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ORIGINAL ARTICLE

Molecular and phytochemical analysis of wild type and olive cultivars grown under Saudi Arabian environment

Abdullah A. Abood[1] · Abdullah M. Al-Ansari[1] · Hussein M. Migdadi[2] · Mohammad K. Okla[1] · Abdulaziz M. Assaeed[2] · Ahmad K. Hegazy[3] · Aref M. Alshameri[1] · Mohamad Altaf Khan[2]

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Abstract This study aimed to assess genetic variability at molecular and phytochemical levels among the four most commonly grown olive cultivars and the wild-type olive of Saudi Arabia. Sixty-six and 80 amplicons were generated from 9 random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) primers, each, producing an average of 95.9 and 86.44% polymorphism for the two markers, respectively. The PIC values were 82.2% for the RAPD and 85.4% for the ISSR markers and the discrimination power for both the markers was 11.1%. The UPGMA cluster analysis based on the RAPD and ISSR data resulted in the aggregation of cultivars and wild accession with a good bootstrapping value according to their origin. Furthermore, a total of 199 compounds were identified in the cultivars based on peak area, retention time, and molecular formula using GC–MS analyses of methanolic and ethanolic extracts. These compounds were classified according to their chemical class; most of them were fatty acids, alcoholic compounds, carboxylic acids, aldehydes, heterocyclic compounds, ketones, alkanes, and phenols. Genetic and phytochemical distances were significantly correlated, based on the Mantel test. The Saudi wild accession also had high numbers of fatty acids and their esters, and can be used in breeding programs for generating new genotypes with interesting characters.

Keywords Genetic variability · *Olea europaea* · *Olea europaea* subsp. *europaea* var. *sylvestris* · Molecular markers · Phytochemicals

Introduction

Olive (*Olea europaea* L.), which belongs to the family *Oleaceae*, is a subtropical, evergreen, oil-producing tree. The genus *Olea* comprises more than 40 species, including the cultivated, wild, and feral forms (Heywood 1978). The number of chromosomes ($2n = 46$) in this genus is very high, which is an indicator of its polyploid (tetraploid) origin (Brousse 1987). Microsatellite patterns and flow cytometry analyses have confirmed the hexaploid, tetraploid, and triploid nature of *O. europaea* (Besnard et al. 2008; Brito et al. 2008). Olive products have been valued since ancient times. The oil extracted from mesocarp of the fruit is a valuable and healthy food. It is also used as lamp fuel and in wool treatment, medicine, and cosmetic and soap production. As a food, it is used in salads, for cooking, and in the preservation of other foods. Table olives are also a typical component of the Mediterranean diet and are consumed after processing and pickling in different ways.

The origin of olive is very ancient and its cultivation goes back to the prehistoric period. Zohary and Hopf (1994) suggested that domestication of olive took place between 5500 and 5700 years ago and the most accepted opinion among researchers is that the olive tree originated in Eastern Mediterranean, more precisely, in the north of

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✉ Hussein M. Migdadi
hmigdadi@ksu.edu.sa; h.migdadi@gmail.com

[1] Department of Botany and Microbiology, College of Science, King Saud University, Riyadh, Saudi Arabia

[2] Department of Plant Production College of Food and Agricultural Sciences, King Saud University, Riyadh, Saudi Arabia

[3] Department of Botany and Microbiology, Faculty of Science, Cairo University, Giza, Egypt

the Dead Sea (Loukas and Krimbas 1983; Zohary and Spiegel-Roy 1975). Cultivated olive harbors enormous genetic variability. To date, more than 2600 different olive cultivars have been described (Rugini and Lavee 1992) and large numbers of mislabeling, homonyms, and synonyms have been reported (Barranco and Rallo 2000). The preservation of this valuable genetic patrimony in olive is important to prevent its erosion, which would lead to an irreversible narrowing of the genetic background, as it is occurring in many other crops.

Before the availability of molecular markers, identification of olive tree cultivars was performed using morphological, agronomical, or biochemical traits (Barranco et al. 2000). In recent times, molecular markers have been widely applied to characterize and identify the olive cultivars. In olive, RAPDs have been extensively used for cultivar identification and was the first class of molecular markers to be considered in olive and have been broadly employed for cultivar identification started by early study of Bogani et al. (1994) and then many studies conducted to characterized olive cultivars utilizing RAPD (Gomes et al. 2008; Martins-Lopes et al. 2009; Muzzalupo and Perri 2009; Brake et al. 2014) and ISSRs markers have been also firstly used by Pasqualone et al. (2001) and followed by many studies of genetic diversity and cultivars fingerprint of olive (Asadiar et al. 2013; Ben-Ali et al. 2015; Brake et al. 2014; Kaya 2015; Linos et al. 2014; Noormohammadi et al. 2012). In addition to these markers AFLPs (Angiolillo et al. 1999; Ipek et al. 2015), microsatellite (Sefc et al. 2000; Cipriani et al. 2002; Erre et al. 2010; Noormohammadi et al. 2014 and Abdessemed et al. 2015), SNP markers (Reale et al. 2006; Salimia et al. 2009; Hakim et al. 2010 and Biton et al. 2015) have been employed in olive inter- and intra-cultivar variability, clarifying varietal synonymy and homonymy cases.

Wild olive grows abundantly in thick forests, and is believed to be indigenous to the Mediterranean Basin (Green 2002). The importance of studying the wild olive germplasm as a valuable source of variability could become an attractive objective in olive breeding programs. In this respect, Erre et al. (2010) attempted to elucidate the genetic relationships within and between the wild and cultivated olives using microsatellites. Genetic diversity and gene flow between wild and cultivated olive has been studied. Lumaret and Ouazzani (2001) reported that the genetic diversity values of cultivars, feral olives, and wild olives in ten forest areas around the Mediterranean basin were 0.286, 0.414, and 0.506, respectively, which is consistent with the interpretation that the domesticated olive represents a sample of the genetic variation in genuine wild olive populations that persist today. Owing to their very long lifespan, these wild trees might be closely related to the Neolithic olives, which are recognized as the crop progenitor.

