.17	0.46	0.47
BSP23	1.11	-	12.55	6.12	7.11	23.10	16.45	-	17.83	1.09	0.66
BSP24	-	-	18.66	5.79	5.93	40.01	8.60	-	7.42	-	0.91
BSP25	-	-	17.15	3.75	6.01	36.59	13.77	-	6.33	-	2.48
BSP26	-	-	15.43	2.61	5.34	40.14	17.33	-	7.42	-	1.17
BSP27	-	-	21.31	3.33	7.18	35.81	8.27	-	6.94	-	2.43
BSP28	-	-	23.10	4.21	6.67	33.74	6.69	-	7.77	-	0.80
BSP29	-	-	20.07	4.67	4.83	36.70	6.87	-	8.04	-	1.18
BSP30	-	-	19.37	4.26	2.36	45.01	8.91	-	8.85	-	2.86
BSP31	-	-	18.86	3.98	5.40	39.33	7.01	-	6.72	-	2.07
Total	0.45	0.29	17.43	5.77	4.63	34.26	12.49	4.96	7.89	0.97	0.99

The persimmon genotypes tested in this study were separated based on both presence or absence and percent of eleven fatty acids found in the genotypes (Table 1). The 18:1 (oleic acid) is the most abundant (34.26%) fatty acid in persimmon leaves, followed by 16:0 (palmitic acid) (17.43%) and 18:2 (linoleic acid) (12.49%), respectively (Table 1). On the other hands, 14:0 (myristic acid), 15:0 (pentadecenoic acid), 22:0 (behenic acid) and 24:0 (lignoceric acid) were found as the minor fatty acids in leaves (Table 1).

16:0 (palmitic acid), 16:1 (palmitoleic acid), 18:0 (stearic acid), 18:1 (oleic acid) and 18:2 (linoleic acid) were detected in all genotypes. However, 21 out of 31 genotypes had not included 14:0 (myristic acid) and 25 out of 31 genotypes had not included 15:0 (pentadecenoic acid) (Table 1). 19:0 (nonadecenoic acid) was found all genotypes, except BSP1, BSP20 and BSP21 genotypes. 22:0 (behenic acid) was not detected in BSP24, 25, 26, 27, 28, 29, 30 and 31 genotypes and also 24:0 (lignoceric acid) was not detected in BSP1, 2, 3, 4, 5, 6, 7, 8, and BSP9 genotypes (Table 1). These results may suggest that presence or absence of linolenic acid (18:3), 14:0 (myristic acid), 15:0 (pentadecenoic acid), 19:0 (nonadecenoic acid), 22:0 (behenic acid) and also 24:0 (lignoceric acid) may be useful in assessing chemotaxonomic relationships among persimmon genotypes. It was previously reported that, the most abundant fatty acids in persimmons were linoleic, palmitic and oleic acid [14,15]. These results are in agreement with our results obtained from persimmon genotypes.

RAPD

Results of RAPD analysis are summarized in Table 2 and Figures 1. A total of 76 visible bands were produced by 9 primers available for analysis. The highest number of 13 bands was produced by OPH-19 followed by 12 in both OPH-18 and OPY-8 and the least of 4 marker levels was produced by OPY-15 (Table 2). There were 8.44 polymorphic bands per primer, of which 8.22 were polymorphic indicating higher genetic variability among *Diospyrus kaki* genotypes. The dendrogram realized from the RAPD markers grouped the 31 genotypes into three major clusters (Figure 1). Cluster 1 formed BSP 24, BSP 25, BSP 26,

BSP 27, BSP 28, BSP 29, BSP 30 and BSP 31. Cluster 2 was divided into 2 sub clusters and BSP 10, BSP 11, BSP 12, BSP 13, BSP 14 and BSP 15 formed a sub clusters. BSP 16, BSP 17, BSP 18, BSP 19, BSP 20, BSP 21, BSP 22 and BSP 23 formed another sub clusters. Cluster 3 formed BSP 1, BSP 2, BSP 3, BSP 4, BSP 5, BSP 6, BSP 7, BSP 8 and BSP 9 genotypes. Overall within a similarity coefficient in the range of 0.0 to 0.25, sub-clustering is in agreement with the genotypic proximity. These high genetic distances present among these genotypes clearly suggest that they must have originated from genetically divergent parents or have a long history of adaptation to their respective micro-climatic regions.

Earlier studies using RAPD [16,17,18] techniques showed large genetic variations present among different *Diospyrus kaki* cultivars and genotypes. The distinctness of the persimmon genotypes revealed by RAPD in the present study can be attributed to their geographical isolation as the genotypes collected are from distant localities, which is in strong contrast to the out breeding and high heterozygosity which have accompanied the long history of cultivation and domestication. Classification of *Diospyrus kaki* genotypes based on phenotypic variations or isoenzyme patterns should be reconsidered in the context of the results obtained from molecular analyses with RAPD. However, the molecular studies with RAPD unraveled considerable differences among genotypes as mentioned above. Thus, RAPD based molecular markers were able to distinguish different genotypes which were indistinguishable by isoenzyme based markers. Additional phylogenetic studies using chloroplast or mitochondrial gene sequences or appropriate nuclear genes like ITS of nrDNA sequences can be helpful to reevaluate the systematic positions of different persimmon genotypes.

