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Original Article

Using Two Retrotransposon Based Marker Systems (IRAP and REMAP) for Molecular Characterization of Olive (*Olea europaea* L.) Cultivars

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Abstract

Olive (*Olea europaea* L.) is one of the most characteristic agricultural trees of the Mediterranean region and has a large number of cultivar diversity. Olive cultivar characterization is very important especially for the fruit productivity and olive oil quality. In the present study, 46 clones belonging to Turkey (eight cultivars, each having five clones) and Italy (two cultivars, each having three clones) were assessed for cultivar characterization via inter-retrotransposon amplified polymorphism (IRAP) and retrotransposon-microsatellite amplified polymorphism (REMAP) marker systems using 10 LTR and 10 ISSR primers. In total, 368 band profiles were obtained, 358 of which are polymorphic (97.28% polymorphism). The cultivars were segregated into three main groups, each group having several branches, where all the clones of each cultivar were belonging to the same main group. The only exception to that was the distribution of the clones of cultivar Yaglik, 'Yaglik 4' and 'Yaglik 5', into different main groups. IRAP and REMAP analysis showed a high level of genetic variability among the olive cultivars in this study and this marker systems would be useful tool for clonal selection programs.

Keywords: LTRs, olive cultivar diversity, molecular marker

Abbreviation list: CTAB (Cetyl trimethyl ammonium bromide); IRAP (Inter-retrotransposon amplified polymorphism); ISSR (Inter simple sequence repeat); LTR (Long terminal direct repeat); REMAP (Retransposon-microsatellite amplified polymorphism).

Introduction

Olive (*Olea europea* L.) has more than 2600 cultivars, and has been cultivated since the ancient times in the Mediterranean area, where it is still the most significant oil-producing crop, the region accounts for not less than 97% of the world production and 91% of world consumption of olive oil (Luchetti, 1993; Rugini and Lavee, 1992; Zohary and Hopf, 1994). The cultivated form of olive (*O. europaea* L. var. *europaea*) is produced from the seedlings of wild form of olive (*O. europaea* L. var. *sylvestris*) by cutting or grafting (Green, 2002), where these two interfertile olive forms produce a large number of varieties with high levels of heterozygosity and genetic diversity among predominantly allogamus cultivars (Angiolillo *et al.*, 1999; Diaz *et al.*, 2006). This variability in olive cultivars makes the cultivar identification extremely difficult, which is actually crucial for the determination of olive productivity and oil quality, i.e., properties inherited to a variety (Fiorino and Rallo, 1999). In this respect, molecular markers are very useful for characterization of olive varieties and detection of synonymous and homonymous. Indeed, wide range of DNA molecular marker types have been used for genetic variability and cultivar identification of olive during the last ten years such as RAPDs (Hess *et al.*, 2000; Bronzini de Caraffa *et al.*, 2002; Martins-Lopes *et al.*, 2007; Zitoun *et al.*, 2008; Awan *et al.*, 2011; Figueiredo *et al.*, 2013), AFLPs (Grati-Kamoun *et al.*, 2006; Montemurro *et al.* 2008; Albertini *et al.*, 2011), ISSRs (Gomes *et al.* 2008; Martin-Lopes *et al.*, 2009; Beiki *et al.*, 2012), SSRs (Muzzalupo *et al.*, 2009; Belaj *et al.*, 2010; Corrado *et al.* 2011; Cicatelli *et al.*, 2013), SNPs (Reale *et al.*, 2006), DArTs (Atienza *et al.*, 2013).

Retrotransposons are mobile genetic elements through the eukaryotic genomes, especially in plants, they are considered to act an important role in genome evolution (Flavell *et al.*, 1992; Vicient *et al.*, 2001; Giordani *et al.*, 2004). They cover about 50% of repetitive DNA of the whole genome in higher plants (Kumar and Bennetzen, 1999). Their unstable genomic locations, flexible copy numbers, length, described and conserved sequences provide them with an advantage to be a more specific genetic marker for plant biodiversity and genome analysis (Queen *et al.*, 2004; Agarwal *et al.*, 2008; Vukich *et al.*, 2009; D'Onofrio *et al.*, 2010; Kalendar *et al.*, 2011). Retrotransposons can be classified into three types according to their structural organization and amino acid similarities. Copia-like (Kumar *et al.*, 1996) and gypsy-like retrotransposons (Suoniemi *et al.*, 1998) belong to long terminal direct repeats (LTRs), they encode proteins similar to the retroviruses, and they are present over

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the plant kingdom. Non-LTR retrotransposons defect terminal repeats and encode proteins with significantly less similarity to those of the retroviruses (Agarwal *et al*., 2008).