Olive oil is distinguished from other edible vegetable oils by its flavor. Its nutritional value is attributed to the presence of high levels of oleic acid and other minor components. It contains more than 180 different aromas and the majority of the volatile compounds consist of aldehydes, esters, hydrocarbons, ketones, and furans (Kalua et al. 2007; Ridolfi et al. 2002). The cultivar, origin, growing season, maturity stage of fruit, storage conditions of fruit, and fruit processing of olives influence the flavor components of olive oil and, therefore, its taste, aroma, and phenolic profile (Dabbou et al. 2010; Gomez-Rico et al. 2006). Phenolic compounds, phenyl ethyl alcohols, flavones, secoiridoids, including oleuropein, lignans, and ligstroside derivatives are other important parameters for determining the quality of olive oil because phenolic structures largely contribute to the olive oil flavor and prevent oxidation of the free fatty acid fraction in the oil (Gallina-Toschi et al. 2005; Servili and Montedoro 2002).

Saudi Arabia is one of the largest consumers of olives and olive oils, but its contribution to the world olive oil production is limited. The climate in the northern part of the country resembles the Mediterranean climate, which favors the growth of olive tree and, thus, the production of olive oils with the same high international quality standards (Al-Ruqaie et al. 2016). In these areas, extensive plantations of exotic and indigenous cultivars of olives have been established (Al-Khalifah et al. 2012).

Studies characterizing the wild-type and other cultivars of olive grown in Saudi Arabia using molecular markers and phytochemical analysis are limited. This study aimed at molecular and phytochemical assessment of the most commonly grown olive cultivars in Saudi Arabia with respect to the wild type.

Materials and methods

Plant materials

Plant samples from four olive cultivars were obtained from the Al-Jouf Olive Research Center. The wild sample was collected from Al-Sodah National Park at Al- BAHA Governorate, in the south west of Saudi Arabia. Fully matured fruits from the cultivars 'Arbosana', 'Koroneiki', 'Picual', 'Arbequina' and the wild accession were harvested at the same time in mid-December from the same olive orchards kept on ice and shipped directly to the lab using ice box. These cultivars were selected because they are some of the most widely planted cultivars in the new orchards of Saudi Arabia, and are highly productive and well-adapted to the modern olive-growing techniques. Only healthy fruits, without any kind of disease or physical damage, were processed. Fruits were crushed to fine paste

using grain chopper (Moulinex grain grinder, France) extracted with ethanol and methanol solvents.

100 g of ground sample with 200 ml of 95% ethanol was mixed for 30 min using a magnetic vibrator. Sodium sulfate anhydrous was used to dehydrate samples then filtered and the ethanol was evaporated using rotary evaporator. Moreover, SPE (C-18: 300 mm and 22 mm L/W) columns were used for sample purification prior GC–MS analysis. Samples were dissolved in 60% ethanol and the supernatant mixed with ether (2:1v/v) and mixed and the supernatant collected three times. After that chloroform was added (2:1v/v) the mixture was shaken and the supernatant contained chemical constituents was collected.

The GC–MS analyses of methanolic and ethanolic extracts were performed using a TSQ^{TM} 8000 Evo Triple Quadrupole GC–MS/MS (Thermo Fisher Scientific, Waltham MA, USA) equipped with an Elite-5 capillary column (30 nm × 0.25 mm ID × 0.25 μm df); the mass detector was operated in the electron impact (EI) mode with full scan (50–550 amu). Helium was the carrier gas, which was used at a flow rate of 1 mL/min. The injector was operated at 290 °C and the oven temperature was programmed to increase from 50 to 200 °C at 8 °C/min (held for 5 min) and further to 290 at 7 °C/min (held for 10 min). The peaks in the chromatogram were identified on the basis of their mass spectra. Mass spectrum obtained from GC–MS was interpreted using the database of National Institute Standard and Technology (NIST). The mass spectra of the phytochemicals were compared with the spectra of known compounds stored in the NIST library.

DNA extraction and PCR amplification

DNA extraction was performed using a modified SDS protocol (Alghamdi et al. 2012). Fresh leaf samples were ground in liquid N_2 and 200 mg of the powder was mixed with 800 μL of the extraction buffer (100 mM Tris–HCl pH 8, 50 mM EDTA pH 8, 1.4 M NaCl, 2% SDS v/v, 2% v/v PVP, and 0.1% mercaptoethanol), and incubated at 65 °C for 30 min. Thereafter, 3 μL RNase1 (10 mg/mL) was added to the extract and it was incubated at 37 °C for 15 min. An equal volume of chloroform–isoamyl alcohol (24:1) was added, mixed, and centrifuged at 13,680×*g* for 20 min. One-third volume of 5 M potassium acetate was added to the supernatant, mixed vigorously, and centrifuged at 13,680×*g* for 20 min. The supernatant was removed and ½ volume of cold isopropanol was added, mixed well, and incubated at 4 °C for 1 h. The samples were then centrifuged at 13,680×*g* for 15 min at 4 °C. The supernatant was decanted and the pellets were allowed to air dry for 10 min. The pellets were re-suspended in 300 μL of TE (10 mMTris, 1 mM EDTA, pH 8.0) and 1/10th volume of 3 M sodium acetate, and 2/3 volume of ice-cold isopropanol were added. The samples were mixed well, incubated at 4 °C for 1 h, and centrifuged at 13,680×*g* for 10 min at 4 °C to pellet the DNA. The supernatant was discarded and the pellets were washed with 80% EtOH and centrifuged at 13,680×*g* for 5 min at 4 °C; the supernatant was discarded again and the tubes were inverted to dry for 30 min. The DNA samples were resuspended in 100 μL of TE and incubated at 4 °C overnight. The quality and concentration of the extracted DNA were determined by electrophoresis on a 0.8% agarose gel and spectrophotometry using Thermo Scientific NanoDrop 2000 (Thermo Fisher Scientific Inc., Waltham, MA, USA). DNA was diluted with TE to a final concentration of 100 ng/μL.

