Turk J Agric For
32 (2008) 363-368

Comparison of Sea Buckthorn Genotypes (*Hippophae rhamnoides* L.) Based on RAPD and FAME Data

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Received: 28.09.2007

Abstract: FAME and RAPD profiles were used to examine biochemical and genetic relationships between 10 selected sea buckthorn genotypes found in the same area of Eastern Anatolia. Fatty acid composition of sea buckthorn berries was determined using gas chromatography. Fatty acid results showed that there were differences between genotypes in both the percent and presence of fatty acids in the berries. Myristic acid was detected only in berries of the ESB8 and ESB9 genotypes. Nervonic acid was detected only in the ESB5 genotype and linoleic acid was detected only in the ESB4 genotype. RAPD data also showed that the ESB4 genotype was in a group distinct from the others. According to these results, it can be concluded that both the absence of linoleic acid and the use of RAPD data could be useful indicators for the characterization and grouping of sea buckthorn genotypes.

Key Words: Biochemical and molecular markers, fatty acids, *Hippophae rhamnoides*, RAPD

Yalancı İğde Genotiplerinin (*Hippophae rhamnoides* L.) RAPD ve Yağ Asidi Profillerine Göre Karşılaştırılması

Özet: Bu çalışmada Doğu Anadolu Bölgesinde bulunan seçilmiş 10 adet yalancı iğde genotipi yağ asiti ve RAPD profillerine göre biyokimyasal ve genetik belirteçler ile karşılaştırılmış ve aralarındaki ilişkiler belirlenmeye çalışılmıştır. Genotiplerin yağ asidi tayini gaz kromatoğrafisi ile belirlenmiş ve genotipler yağ asitlerinin meyvelerinde var-yok durumu ve oranı bakımından farklılıklar göstermişlerdir. Myristik asit sadece ESB8 ve ESB9 genotiplerinde, nervonik asit ESB5 genotipinde ve linoleik asit ise ESB4 genotipinde belirlenmiştir. RAPD sonuçları da ESB4 genotipinin diğer genotiplerden ayrı bir grupta yer aldığını ortaya koymuştur. Elde edilen sonuçlara göre, hem linoleik asitin meyvelerde bulunup bulunmaması ve hem de RAPD verilerinin yalancı iğde genotiplerini karakterize etmede kullanılabileceği ortaya çıkmıştır.

Anahtar Sözcükler: Biyokimyasal ve moleküler belirteçler, yağ asitleri, *Hippophae rhamnoides*, RAPD

Introduction

Hippophae rhamnoides L., also known as sea buckthorn, is a member of the family *Elaeagnaceae*. It is a fascinating plant that grows widely in various regions of Asia, Europe, and North America, from longitude 2° to 123° E, latitude 27° to 69° N, and from 0 to 3300 m above sea level. Sea buckthorn has proven highly adaptable to extreme conditions, including temperatures ranges of –43 to 40 °C, drought, high altitude, salinity, alkalinity, and inundation (Ruan and Li, 2002). The broad geographical distribution and diverse growing conditions may contribute to the extensive diversity of sea buckthorn.

Turkey has important sea buckthorn populations, which are generally wild and distributed throughout north and east Anatolia, and is known locally as 'Yalancı iğde' or 'Karga dikeni' (Baytop, 1999; Çakır, 2004).

The characterization of germplasm is essential for identifying individual genotypes as well as the 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 help

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in elucidating the genetic and biochemical diversity among the accessions. The comprehensive information obtained from such an exercise would help breeders, geneticists, and conservationists to effectively utilize the valuable genetic resources (Vijayan et al., 2005).