IRAP and REMAP marker systems, in contrast to other techniques, characterize large genetic dissimilaries in the cultivars. Integration of retrotransposon creates new links between genomic DNA and their conserved ends, for this reason, they can be used as useful molecular markers. Retrotransposon-based marker systems are an important source of plant genetic diversity and this system mostly use PCR to reproduce a segment of genomic DNA at this link (Kalendar and Schulman, 2006). Therefore, genetic differentiation perseveres through the old copies, but insertion of new copies arises. The ancestral and reproduced typical locus differentiations become potential as the lack of the introduced sequence can be, with high reliance, conceived ancestral. Basicly, the presence of a fixed retrotransposon in relevant taxa recommends their orthologues integration while the lack of specific elements shows the plesiomorphic condition prior to integration in more different taxa. A phylogenetic tree of species based on the presence of retrotransposons dispersion and its irreversible facts during evolution can build this presence/absence analyses. This is why retrotransposons are accepted to show strong synapomorphies (Shedlock and Okada, 2000).

Inter-retrotransposon amplified polymorphism (IRAP), where the fragments are amplified with LTR primers while in the retrotransposon-microsatellite amplified polymorphism (REMAP), where the fragments are amplified with a LTR and an ISSR primer. Both retrotransposon-based marker systems based on the position of the given LTRs within the genome (Kalendar *et al*., 1999). The IRAP and REMAP marker systems have been developed essentially for BARE-I retrotransposon of barley (Kalendar *et al*., 2000), and both have been used to detect similarity of rice (Branco *et al*., 2007), Triticum (Pagnotta *et al*., 2009), grapevine (D'Onofrio *et al*., 2010), Citrus (Biwas *et al*., 2010), Japanese apricot (Yuying *et al*., 2011), potato (Sharma and Nandieni, 2014) and Hordeum (Cabo *et al*., 2014).

There are only few reports available on application of retrotransposon-based marker systems for molecular identification in olive. The first study, reported by Hernandez *et al*. (2001), presents the first evidence of a retrotransposon-like element in olive using SCAR-markers. Giordani *et al*. (2004) and Koksal *et al*. (2014) reported genetic diversity in olive cultivars using retrotransposon-based marker system, as well. They used the IRAP (Koksal *et al*, 2014) and REMAP (Giordini *et al*., 2004) marker systems for molecular characterisation of olive cultivars. These reports can be considered as the first comprehensive research, where retrotransposon-based marker technique is used on olive genome. However, these short presentations have not generated yet a published research article.

Materials and Methods

Plant Material

46 olive clones belonging to 10 olive cultivars were analysed. 8 cultivars were obtained from different cities of Turkey (Balıkesir cv. 'Edincik'; Bursa-Gemlik cv. 'Gemlik'; Hatay cv. 'Edremit'; Mardin cv. 'Halhali'; Mugla cv. 'Domat'; Samsun cv. 'Alacam'; cv. 'Tekir' and cv. 'Yaglik', obtained from Olive Research Institute, Izmir) while the other 2 (cv. 'Canino' and cv. 'Frantoio') were obtained from CNR (National Research Council) / IVALSA (Trees and Timber Institute), Italy.

DNA Extraction

The total genomic DNA was extracted by using CTAB method (Doyle and Doyle, 1987) after grinding the young leaf tissue to a fine powder. DNA sample concentration was determined using a nanodrop spectrophotometer (BioSpec-nano; Shimadzu-Biotech). DNA samples were diluted to 50 ng/µl prior to IRAP and REMAP PCR amplifications.

IRAP (Inter-Retroelement Amplified Polymorphisms) PCR

IRAP-PCR DNA amplification was performed using 10 IRAP primers (LTR 1-13; Smykal *et al*., 2011; Table 1). Amplifications were performed according to Kalendar *et al*. (2011) in a 25 µl reaction volume, containing PCR Buffer (1x final concentration, invitrogen), 2,5 mM $MgCl_2$, 0,4 mM of each dNTP, 0,4 mM IRAP primer, 50 ng genomic DNA, and 2 unit Taq DNA polymerase. Amplification conditions (thermocycler Model-9700, Perkin-Elmer, Boston, MA, USA) were as follows: initial denaturation at 95 °C for 3 min, 35 cycles at 95 °C for 15 sec, 55 °C for 30 sec, a ramp to 72 °C reaching in 3 min, followed by a 10 min lag at this temperature, and an indefinite holding at 4 °C, respectively. Amplicons were separated on 1.5% agarose gel at 80 V. They were then stained with 0.5 µg/ml ethidium bromide solution, visualized by illumination under UV light, and documented using a gel documentation and image analysis system (BIORAD, Molecular Imager®, ChemiDocTM XRS+ with Image LabTM Software).