Polymerase chain reactions were performed in 20-μL volumes containing 1X GoTaq Green Master Mix (Promega Corporation, Madison, WI, USA), 0.1 μM of each primer, 50 ng DNA templates, and nuclease-free water. The PCR amplification was performed in a TC-5000 thermal cycler (Bibby Scientific, Staffordshire, UK) using the following thermal profile: denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 35 °C for 1 min for RAPD reactions and 50 °C for ISSR reactions, and elongation at 72 °C for 1 min, followed by a final elongation step at 72 °C for 7 min. The amplified DNA fragments were separated by electrophoresis at 80 V for 100 min on a 1.5% agarose gel in TBE buffer (0.1 M Tris base, 0.1 M boric acid, 2 mM EDTA) and visualized by staining with acridine orange (10 mg/mL). The amplification reactions were repeated at least twice and only reproducible and intense bands were scored. The gel was viewed under ultraviolet light and photographed using Bio-Rad Gel Doc EZ System. The molecular sizes of the amplified products were estimated by using a 100-bp DNA ladder (Sigma Chemical Company, Darmstadt, Germany).

Data analysis

The DNA profiles were scored visually from gel photographs. The clear and reproducible amplified bands were chosen for the analyses. Polymorphism information content (PIC) value was estimated using the following equation described by Anderson et al. (1993): $PIC = 1 - \sum_{j=1}^{n} Pij^2$, where *Pij* is the frequency of the *i*th allele for marker *j* and the summation extends over *n* amplicon calculated for each locus. The discrimination power was calculated by dividing the number of polymorphic markers amplified for each primer by the total number of polymorphic bands obtained (Khierallah et al. 2011). The presence of a band was designated as "1" and the absence of a band was recorded as "0". The data obtained by scoring the RAPD and ISSR profiles, both individually as well as collectively, were

subjected to the calculation of a similarity matrix using Jaccard's coefficients. Cluster analysis was performed to construct dendrograms with the unweighted pair-group method by arithmetic averages (UPGMA) from the similarity data matrices using PAST3.11 software (Hammer et al. 2001). The co-phenetic correlation coefficient was used to check the goodness of fit a cluster analysis to the associated similarity matrix. A bootstrap analysis of 1000 replicates was performed using PAST3.11 software to estimate structural stability of clusters. Mantel test (Mantel 1967) was also performed using the PAST3.11 software in order to investigate the relationship between the genetic and phytochemical distances of the cultivars.

Results

Molecular analysis

Out of the 25 primers used, 9 primers that produced unambiguous fragments with repeatable patterns when tested two times with the same cultivar were considered reproducible amplicons and were used in the analysis (Table 1). A total of 66 amplicons (loci) were obtained, out of which 64 amplicons were polymorphic; the average number of polymorphic amplicons obtained per primer was 7.11 and the range was from 3 (for primer P9) to 13 (for primer P6). The polymorphism percentage ranged from 75 to 100% with an average of 95.89%. Across cultivars, the primers produced 159 bands with an average of 17.67 bands per primer, ranging from 9 for primer P1 to 33 bands for primer P6. The polymorphic information content (PIC) ranged from 66 for primer P9 to 91 for P6, with an average of 82.22%. Primer P6 had the highest discrimination power (DP) with a value of 20% and P9 had the lowest (5%) value. All the primers showed an average DP of 11.11%. A total of 80 reproducible ISSR amplicons were generated from 9 primers out of the 16 ISSRs screened, of which 70 were polymorphic, and ranged from 2 for primer P9 to 11 amplicons for primer P6; this accounted for a high percentage of polymorphism (86.44%), ranging from 50% for primer P9 to 100% for primers P4, P5, and P8. The primers produced 236 bands across cultivars with an average of 26.22 bands per primer ranging from 13 bands for primer P8 to 39 bands for primer P1. The PIC ranged from 74 for primer P9 to 91 for primer P1, with an average of 85.44%. Primer P6 had the highest discrimination power with a value of 16% and P9 had the lowest (3%) value. All the primers showed an average DP of 11.11% (Table 2).

Based on the Jaccard's similarity coefficient, a genetic similarity matrix was constructed using the RAPD and ISSR data to assess the genetic relatedness among the four olive cultivars and one Saudi wild olive accession. The RAPD data showed a similarity coefficient ranging from 0.05 (between Arbosana and Koroneiki cultivars) to 0.58 (between Arbequina and Picual cultivars). All the cultivars and wild accession showed an overall genetic similarity value of 0.31. The UPGMA cluster analysis of the cultivars and wild accession based on the RAPD data was cut at a similarity of 0.45 (which represented 50% of the distance from the maximum similarity of 0.58 to the minimum of 0.12). Cutting the dendrogram at this similarity value resulted in aggregating three cultivars, namely Arbequina, Picual, and Arbosana, with a good bootstrapping value (96), whereas Koroniki and wild accession failed to form a cluster and were individually separated (Fig. 1). However,

Table 1 Primers used for RAPD analyses: total number and polymorphic amplicons, % of polymorphism obtained, total number of bands across cultivars, polymorphism information content (PIC) and discrimination power (DP)

RAPD primers	5′-3′ Sequence	Total number of amplicons	Polymorphic amplicons	Polymorphism (%)	Total bands	PIC	DP
P1	CACACTCCAG	7	7	100	9	84	11
P2	ACGACCGACA	7	7	100	15	82	11
P3	AGGTGACCGT	8	8	100	22	86	13
P4	GGCTCATGTG	6	6	100	14	82	9
P5	GGACTGCAGA	8	7	88	21	85	11
P6	AGTCAGCCAC	13	13	100	33	91	20
P7	AAAGCTGCGG	7	7	100	19	84	11
P8	GTCAGGGCAA	6	6	100	16	80	9
P9	CCTTGACGCA	4	3	75	10	66	5
Total		66	64	–	159	–	–
Mean		7.34	7.11	95.89	17.67	82.22	11.11
Min		4	3	75	9	66	5
Max		13	13	100	33	91	20

Table 2 Primers used for ISSR analyses: total number and polymorphic amplicons, % of polymorphism obtained, total number of bands across cultivars, polymorphism information content (PIC) and discrimination power (DP)

ISSR primers	5′-3′ Sequence	Total number of amplicons	Polymorphic amplicons	Polymorphism %	Total bands	PIC	DP
P1	$(AAC)_7G$	13	10	77	39	91	14
P2	$(AAC)_7A$	8	7	88	20	84	10
P3	$(GTT)_7C$	10	9	90	30	88	13
P4	$(GTT)_7T$	10	10	100	26	89	14
P5	$(CA)_{10G}$	9	9	100	27	88	13
P6	$(CA)_{10}A$	13	11	85	36	91	16
P7	$(CA)_{10}T$	8	7	88	28	87	10
P8	$(TG)_{10}C$	5	5	100	13	77	7
P9	$(TG)_{10}A$	4	2	50	17	74	3
Total		80	70	–	236	–	–
Mean		8.89	7.78	86.44	26.22	85.44	11.11
Min		4	2	50	13	74	3
Max		13	11	100	39	91	16

at 60% of similarity index, all the cultivars and wild accession were identified and individually separated.