Table 2. Primers employed with the number of RAPD markers obtained, their sequence, the size of the fragments, and the percentage of polymorphic markers (P) for each primer

Primer code	Sequence 5'→3'	Size (bp) Max-min	Polymorphic Bands	Monomorphic Bands	Total	P(%)
OPA- 1	CAGGCCCTTC	950- 750	4	2	6	66.6
OPH- 14	ACCAGGTTGG	1200-500	9	0	9	100
OPH- 18	GAATCGGCCA	4000- 500	12	0	12	100
OPH- 19	CTGACCAGCC	2000- 450	13	0	13	100
OPW-13	CACAGCGACA	1200- 300	10	0	10	100
OPW-20	TGTGGCAGCA	1500- 700	7	0	7	100
OPY- 1	GTGGCATCTC	1500- 600	11	0	11	100
OPY- 8	AGGCAGAGCA	4000- 700	12	0	12	100
OPY- 15	AGTCGCCCTT	1200- 700	4	0	4	100
Total		4000- 300	74	2	76	97.36

Relationships between FAME and RAPD Data

As mentioned above, the dendrogram realized from the RAPD markers grouped the 31 genotypes into three major clusters (Figure 1). Cluster 1 included 8, cluster 2 included 14 and cluster 3 included 9 genotypes. Some relationships between RAPD and FAME data were found. The linolenic acid (18:3) was detected only in genotypes in cluster 3. Moreover, all genotypes in cluster 3 were absent of lignoceric acid (24:0). Behenic acid (22:0) was detected only in genotypes in cluster 1. Thus, RAPD data of used persimmon genotypes supported these FAMEs findings. In a previous study it was found that the presence or absence of linoleic acid may have useful biochemical indicator for chemotaxonomic classification of pomegranate cultivars [10]. Both FAME and RAPD profiles have been used to study phenotypic and genetic diversity in many plant species such as accessions of *Leucaena* [19],

Hypericum [18] and *Punica* [10] showed that there are some level of similarities between both used techniques which support our findings.

In conclusion, differences in fatty acid patterns illustrate some chemotaxonomic relationships between the genotypes studied. The results demonstrated that RAPD analyses are also found to be useful for differentiation of persimmon (*Diospyros kaki* Thunb.) genotypes tested in the present study. However more cultivars are needed to determine of the degree of relationships of RAPD and FAME data which can contribute to delimiting taxonomic classes within persimmon.

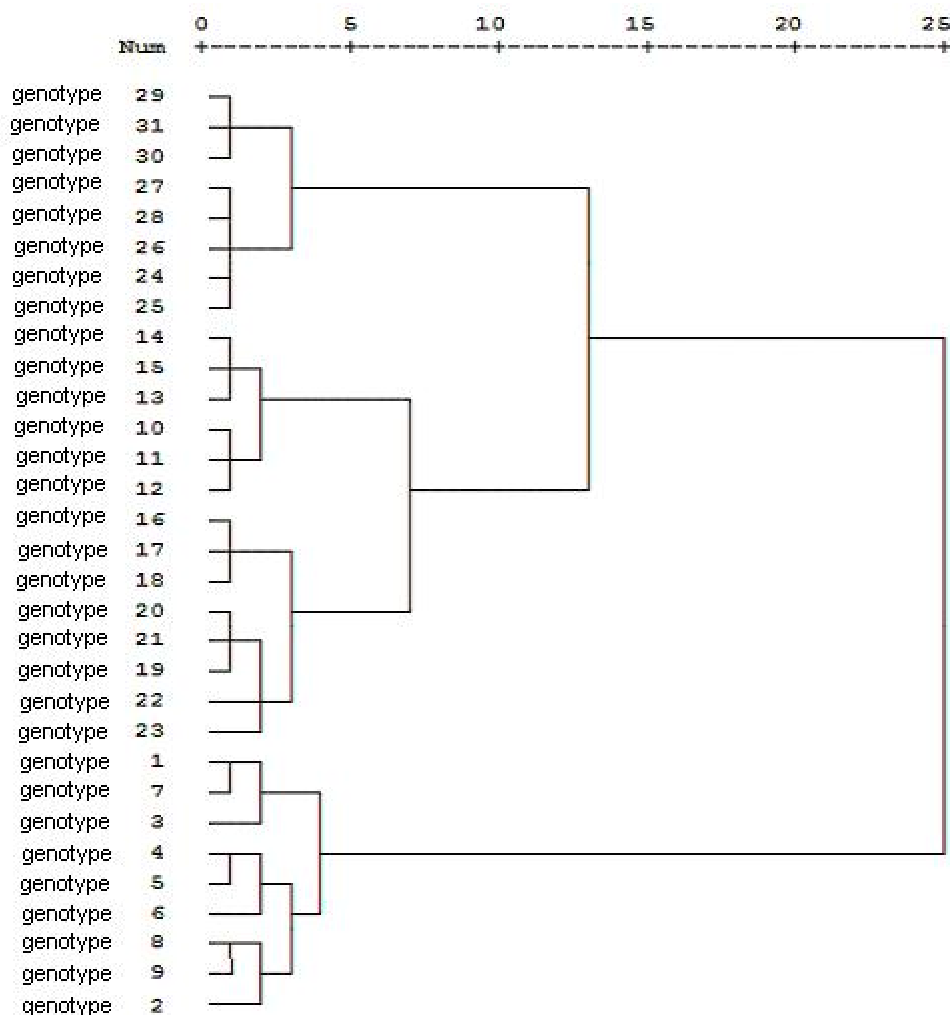

Figure 1. UPGMA dendrogram showing the relationship of persimmon genotypes

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