REMAP (Retrotransposon-Microsatellite Amplified Polymorphism) PCR

REMAP-PCR DNA amplification was performed using a combination of 10 LTR primers (0.2 mM for each reaction) and 10 ISSR primers, each primer at the concentration of 0.2 mM for each reaction (Martins-Lopes *et al*., 2009; Smykal *et al*., 2011; Table 2). Amplification conditions and separating were the same as for IRAP PCRs (see above). DNA fragments of IRAP and REMAP PCRs were scored by their presence (1) or absence (0), and the ones at low intensities were scored only if they were reproducible in both the PCR runs. Cluster analysis was performed to construct dendrograms, with the unweighted pair-group method by arithmetic averages (UPGMA) from the similarity data matrices using Jaccard's coefficient (D-UPGMA, 2002).

Results and Discussion

Molecular fingerprinting of forty six clones belonging to ten cultivars was carried out using IRAP and REMAP analysis, and very high polymorphism (97.28%, in average) was detected by both the methods. The total of 368 reproducible bands, ranging from 125 to 3600 bp, were scored. 126 bands were obtained by IRAP and 242 were by REMAP techniques, with a similar polymorphism rates of 96.82% (122 polymorphic bands) and 97.52% (236 polymorphic bands), respectively. The highest polymorphism rate was obtained by REMAP PCR 2 amplification, and produced 23 polymorphic bands (Fig. 1).

The dendrogram of 46 clones belong to 10 olive cultivars amplified by the IRAP markers is shown in Fig. 2. The genetic similarities ranged from 0,013 (between Y1 cv. 'Yaglik' and T4-T5 cv. 'Tekir'; between H1 and H2 cv. 'Edremit') to 0,75 (between B1 cv. 'Edincik' and S1 cv. 'Alacam'), while T3 and T4 cv. 'Tekir' were shown to be similar. The cultivars were grouped into eight clusters; *Cluster I*, B1-5, (Balıkesir cv. 'Edincik'); *Cluster II*, G1-5 (Bursa-Gemlik cv. 'Gemlik'), H1-5 (Hatay cv. 'Edremit'); *Cluster III*, Ma