For the ISSR data, the similarity coefficient ranged from 0.36 (between Saudi wild accession and both Picual and Arbozana cultivars) to 0.66 (between Picual and Arbosana cultivars). All the cultivars and wild accession showed an overall genetic similarity value of 0.43. The UPGMA cluster analysis of the cultivars and wild accession based on the ISSR data was cut at a similarity of 0.58 (which represented 50% of the distance from the maximum similarity of 0.66 to the minimum of 0.38). Cutting the dendrogram at this similarity value resulted in aggregating Picual and Arbosana with a good bootstrapping value (97), whereas the other cultivars and the wild accession failed to form a cluster and were individually separated (Fig. 2).

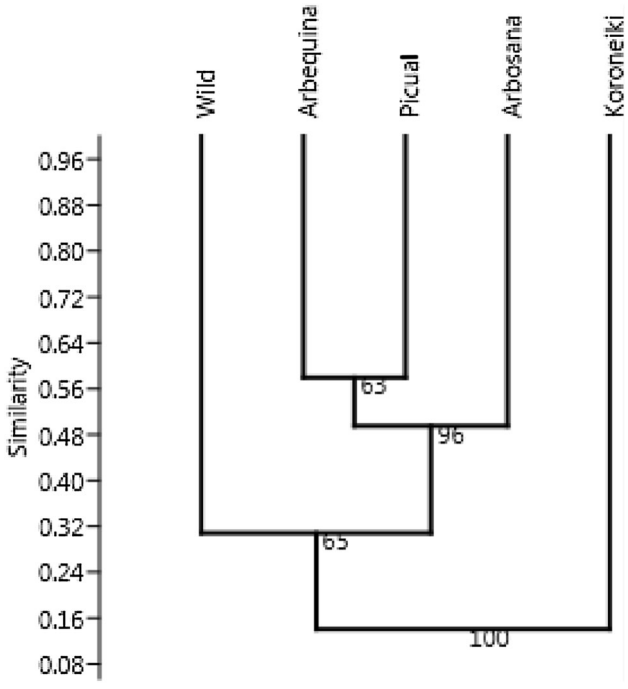

Fig. 1 UPGMA dendrogram based on Jaccard's coefficient illustrating the genetic similarities among olive cultivars and wild accession based on RAPD data

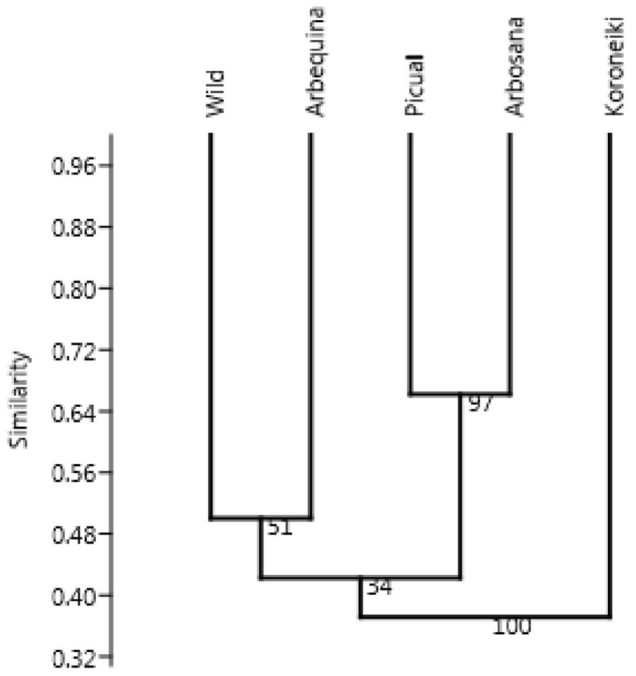

Fig. 2 UPGMA dendrogram based on Jaccard's coefficient illustrating the genetic similarities among olive cultivars and wild accession based on ISSR data

However, at 68% similarity index, all the cultivars and the wild accession were identified and individually separated.

Phytochemical analysis

A large number of phytochemical compounds were identified in the methanolic and ethanolic extracts of the five olive cultivars, including one wild-type olive accession, using GC–MS analysis. A total of 199 compounds were identified based on peak area, retention time, and molecular formula (Supplementary Tables 1 and 2). The compound with the lowest retention time (4.82 min) was propanal, 2-methyl, whereas dihydrotorulosol and silane, (3.beta.)-gorgost-5 compounds had the highest retention time (57.62 min) (Supplementary Table 1). There were significant differences in the compositions of the phytochemicals in the olive genotypes based on the extraction solvent. The main compounds in the Arbequina cultivar included 2,6,10,14,18,22-tetracosahexaene (56.39%) and 2,6,10,15,19,23-hexamethyl-2,6 (56.81%) in the ethanolic and methanolic extraction solvents, respectively. In the wild accession, 72.26% of the constituents were (10.-beta.h)-des-a-lupane and octane nitrile arneel (18.02%) in ethanol and methanol, respectively. The main constituents of the Picual genotype included 2,6,10,14,18,22-tetracosahexaene (27.66%) in ethanol and *n*-hexadecanoic acid (19.82%) in methanol. The compounds 2,6,10,14,18,22-tetracosahexaene in ethanol and 1,1-heptanediol, diacetate in methanol were the main compounds in the Koroneiki genotype, present at 53.92 and 19.73%, respectively.