Recent advances in the field of horticultural and biological sciences are creating exciting possibilities for the rapid and accurate determination of biochemical and genetic variations between plant species and cultivars. Fatty acid methyl ester (FAME) analysis and nucleic acid-based techniques, such as RAPD (random amplified polymorphic DNA), have been utilized to determine the phylogenetic relationship within and between plant species, in addition to morphological characters, since the 1990s (Williams et al., 1993, Wolff and Morgan-Richards, 1999; Özen et al., 2004; Ağar et al., 2006). Earlier classifications and evaluations of the sea buckthorn were performed based primarily on phenotypic expressions of the plants, such as growth form, leaf morphology, and fruit properties (Sabir et al., 2003; Sabir et al., 2005). Nevertheless, information from these environmentally influenced morphological characteristics is not sufficient to identify sea buckthorn 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. Although no comparative biochemical and genetic work has been carried out to date, biochemical identification of sea buckthorn was performed in Finland (Tiitinen et al., 2006), Pakistan (Sabir et al., 2005), and Poland (Kawecki et al., 2004). The genetic identification of sea buckthorn using RAPD data has been performed in China (Sheng et al., 2006) and India (Singh et al., 2006). In addition, according to a literature search, no comparative study has been carried out with FAME analysis of the berries of different sea buckthorn genotypes.

In our previous paper (Ercişli et al., 2007a) the chemical content, including fatty acid composition, of 10 selected sea buckthorn genotypes was reported. Since FAME and RAPD technique may provide a rapid means of assessing diversity among closely related genotypes (Ercişli et al., 2007b), we have chosen these techniques for the present study to assess the diversity among 10 sea buckthorn genotypes. The data might provide a scientific basis for the future selection and management of the germplasm.

Materials and Methods

Sample Collection

Leaf samples for RAPD analysis were collected from 10 selected sea buckthorn genotypes (ESB1, ESB2, ESB3, ESB4, ESB5, ESB6, ESB7, ESB8, ESB9, and ESB10) that belonged to *Hippophae rhamnoides* L. and were found in the town of Uzundere (lat 40°33´ N, long 41°35´ E, and 1025 m asl) in Erzurum province in Eastern Anatolia, Turkey. Seventeen leaves from the tops of 90-day-old primary branches were collected from each genotype separately and stored immediately at –80 °C for DNA extraction. FAME analysis was conducted on the berries and the berry sampling period was estimated to be a reasonable time for commercial harvesting. Before the last week of August the berries were unpalatable and after the first week of September the berries started to be difficult to harvest due to loss of firmness.

Determination of Fatty Acids

Fatty acid composition was analyzed according to a previous method (Ağar et al., 2006) and fatty acids were separated by gas chromatography (HP6890, Hewlett Packard, Palo Alto, CA, USA) with a fused-silica capillary column (25 m × 0.2 mm) and cross-linked 5% phenyl methyl silicone.

DNA Extraction

Genomic DNA was extracted from powdered leaf materials using a modified method described by Lin et al. (2001). The purity and quantity of DNA were measured spectrophotometrically in both 260 and 280 nm wavelengths (Thermo Nicolet 100 UV, London, UK). The quantity of DNA measured at 260/280 nm was between 1.4 (ESB1) and 1.6 (ESB10).

RAPD Analysis

Fifty-two primers (Operon Technologies Inc., Alameda, CA, USA) were used to generate RAPD profiles. PCR amplification reactions were carried out in 30 µl of final reaction mixture containing 3.0 µl of 10× buffer, 1.2 µl of dNTPs (10 mM), 1.2 µl of magnesium chloride (25 mM), 2.0 µl of primer (5 µM), 0.4 µl of *Taq* polymerase (5 units), 19.2 µl of water, and 3.0 µl of sample DNA (100 ng μl^{-1}). The thermal cycler (Eppendorf Company, Hamburg, Germany) was programmed as follows: 2 min at 95 °C; 2 cycles of 30 s at 95 °C, 1 min at 37 °C, and 2 min at 72 °C; 2 cycles of 30 s at 95 °C, 1 min at 35 °C, and 2 min at 72 °C; 41 cycles of 30 s at 94 °C, 1 min at 35 °C, and 2 min at 72 °C; followed by

a final 5 min extension at 72 °C and then it was lowered to 4 °C.