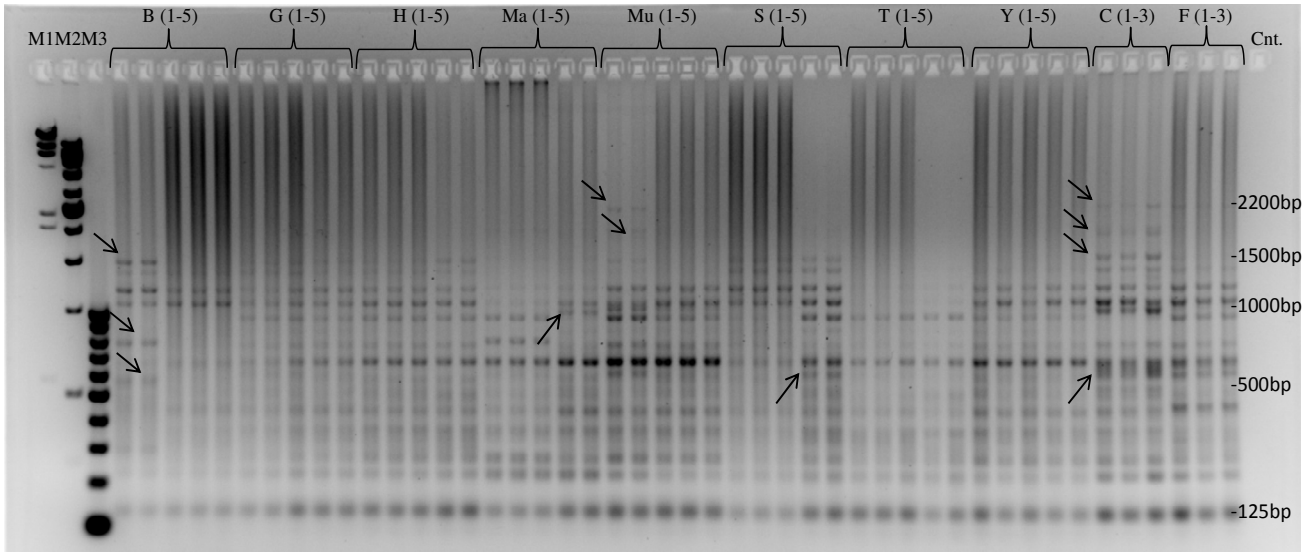

Fig. 1. REMAP 2 amplification products obtained in 1,5% agarose gel from fourty-six clones belong to ten olive cultivars with LTR 1 primer and ISSR 2 [B, 'Burhaniye'; G, 'Gemlik'; H, 'Hatay'; Ma, 'Mardin'; Mu, 'Mugla'; S, 'Samsun'; T, 'Tekir'; Y, 'Yaglik'; C 'Canino' and F 'Frantoio'; M1, Lambda DNA/Hind III marker (vivantis); M2, 1kb ladder (GeneRuler™), M3, 100bp ladder (GeneRuler™); Cnt. (- Control), Some of polymorphic band profiles were shown with arrows]

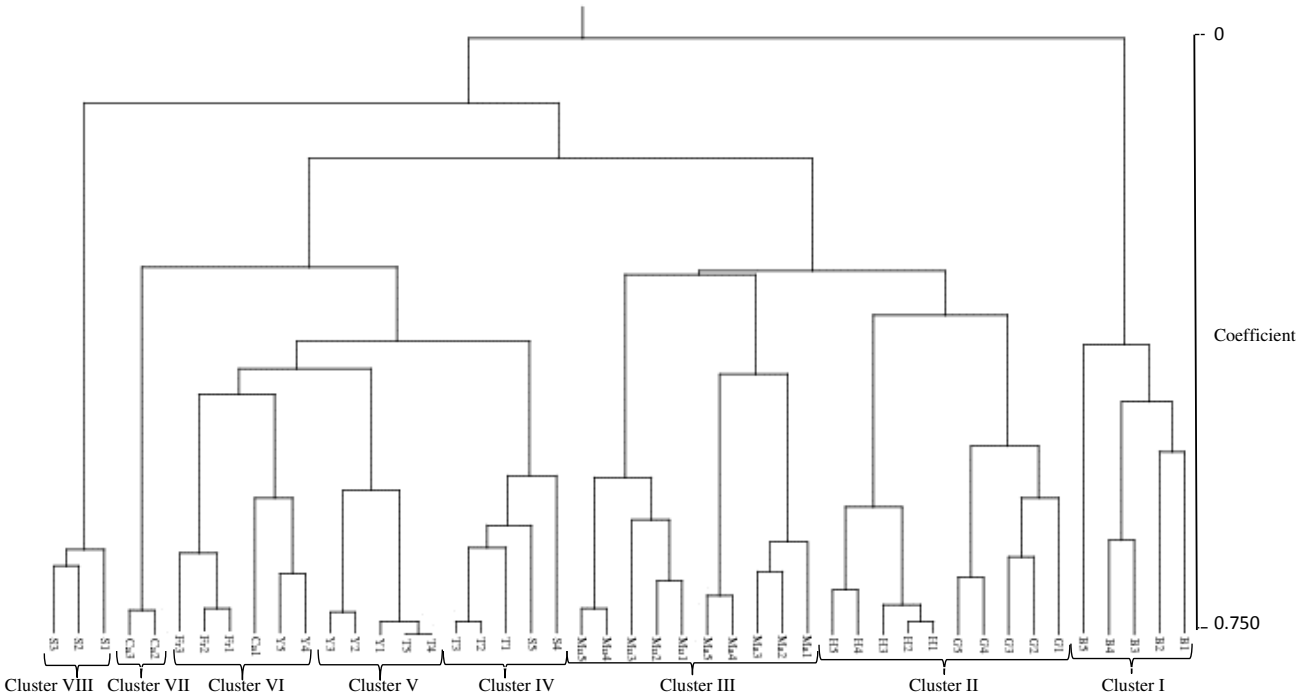

Fig. 2. UPGMA dendrogram based on Jaccard's coefficient illustrating the genetic similarities and distance among olive cultivars obtained by IRAP data, generated by the UPGMA cluster analysis (NTSYS). Cluster I, B1-5, (Balıkesir cv. 'Edincik'); Cluster II, G1-5 (Bursa-Gemlik cv. 'Gemlik'), H1-5 (Hatay cv. 'Edremit'); Cluster III, Ma 1-5 (Mardin cv. 'Halhali'), Mu 1-5 (Mugla cv. 'Domat'); Cluster IV, S4-5 (Samsun cv. 'Alacam'), T1-3 (cv. 'Tekir'); Cluster V, T4-5 (cv. 'Tekir'), Y1-3 (cv. 'Yaglik'); Cluster VI, Y4-5 (cv. 'Yaglik'), Ca1 (cv. 'Canino'), Fr1-3 (cv. 'Frantoio'); Cluster VII, Ca1-3 (cv. 'Canino') and Cluster VIII, S1-3 (Samsun cv. 'Alacam')