The phytocompounds identified were classified into different chemical classes (Table 3). A total of 32 fatty acids, 7 fatty acid esters, 17 alcoholic compounds, 22 carboxylic acids, 16 aldehydes, 17 heterocyclic compounds, 15 ketones, 7 esters, 6 ethers, 11 alkanes, 3 phytosterols, 3 sugars and 5 steroid compounds, two compounds each of alkenes, phenols, urea, one compound from amine, organosilicone, and nitriles were determined in the fruits and correspondent oils. Thirty-two residues were classified as unknown constituents. The most abundant compounds from each class with values more than 3% are presented in Table (3). The main constituents of the extracts were the heterocyclic compound 10.beta.h-des-a-lupane (72.26), the steroid 2,6,10,15,19,23-hexamethyl-2,6 (60.11%), the aldehyde propanal, 2-methyl-1 (37.8%) and the carboxylic acid quinic acid 1,3,4,5-tetrahy (26.57%). DOCOSANOIC ACID was the most abundant fatty acid (19.07%) and the BETA-SITOSTEROL (17.04%) was the most abundant phytosterols. A typical chromatogram of one genotype is shown in Fig. (3). GC–MS analysis revealed that the ethanolic and methanolic extracts were predominantly composed of fatty acids, carboxylic acids, heterocyclic compounds, and alcohol and aldehyde compounds. The aldehydes (14-heptadecenal and octadecanal), ester (eicosyl acetate), fatty acid (9-octadecenoic acid), and heterocyclic compound (2,6,10,14,18,22-tetracosahexaene were detected in all the genotypes. However, hexadecanal and propanal, 2-methyl aldehyde compounds, hexadecanoic acid, nonadecanoic acid, and nonadecanoic acid-ethyl ester fatty acids, 1,2-benzenedicarboxylic acid, and oxalic acid-isohexyl pentyl two carboxylic acids, octadecane alkane and methyl 3′-methyllecanorate ester were predominant in four out of five genotypes. The Arbosana genotype recorded the highest total ion chromatogram (TIC) in the ethanolic and methanolic extracts (82 compounds) followed by that in the wild genotype (74 compounds) and Arbequina (62 compounds), whereas Koroneiki and Picual recorded 40 and 50 compounds, respectively.

The GC–MS analysis also showed considerable variation in alcoholic compounds in the different genotypes investigated; the wild accession had 8 alcoholic compounds and Arbequina recorded only two alcoholic compounds. 11-tridecenol and vitamin E were recorded in three genotypes. The most dominant aldehydes were octadecanal and 14-heptadecenal in all the genotypes, followed by propanal and hexadecanal in four out of five genotypes. Out of 10 alkane compounds, octadecane was dominant in four genotypes. Two carboxylic acid compounds, benzenedicarboxylic acid and oxalic acid, and isohexyl pentyl were recorded in four genotypes. Fatty acid constituents showed high variability among the genotypes, ranging from single fatty acid specific for a genotype to one fatty acid (octadecenoic acid) dominant in all the genotypes. Nonadecanoic acid, hexadecanoic acid, and ethyl ester were common in four genotypes. Arbequina (14 fatty acid compounds) and the wild accession contained 12 fatty acids, whereas Picual and Koroneiki had 8 fatty acids. The wild accession and Arbosana recorded 8 and 7 heterocyclic compounds, respectively, whereas the other three genotypes produced five compounds each. 2,6,10,14,18,22-tetracosahexaene (squalene 30-carbon organic compound) was present in all the genotypes. Three ketone compounds were present in Koroneiki and 6 compounds were present in both Arbosana and the wild genotype. Octenone was recorded in two genotypes (Koroneiki and Wild) and bicyclooctenone was recorded in wild and Arbosana, whereas 13 other ketones were recorded in the different genotypes individually.

Cluster analysis showed that Spanish cultivars namely Arbequina, Picual, and Arbosana were aggregating and form one cluster and the Saudi wild accession and Koroniki cultivar failed to form a cluster and were individually separated (Fig. 4). The principal component analysis (PCA) results revealed that a considerable amount of variation (90.24%) was explained by the first three axes.

Table 3 Major classes of the secondary chemical constituents, number of compounds and percentage of the most abundant compounds with retention time and molecular weight determined in olive cultivars and wild accession

Chemical class/compound	RT	Area	Area (%)	MW
Alcohol (17 compounds)				
1-Octadecanol	22.12	42,461	3.27	270.5
1-Hexadecanol	32.42	39,380	3.8	242.45
DL-3,4-dimethyl-3,4-hexanediol	27.10	32,680	4.88	146.23
Vitamin E	53.70	75,700	5.87	430.72
1,2,3-Propanetriol	17.76	208,090	7.84	92.09
Aldehyde (16 compounds)				
4-Hydroxytetradec-2-ynal	47.88	72,051	3.77	224.34
7-Octadecenal	23.66	84,465	5.42	266.47
Octadecanal	52.62	191,624	11.55	268.49
Propanal, 2-methyl-I	4.82	636,539	37.8	72.11
Alkane (10 compounds)				
1,3,4-Trimethyl-1,3-cyclohexan	26.94	51,431	3.3	124.23
Dodecane, 1,1′-oxybis-	54.7	111,361	4.78	354.66
2L,4L-dihydroxyeicosane	22.16	21,110	5.13	314
D-Manno-(E)-tetradec-6-en-1,2,	26.64	87,287	6.72	356.5
Carboxylic acid (22 compounds)				
Oxalic acid, isohexyl pentyl E	10.28	48,551	3.11	244.33
Benzenepropanoic acid	22.76	54,531	5.27	428.75
Butanoic acid	26.68	33,054	5.65	88.11
Benzoic acid	30.36	25,960	8.24	122.12
Silane, [[(3.beta.)-gorgost-5-]]	57.62	188,197	9.84	472.86
Cantharic acid	22.94	167,915	12.92	196.2
Quinic acid 1,3,4,5-tetrahy	28.84	414,229	26.57	192.17
Cyclopropanepentanoic acid	37.72	2199	7.09	310
Ester (7 compounds)				
Vitamin E acetate	53.62	84,397	3.95	472.74
2-Octenyl acetate	21.76	80,782	6.22	170.25
Ether (6 compounds)				
Severine	45.38	64,047	3.52	473.7
19-Norambrox	55.94	82,277	4.53	–
Isocineole	16.88	48,319	11.81	154.25
Fatty acid/fatty acid ester (39 compounds)				
4,8-Decadienoic acid, 2-acetyl	31.22	48,640	3.12	280.41
N-Hexadecanoic acid	35.94	64,462	3.55	256.43
(E)-9-Octadecenoic acid ethyl	39.68	74,257	3.94	310.52
Tetradecanoic acid	23	21,259	4.24	228.38
3,5,7-Trioxononanoic acid *N*	53.8	108,672	4.66	200.19
(E)-9-Octadecenoic acid	38.52	52,148	5.66	282.47
15-Tetracosenoic acid, methyl	38.5	35,769	5.88	380.66
Docosanoic acid 1-methyl-butyl	27	158,721	5.98	410.73
13-Docosenoic acid	37.04	28,670	9.1	338.58
9-Hexadecenoic acid	39.22	69,508	10.5	254.41
Docosanoic acid	26.98	78,002	19.07	340.59
Hexadecanoic acid, ethyl ester	36.54	84,679	4.49	284.48
Heptadecanoic acid, methyl EST	35.24	33,151	5.01	284.48
Heterocyclic compound (17 compounds)				