Electrophoresis

The PCR products (27 µl) were mixed with 6× gel loading buffer (3 µl) and loaded onto an agarose (1.5% w/v) gel electrophoresis in 0.5× TBE (Tris-Borate-EDTA) buffer at 70 V for 150 min. The gel was stained in ethidium bromide solution (2 µl of EtBr 100 ml^{-1} of 1× TBE buffer) for 40 min and visualized under UV in a Bio Doc image analysis system with the UVIsoft analysis program (Cambridge, UK).

Data Analysis

For FAME analysis, fatty acids of each genotype were scored as present (0.1%-100%) or absent (0%). FAME data were subjected to analysis of variance (ANOVA). The means were separated by Duncan's multiple range test at the significance level of $P < 0.05$. PCR products were scored as the presence of (1) or absence of (0) bands for each of the 10 accessions analyzed. Only reproducible bands were scored. Data were used to calculate a Jaccard's similarity index from which a UPGMA dendrogram was constructed. All of the experiments in this study were repeated at least twice.

Results

FAME

The fatty acid composition in the mesocarp of the berries of 10 different sea buckthorn genotypes is summarized in Table 1. The sea buckthorn genotypes tested in this study were separated based on the presence or absence of and percentage of 7 fatty acids found in the genotypes.

Great variation in fatty acid percentage was observed among the genotypes ($P < 0.05$) (Table 1). The genotypes contained significant amounts of palmitoleic acid (21.8%-51.0%), palmitic acid (19.9%-35.8%), and oleic acid (14.3%-36.7%), which are the major fatty acids found in sea buckthorn berries. On the other hand, linoleic acid (0.0%-6.5%) and stearic acid (0.0%-1.8%) were determined to be minor fatty acids in the berries (Table 1). Among the genotypes, myristic acid was only detected in the ESB8 and ESB9 genotypes, and nervonic acid was only found in the ESB5 genotype. Linoleic acid was not detected in the ESB4 genotype, while all the other genotypes studied contained linoleic acid (Table 1).

RAPD

Results of RAPD analysis are summarized in Table 2, and in Figures 1 and 2. The primers, which generated clear amplification products with the genomic DNA, have been selected for further work. Ten of the previous 52 primers were used in this study for their ability to amplify sea buckthorn genomic DNA from 10 sea buckthorn genotypes.

The 10 random primers generated a total of 116 RAPD bands. OPA1, OPB11, OPH8, OPW13, OPW17, OPW18, OPW20, OPBA03, OPBB14, and OPBD07 produced 17, 16, 15, 8, 6, 5, 6, 14, 6, and 14

Table 1. Fatty acid content (%) of the sea buckthorn genotypes.

	Genotypes										
Fatty acids	ESB1	ESB2	ESB3	ESB4	ESB5	ESB6	ESB7	ESB8	ESB9	ESB10	Mean
Myristic	0.0	0.0	0.0	0.0	0.0	0.0	0.9	0.9	0.0	0.0	0.18
Palmitic	19.9b	25.8ab	30.5ab	28.9ab	25.4ab	24.9ab	29.0ab	28.0ab	33.1ab	35.8a	28.13
Palmitoleic	51.0a	40.6b	33.0c	33.5c	34.9c	41.3b	42.9b	32.6c	21.8d	23.2d	35.48
Stearic	0.0	1.4	1.8	1.5	1.4	1.1	1.0	1.3	0.0	0.0	0.95
Oleic	14.9c	19.4bc	20.2bc	26.5b	24.6ab	20.5bc	14.3c	22.7bc	36.7a	29.1ab	22.89
Linoleic	6.5a	4.6ab	6.1ab	0.0b	5.0ab	2.7ab	3.7ab	3.8ab	1.8ab	5.4ab	3.96
Nervonic	0.0	0.0	0.0	0.0	1.6	0.0	0.0	0.0	0.0	0.0	0.16
Total peak area	92.3	91.8	91.6	90.4	92.9	90.5	91.8	89.3	93.4	93.5	

These results were reported previously by Ercişli et al. (2007a).