1-5 (Mardin cv. 'Halhali'), Mu 1-5 (Mugla cv. 'Domat'); *Cluster IV*, S4-5 (Samsun cv. 'Alacam'), T1-3 (cv. 'Tekir'); *Cluster V*, T4-5 (cv. 'Tekir'), Y1-3 (cv. 'Yaglik'); *Cluster VI*, Y4-5 (cv. 'Yaglik'), Ca1 (cv. 'Canino'), Fr1-3 (cv. 'Frantoio'); *Cluster VII*, Ca2-3 (cv. 'Canino') and *Cluster VIII*, S1-3 (Samsun cv. 'Alacam'). However, each cluster divided into some sub-clusters, for example *Cluster II* and *III* divided into two sub-clusters (Fig. 2). On the other hand, according to the dendrogram of the REMAP markers, the cultivars were grouped into five clusters (Fig. 3; *Cluster I*, B1-5, (Balıkesir cv. 'Edincik'); *Cluster II*, G1-5 (Bursa-Gemlik cv. 'Gemlik'), H1-5 (Hatay cv. 'Edremit'); *Cluster III*, Ma 1-5 (Mardin cv. 'Halhali'), Mu 1-5 (Mugla cv. 'Domat'); *Cluster IV*, S1-5 (Samsun cv. 'Alacam'), T1-5 (cv. 'Tekir'), Y1-3 (cv. 'Yaglik') and *Cluster V*, Y4-5 (cv. 'Yaglik'), Ca1-3 (cv. 'Canino'), Fr1-3 (cv. 'Frantoio'). The genetic similarities ranged from 0,054 (between Ma3 and Ma cv. 'Halhali') to 0,735 (between B1 cv. 'Edincik' and H2 cv. 'Edremit'). However, each cluster divided into some sub-clusters, for example, *Cluster II, III* and *IV* divided into three sub-clusters (Fig. 3).

Genetic similarities/varieties were obtained with combined (IRAP and REMAP) UPGMA algorithm using Jaccard's coefficient (Fig. 4). The genetic similarities ranged from 0,068 (Ma 1 – Ma 2, Mardin cv. 'Halhali') to 0,705 (B1, Balıkesir cv. 'Edincik' – S1, Samsun cv. 'Alacam'). The cultivars were grouped into four major clusters; *Cluster I*, B1-5, (Balıkesir cv. 'Edincik'); *Cluster II*, G1-5 (Bursa-Gemlik cv. 'Gemlik'), H1-5 (Hatay cv. 'Edremit'), Ma 1-5 (Mardin cv. 'Halhali'), Mu 1-5 (Mugla cv. 'Domat'); *Cluster III*, S1-5 (Samsun cv. 'Alacam'), T1-5 (cv. 'Tekir'), Y1-3 (cv. 'Yaglik') and *Cluster IV*, Y4-5 (cv. 'Yaglik'), Ca1-3 (cv. 'Canino'), Fr1-3 (cv. 'Frantoio'). However, each cluster divided into some sub-clusters, for example *Cluster I* and *IV* divided into two sub-clusters, *Cluster II* divided into four sub-clusters, *Cluster III* divided into three sub-clusters (Fig. 4).

Some close relationships between cultivars were constant in all IRAP and REMAP analyses performed; for instance, cv. 'Gemlik' and cv. 'Edremit' (similarity ranges from 0.393 to 0.581); cv. 'Halhali' and 'Domat' (similarity ranges from 0.319 to 0.587) and Italian