Table 3 continued

Chemical class/compound	RT	Area	Area (%)	MW
Stigmast-5-en-3-ol, (3.beta.,2)	55.98	107,928	4.63	414.72
2H-Pyran-2-one, 5-ethylidenete	29.66	74,200	4.76	170.21
Stigmasterol, 22,23-dihydro	55.92	96,820	5.13	414.72
Adenosine 9-beta-D	18.62	96,495	5.31	267.24
Fluorenone oxime 9-fluoreno	41.18	131,237	5.63	195.22
Cytidine cyd cytos	28.82	194,693	10.71	243.22
2,6,10,14,18,22-tetracosahexae	49.62	357,013	27.66	410.73
(10.beta.h)-des-a-lupane	57.58	1,363,225	72.26	412.75
Ketone (16 compounds)				
Cyclohexan-1,4,5-triol-3-one-1	17.8	57,507	3.16	190.15
2-Cyclopenten-1-one, 2-hydroxy	8.06	88,703	3.33	98.1
2-Cyclopenten-1-one	8.08	55,876	3.53	82.1
Spiro[4.5]dec-6-ene-1,4-dione	30.36	79,723	4.81	164.2
Nitriles (2 compounds)				
Octanenitrile arneel	23.22	327,558	18.02	125.22
Phenol (2 compounds)				
6-Amino-1-[2-(3,4-diethoxy-phe	30.36	116,713	3.39	307.37
1,3-Benzenediol	19.16	85,295	5.39	110.11
Phytosterols (2 compounds)				
Stigmasterol	55.88	102,892	6.2	412.7
Gamma-sitosterol	55.92	150,662	8.95	414.72
Beta-sitosterol	55.94	219,994	17.04	414.72
Steroid (5 compounds)				
L-Gala-L-ido-octose	29	25,786	3.9	240.21
Ursodeoxycholic acid	53.46	126,365	12.2	392.58
2,6,10,15,19,23-Hexamethyl-2,6	49.5	351,622	60.11	410.72

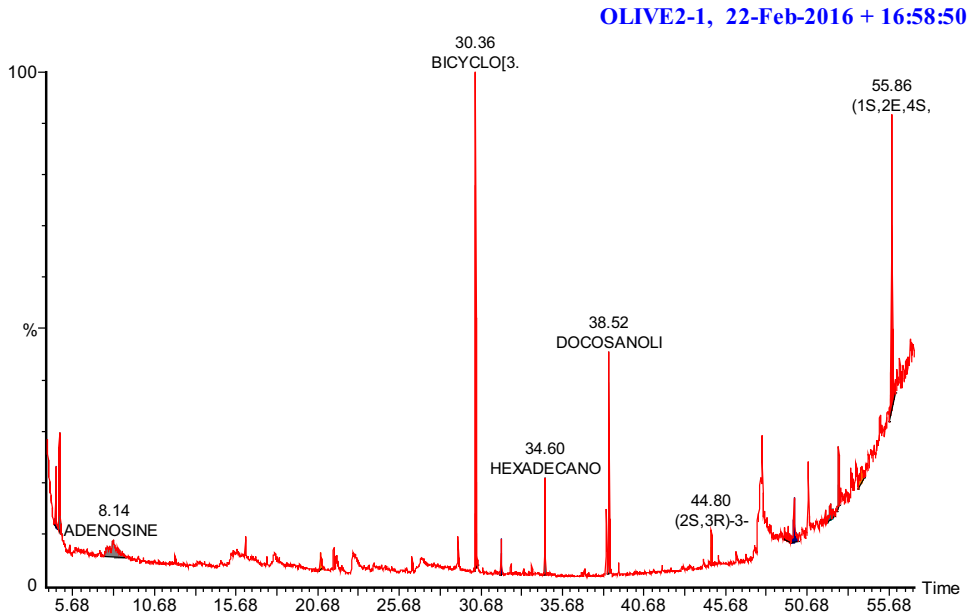

Fig. 3 Chromatogram obtained from the GC–MS with the ethanol extract of Saudi wild accession olive oil

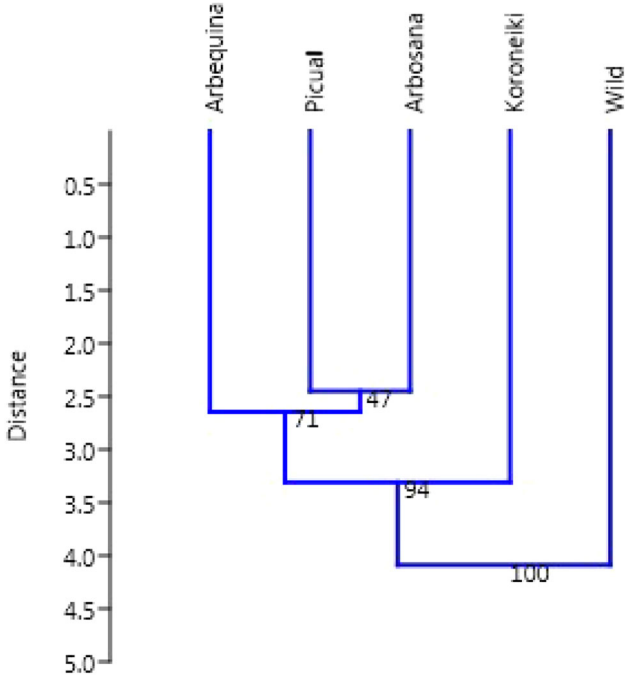

Fig. 4 UPGMA dendrogram based on Euclidian distance coefficient illustrating the genetic similarities among olive cultivars and wild accession based on phytochemicals constitutes data

Axes 1, 2 and 3 explained 50.36, 24.15 and 15.73% of the total variation, respectively (Table 4). The first principal component was positively correlated with ketone, alcohol, carboxylic acid and aldehyde. Fatty acid, carboxylic acid and steroid were the components positively correlated with the second axis and the third axis was positively correlated with alkane and carboxylic acid.