*Values in the same line with different lower-case letters are significantly different at $P < 0.05$.

Table 2. Number of amplification products generated with 10 arbitrary oligonucleotide primers in sea buckthorn (*Hippophae rhamnoides* L.).

Primers	Sequence 5'→3'	Size (bp) min-max	Polymorphic Bands	Total	P(%)
OPA1	CAGGCCCTTC	500-4000	17	18	94.45
OPB11	GTAGACCCGT	800-4000	16	17	94.11
OPH18	GAATCGGCCA	700-3000	15	15	100.00
OPW13	CACAGCGACA	750-2000	8	9	88.88
OPW17	GTCCTGGGTT	650-2000	6	6	100.00
OPW18	TTCAGGGCAC	1000-3000	5	5	100.00
OPW20	TGTGGCAGCA	750-2000	6	7	85.71
OPBA03	GTGCGAGAAC	500-4000	14	16	87.50
OPBB14	GTGGGACCTG	500-1400	6	7	85.71
OPBD17	GAGCTGGTCC	900-4000	14	16	87.50
Total		500-4000	107	116	92.24

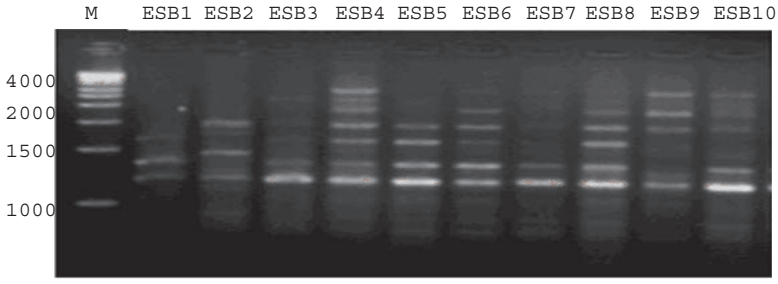

Figure 1. RAPD bands obtained by amplification with primer OPA-1: molecular marker (M).

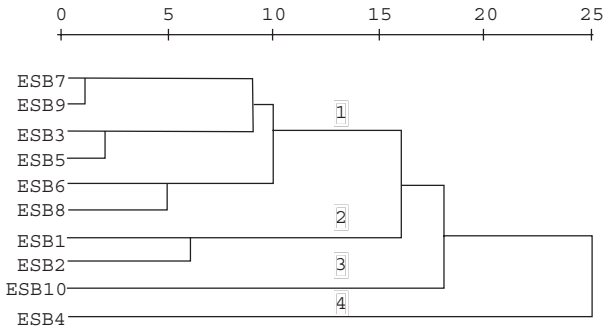

Figure 2. UPGMA dendrogram showing the relationships between *H. rhamnoides* genotypes.

polymorphic bands, respectively (Table 2). OPA1, OPB11, OPW13, OPW20, OPBA03, OPBB14, and OPBD07 produced 1, 1, 1, 1, 2, 1, and 2 monomorphic bands, respectively (Table 2). In total, 92.24% of the bands were polymorphic and the amplicons varied in size from 500 to 4000 bp (Table 2, Figure 1). Banding patterns of the 10 sea buckthorn genotypes using the primer OPA-1 are illustrated in Figure 1.

RAPD analysis showed that there were distinct genetic differences between the genotypes. All 10 genotypes of *H. rhamnoides* were clearly differentiated based on a dendrogram constructed using Jaccard's similarity index with UPGMA (Figure 2). The dendrogram produced from the RAPD markers grouped the 10 genotypes into 4 major clusters. Cluster 1 was divided into 2 sub-clusters: ESB7, ESB9, ESB3, and ESB5 formed 1 sub-cluster, and ESB6 and ESB8 formed the other sub-cluster. Cluster 2 consisted of ESB1 and ESB2. Clusters 3 and 4 consisted of ESB10 and ESB4, respectively (Figure 2). The greatest similarity was observed between genotypes ESB7 and ESB9, and the greatest dissimilarity was observed between genotypes ESB4 and ESB5 (Figure 2).