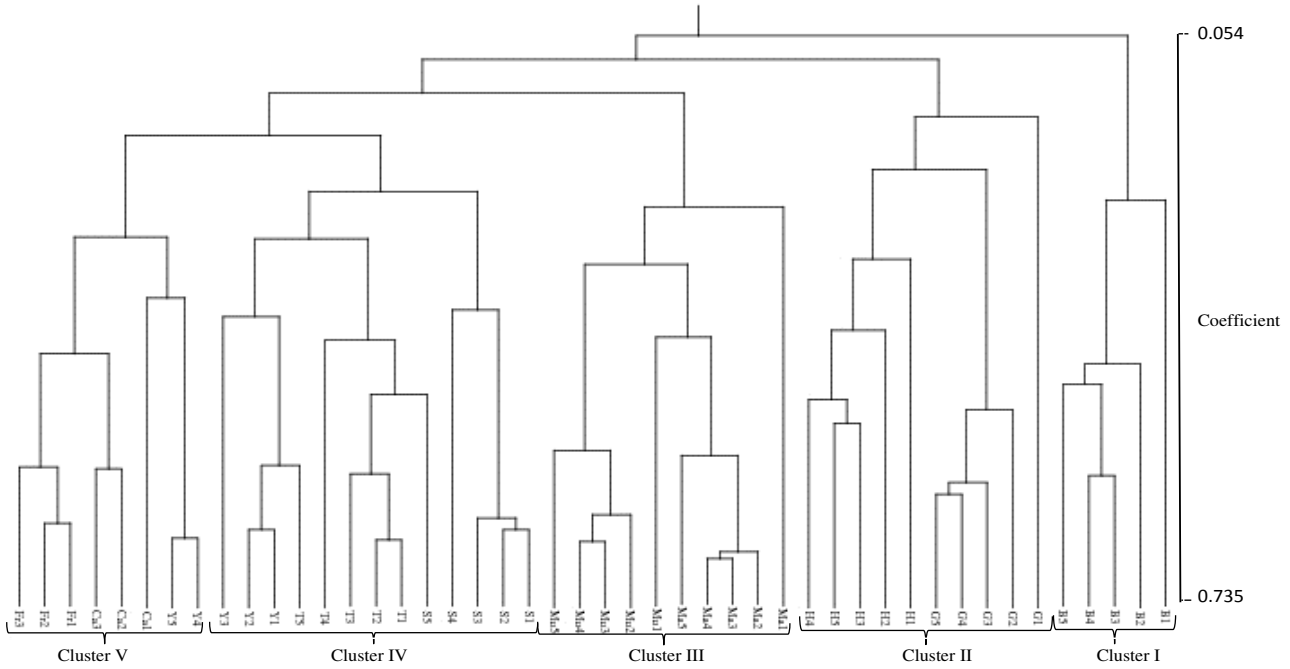

Fig. 3. UPGMA dendrogram based on Jaccard's coefficient illustrating the genetic similarities and distance among olive cultivars obtained by REMAP data, generated by the UPGMA cluster analysis (NTSYS). Cluster I, B1-5, (Balıkesir cv. 'Edincik'); Cluster II, G1-5 (Bursa-Gemlik cv. 'Gemlik'), H1-5 (Hatay cv. 'Edremit'); Cluster III, Ma 1-5 (Mardin cv. 'Halhali'), Mu 1-5 (Mugla cv. 'Domat'); Cluster IV, S1-5 (Samsun cv. 'Alacam'), T1-5 (cv. 'Tekir'), Y1-3 (cv. 'Yaglik') and Cluster V, Y4-5 (cv. 'Yaglik'), Ca1-3 (cv. 'Canino'), Fr1-3 (cv. 'Frantoio')