The detected compounds are reported to be potential therapeutic agents and of use in medical and drug formulations (Supplementary Table 3). Genetic and phytochemical distance differences among cultivars were concordant based on the Mantel test ($r = 0.75$, $P = 0.006$ for phytochemical and RAPD distances, $r = 0.82$, $P = 0.01$ for phytochemical and ISSR distances and $r = 0.94$, $P = 0.01$ for RAPD and ISSR distances) indicating that these two analysis (genetic and phytochemicals) grouped the genotypes (wild accession and cultivars) in a similar manner.

Discussion

Olea europaea L. is one of the most important trees cultivated for thousands of years in the Mediterranean area, and is known to have large genetic variability. It exists in two forms, wild (*O. europaea* subsp. *europaea* var. *sylvestris*) and cultivated (*O. europaea* subsp. *europaea* var. *europaea*). The variety '*europaea*' is propagated by cutting or grafting, whereas '*sylvestris*' is reproduced from seeds (Green 2002).

Although phenotypic characters and biochemical profiles are valuable in identifying the genotypes, they are controlled by minor polygenic traits and can be affected by environmental factors and cultivation conditions. RAPD and ISSR are multi-locus profiling techniques extensively used in genome studies and marker assisted selection. They are able to distinguish genotypes below the species level,

Table 4 Eigen values, percentage variations and loadings of the phytochemical classes on the plane of the first three principal components

	PC 1	PC 2	PC 3
Eigen value	25.28	12.12	7.90
Percent of variance	50.36	24.15	15.73
Alcohol	0.43	−0.17	−0.15
Aldehyde	0.35	−0.17	−0.21
Alkane	0.20	−0.03	0.48
Alkene	0.11	−0.07	0.23
Carboxylic acid	0.39	0.27	0.56
Ester	0.05	0.05	0.17
Ether	0.24	0.00	−0.43
Fatty acid	0.02	0.87	−0.18
Heterocyclic compound	0.25	0.04	−0.24
Ketone	0.60	−0.03	−0.05
Phenol	0.00	−0.10	−0.01
Phytosterols	−0.05	−0.03	0.12
Steroid	0.11	0.28	−0.06
Sugar	0.04	−0.11	0.08

such as cultivars and clones, and have been used in numerous diversity studies (Karp et al. 1997; Pasqualone et al. 2016). They have been used alone or in combination to analyze clonal variation and genetic variability in olive cultivars. The combined use of RAPD and ISSR markers facilitates a high level of genomic coverage as RAPD markers are potentially associated with functionally important loci (Penner 1996) and ISSR markers amplify the hypervariable non-coding regions (Esselman et al. 1999).

In the present study, 9 RAPD primers generated 66 amplicons (loci) with an average of 95.9% polymorphism, 82.2% PIC, and 11.1% DP values. In the ISSR analysis, 9 primers generated a total of 80 amplicons with an 86.44% polymorphism, 85.4% PIC, and 11.1% DP values. Previous studies have also indicated high polymorphism among olive cultivars grown in different parts of the world and have shown that olive germplasm encompassed high genetic variability, in Jordan (Brake et al. 2014), Syria and other Mediterranean regions (Belaj et al. 2003a, c), Cyprus (Banilas et al. 2003), Greece (Hagidimitriou et al. 2005), Morocco and western countries of the Mediterranean Basin (Essadki et al. 2006), Egypt (Hegazi et al. 2012), Iran (Noormohammadi et al. 2012), Portugal (Cordeiro et al. 2008; Martins-Lopes et al. 2007), Italy (Ganino et al. 2007), Spain (Belaj et al. 2004; Caraffa et al. 2002; Gomes et al. 2009; Sanz-Cortes et al. 2001), Turkey (Kaya 2015), and Pakistan (Irshad et al. 2014). This high genetic diversity could be due to a diverse germplasmic origin that resulted in a predominant allogamous species with a high degree of outcrossing (Bartolini et al. 1998) and the complexity of the olive genome (23 pairs of chromosomes, which is believed to have been originated by allopolyploidy (Zohary and Spiegel-Roy 1975), resulted in new cultivars found throughout the Mediterranean amid low breeding pressures (Besnard et al. 2001; Contento et al. 2002). Variations reported in the olive cultivars by different researchers may be related to the variations in the loci studied as well as to the number of genotypes and their localities (Lopes et al. 2004).

The UPGMA cluster analysis of the cultivars and wild accession based on the RAPD and ISSR data resulted in aggregation with a good bootstrapping value according to the origin. Picual, Arbosana, and Arbequina formed a single cluster using the RAPD data, whereas Picual and Arbosana formed one cluster using the ISSR data; these three cultivars were introduced from Spain. Koroneiki, the cultivar from Greece, and the wild Saudi accession failed to aggregate and were individually separated in both the DNA-based marker techniques (RAPD and ISSR). Although, there were differences in the number of amplicons (66 and 80) and polymorphism percentage (96 and 86%) generated by RAPD and ISSR, respectively, and considering the fact that the ISSR primers target specific genomic regions, whereas the RAPD primers amplify arbitrary regions (Martins-Lopes et al. 2007), there is a clear structure with the origin of the cultivars that has been observed in this study. These results are in agreement with those of several other studies conducted in both Jordan (Hassawi and Hadeib 2004) and the Mediterranean (Belaj et al. 2001, 2003a, b, 2004; Sanz-Cortes et al. 2001), where a good correlation between the banding patterns of olive cultivars and their geographical origin was obtained using RAPD markers. Moreover, the results also supported the hypotheses of autochthonal origin as well as the limited diffusion of olive cultivars from their zones of cultivation (Belaj et al. 2001; Besnard et al. 2001). However, Besnard et al. (2001), Caraffa et al. (2002), Khadari et al. (2003), Martins-Lopes et al. (2007), Poljuha et al. (2008), and Brake et al. (2014) observed no clear correlation between the olive genotypes and their geographical origin.