Discussion

FAME

In the present study fatty acid content and RAPD data were used for the first time as biochemical and molecular markers, respectively, to differentiate sea buckthorn genotypes grown in Turkey.

According to the fatty acid data it can be said that the sea buckthorn genotypes are separated based on the presence or absence of and percent of fatty acids. Among the genotypes, palmitoleic acid was the predominant fatty acid. These results suggest that the presence of myristic acid and nervonic acid, and absence of linoleic acid might be useful in assessing chemotaxonomic relationships between sea buckthorn genotypes. In a previous study it was found that the absence of linoleic acid might be a useful biochemical indicator for the chemotaxonomic classification of pomegranate cultivars, and the RAPD data collected from the studied pomegranate cultivars also supported this finding (Ercişli et al., 2007b). It can therefore be concluded that among the fatty acids, linoleic acid may be used as biochemical marker in this fruit species; however, more fruit species need to be studied in order to confirm this hypothesis. It was previously demonstrated that the content and composition of fatty acids can serve as taxonomic markers in higher plants (Aitzetmuller, 1993).

The presence of palmitoleic acid (16:1) in various sea buckthorn varieties and species was reported earlier. Ranjith et al. (2006) investigated the fatty acid composition of different sea buckthorn species grown in India and found that palmitoleic acid was the dominant fatty acid (32%-53%). Bekker and Glushenkova (2001) reported palmitoleic acid and palmitic acid were major fatty acids in the mesocarp of *H. rhamnoides* from Uzbekistan. These results are in agreement with the present results obtained from the berries of sea buckthorn genotypes. The current fatty acid results also indicate the potential for breeding and industrial applications to evolve 16:1-rich varieties as well.

The RAPD analysis indicated that *H. rhamnoides* genotypes possess abundant genetic diversity and distinct genetic variation. The genotypes ESB7 and ESB9 are genetically closer than the other genotypes. Earlier studies using RAPD (Ruan et al., 2004) and ISSR (Liu et al., 2007; Chen et al., 2008) showed large genetic variation among different sea buckthorn genotypes and groups. In particular, Liu et al. (2007) reported that Russian sea buckthorn genotypes had a higher polymorphism ratio than China's sea buckthorns. This result supports the present findings because we also obtained a high polymorphism ratio among the genotypes, suggesting that these genotypes may be close to the Russian sea buckthorn group, not to the Chinese group.

In most cases RAPD data were not in agreement with FAME results. For example, stearic acid was not detected in the ESB1, ESB9, or ESB10 genotypes, but these genotypes were within different clusters in the dendrogram (Figure 2). Genetically, the closest genotypes, ESB7 and ESB9, had very different fatty acid profiles.

The ESB4 genotype had a distinct RAPD profile and this genotype formed a single cluster in the dendrogram (Figure 2). It was interesting that linoleic acid was not detected in this genotype. Therefore, the FAME data supported the RAPD data for this genotype.

FAME and RAPD profiles have been used to study chemical and genetic diversity, respectively, in many plant species, such as accessions of *Hypericum* (Özen et al., 2004) and *Punica* (Ercişli et al., 2007b), demonstrating that there is some level of similarity between both techniques, which supports our findings.

In conclusion, the chemotaxonomic importance and potential of fatty acids in sea buckthorn genotypes were confirmed by the present study. Some indicators (absence of linoleic acid) were found for determining the degree to which fatty acids can contribute to delimiting taxonomic classes within this species. The differences in fatty acid patterns illustrated some chemotaxonomic relationships between the genotypes studied; however, further studies are required, in particular delineation of the worldwide fatty acid patterns of this species, in order to confirm our results. The results demonstrated that RAPD analysis was useful for the differentiation of the sea buckthorn (*H. rhamnoides* L.) genotypes tested in the present study; however, more cultivars need to be studied in order to determine the degree of the relationship between RAPD and FAME data, which might contribute to delimiting the taxonomic classes within sea buckthorn.

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