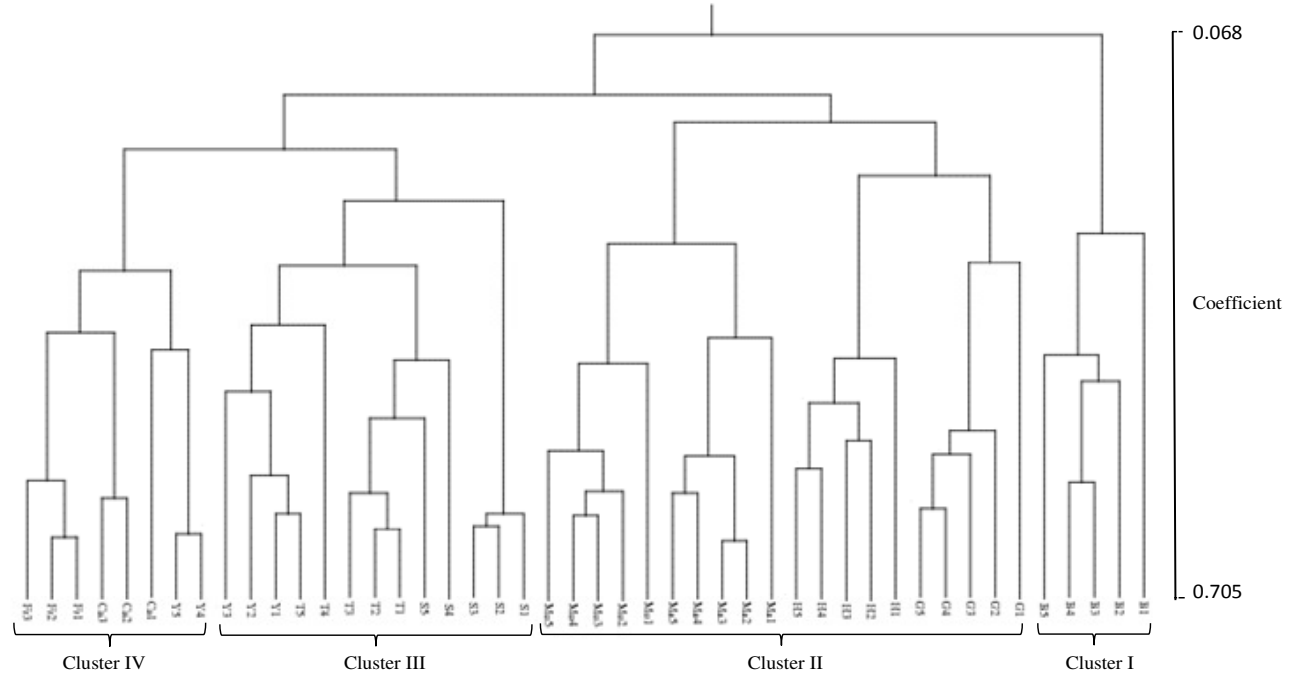

Fig. 4. UPGMA dendrogram based on Jaccard's coefficient illustrating the genetic similarities and distance among olive cultivars obtained by combined IRAP and REMAP data, generated by the UPGMA cluster analysis (NTSYS). Cluster I, B1-5, (Balıkesir cv. 'Edincik'); Cluster II, G1-5 (Bursa-Gemlik cv. 'Gemlik'), H1-5 (Hatay cv. 'Edremit'), Ma 1-5 (Mardin cv. 'Halhali'), Mu 1-5 (Mugla cv. 'Domat'); Cluster III, S1-5 (Samsun cv. 'Alacam'), T1-5 (cv. 'Tekir'), Y1-3 (cv. 'Yaglik') and Cluster IV, Y4-5 (cv. 'Yaglik'), Ca1-3 (cv. 'Canino'), Fr1-3 (cv. 'Frantoio')