The dendrogram topology showed a clear separation of the cultivars from the Saudi wild olive, which agreed with the results of Erre et al. (2010) who examined the genetic relationships among and within the wild and cultivated olives and found that the differences on the allelic composition and heterozygosity levels were clear between the wild and cultivated trees. Noormohammadi et al. (2012) have reported high allelic variation and intra- and inter-population genetic diversity in wild olive trees of Iran using RAPD and ISSR markers, which is also supported by the results of Baldoni et al. (2006), who reported that the observed patterns of genetic variation were able to distinguish the wild olives from the cultivated populations and the continental olives from those found in the insular regions. The ISSR analysis also revealed that the cultivated olives from different Mediterranean countries are nested within the wild populations, indicating that either the wild and domesticated olives exchanged genetic material through hybridization or the olive tree domestication occurred more than once (Vargas and Kadereit 2001). In this study, phytochemical cluster analysis confirmed overlapping variability in the cultivars and wild accession corresponds to the geographical origin of cultivars. Similarity among cultivars within the same cluster (Picual, Arbosana, and Arbequina) is high, comparing to Saudi wild accession and Koroneiki (which showed the lowest similarity) indicated that these cultivars may have similar adaptive characteristics or were of the same origin.

In the present study, a large number of phytochemical compounds were identified in olive cultivars and the Saudi wild-type accession, which agreed with the detection of a high number of phytochemical compounds in olive oil, leaves, and other parts (Morales et al. 1994; Perez et al. 2014, 2016; Reboredo-Rodríguez et al. 2013). The identified phytocompounds were classified according to their

nature into different groups, including fatty acids, alcoholic, aldehydes, phenols, phytosterols, ketones, esters, alkanes, phytosterols, sugars, and steroid compounds. Aroma, taste, and color of olive oil are considered as the main sensory properties reflective of the oil quality, which may change over time and with location (Kalua et al. 2007). Carbonyl compounds, alcohols, esters, and hydrocarbons are the main compounds found in the volatile fraction of virgin olive oil (Flath et al. 1973). The volatile compounds can be highly useful as biomarkers of the quality of virgin olive oil and show correlations with the sensory characteristics (Issaoui et al.2015). Leon et al. (2011) suggested the existence of a strong genetic influence on the fatty acid composition and several minor components and related characteristics.

The volatile composition of olive oil depends on several factors, such as on the levels and activity of the enzymes involved in various pathways (Angerosa 2002), which are genetically determined (Campeol et al. 2001), ripening cycle of the fruit (Lazzez et al. 2008), processing equipment (Di Giovacchino et al. 2001), extraction method and storage conditions (Vekiari et al. 2007), and climate and soil type (Ranalli et al. 1999). Phenolic compounds are also used as quality markers for olive oil and as traits in new cross-breeding programs because of their health promoting and organoleptic properties (Leon et al. 2011). Many studies have used the profiles of fatty acids and other minor components to characterize oils in several areas of the world, including Portugal (Matos et al. 2006), Tunisia (Haddada et al. 2008; Zarrouk et al. 2008), Spain (Pardo et al. 2010), Jordan (Al-Ismail et al. 2011), and Greece (Longobardi et al. 2012). Moreover, stigmasterol and the main fatty acids (palmitic, oleic, and linoleic acids) can be used to validate the different varieties. In addition, α-tocopherol can be used as a differentiator in bitter spicy oils (Lopez-Cortes et al. 2013).

It has been shown that olive oil consumption may reduce the risk of many diseases associated with oxidative damage, such as coronary heart diseases and cancers (Newmark 1997). The compounds detected in the present study are reported to be potential therapeutic agents, with analgesic, anti-inflammatory, and antimicrobial effects. The results obtained were consistent with many reports that have indicated that polyphenols possess potent antioxidant and anti-cancer, anti-carcinogenic, anti-bacterial, anti-viral, and anti-inflammatory activities and play a vital role in the metabolism of plants (Tapiero et al. 2002). The high demand for olive oil is based on dietary habits correlated with health benefits (Allalout et al. 2011). Monounsaturated fatty acids, tocopherols, and phenolic compounds have great importance in biological systems and act as natural antioxidants (Bendini et al. 2007). Epidemiological studies of cancer (breast and pancreatic) have demonstrated that decreased risk or no enhancement in the risk of cancer is associated with increased dietary intake of olive oil despite the higher proportion of overall lipid intake (Newmark 1997). Furthermore, phytosterols play a vital role in decreasing the blood cholesterol levels because of their antioxidant activities and their impact on health (Ostlund 2004).

Among the olive oil varieties included in the present study, were Spanish sweet oil producing variety, Arbequina, which is used as the standard in fatty acid composition (Lopez-Cortes et al. 2013) and bitter spicy oil producing variety, Picual, which is considered as the standard for the bitter spicy olive oils having the highest content of phenolic compounds (Nieto et al. 2010). In the present study, the Saudi wild accession was also recorded to have a high number of fatty acids and their esters, which can be used for generating new genotypes with interesting characters.

Conclusion

Both DNA-based markers (RAPD and ISSR) showed high allelic variation and have the power to discriminate the olive cultivars and the Saudi wild accession. A large number of phytochemical compounds were identified and the wild accession should be given more attention to understand why it was so different from the olive cultivars. Concordance of genetic and phytochemical analysis indicated that these two analyses grouped the genotypes (wild accession and cultivars) in a similar manner. Such information may prove useful in the selection of optimal varieties and help promote continued progress in olive breeding strategies.

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Compliance with ethical standards

Conflict of interest Authors declare no conflict of interest of any type.

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