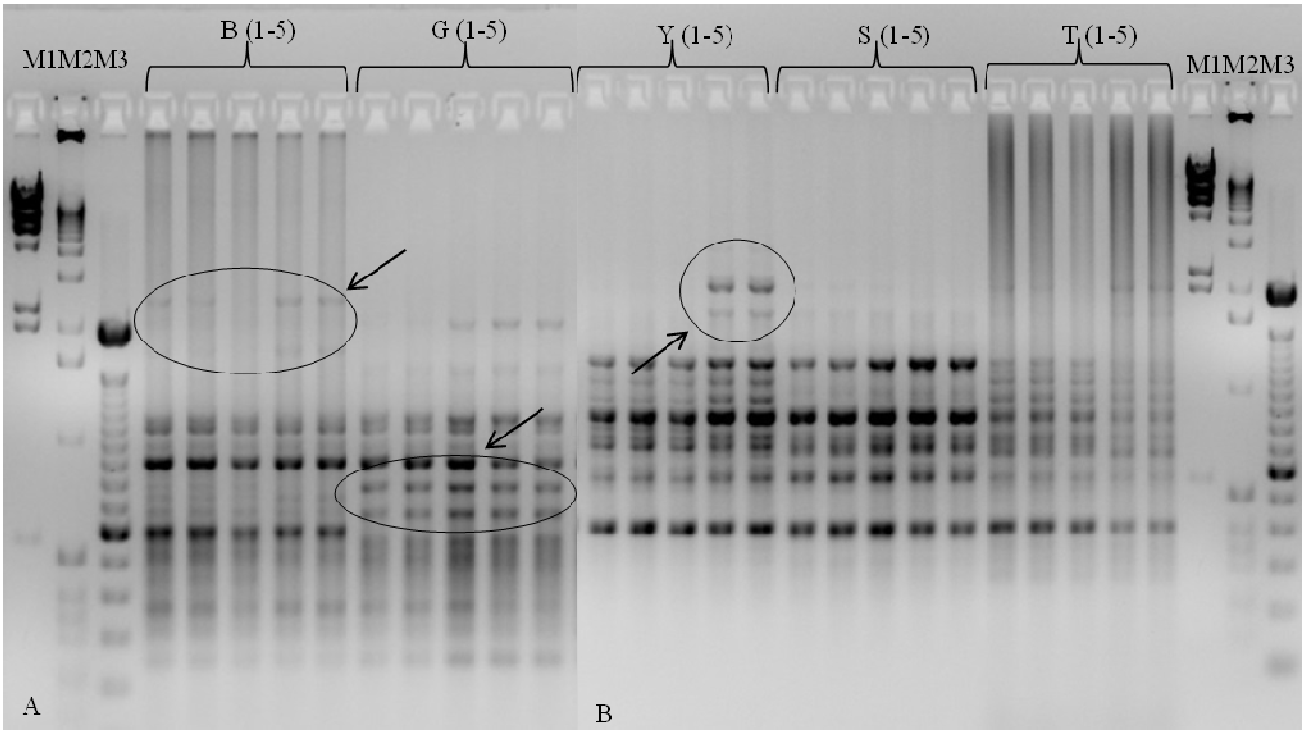

Fig. 5. REMAP 4 using LTR 1 and ISSR 4 primers (A) and IRAP 2 using LTR 2 primer (B) amplification products obtained in 1,5% agarose gel [B, 'Burhaniye'; G, 'Gemlik'; S, 'Samsun'; T, 'Tekir'; Y, 'Yaglik'; M1, Lambda DNA/Hind III marker (vivantis); M2, 1kb ladder (GeneRulerTM), M3, 100bp ladder (GeneRulerTM); Cnt. (- Control), Some of polymorphic band profiles were shown with circles and arrows]

Table 1. LTR and ISSR primers (Martins-Lopes *et al*., 2009; Smykal *et al*., 2011)

LTR Primers	Sequence 5'-3'	Gene Bank accession number	ISSR Primers	Sequence 5'-3'	Gene Bank accession number
LTR 1	ACCCCTTGAGCTAACTTTTGGGGTAAG	GU735096	ISSR 1	$(AG)_8T$	UBC 807
LTR 2	CTTGCTGGAAAGTGTGTGAGAGG	GU929874	ISSR 2	$(AG)_8G$	UBC 809
LTR 3	TGTTAATCGCGCTCGGGTGGGAGCA	DQ767972	ISSR 3	$(GA)_8T$	UBC 810
LTR 4	AGCCTGAAAGTGTTGGGTTGTCG	GU980589	ISSR 4	$(GA)_8C$	UBC 811
LTR 5	CTGGCATTTCCATTGTCGTCGATGC	GU980588	ISSR 5	$(CA)_8A$	UBC 817
LTR 6	GCATCAGCCTGGACCAGTCCTCGTCC	GU929878	ISSR 6	$(TC)_8C$	UBC 823
LTR 7	CACTTCAAATTTTGGCAGCAGCGGATC	GU735096	ISSR 7	$(AC)_8C$	UBC 826
LTR 8	TCGAGGTACACCTCGACTCAGG	GU929877	ISSR 8	$(AG)_8CTT$	UBC 846
LTR 9	ATTCTCGTCCGCTGCGCCCCTACA	GU980590	ISSR 9	$(CA)_8AGT$	UBC 855
LTR 10	TGAGTTGCAGGTCCAGGCATCA	GU980587	ISSR 10	$(GT)_8CTA$	UBC 856

Table 2. REMAP primer combinations

	Primer Combination		Primer Combination
REMAP 1	LTR 1 and ISSR 1	REMAP 11	LTR 2 and ISSR 2
REMAP 2	LTR 1 and ISSR 2	REMAP 12	LTR 3 and ISSR 3
REMAP 3	LTR 1 and ISSR 3	REMAP 13	LTR 4 and ISSR 4
REMAP 4	LTR 1 and ISSR 4	REMAP 14	LTR 5 and ISSR 5
REMAP 5	LTR 1 and ISSR 5	REMAP 15	LTR 6 and ISSR 6
REMAP 6	LTR 1 and ISSR 6	REMAP 16	LTR 7 and ISSR 7
REMAP 7	LTR 1 and ISSR 7	REMAP 17	LTR 8 and ISSR 8
REMAP 8	LTR 1 and ISSR 8	REMAP 18	LTR 9 and ISSR 9
REMAP 9	LTR 1 and ISSR 9	REMAP 19	LTR 10 and ISSR 10
REMAP 10	LTR 1 and ISSR 10		

cultivars 'Canino' and 'Frantoio' (similarity ranges from 0.245 to 0.379). On the contrary, B1-5, Balıkesir cv. 'Edincik' (*Cluster I*) indicated independent branches from the other cultivars (Fig. 1) and this cultivar had many polymorphic bands in the most of PCR gel analysis (Fig. 5A). On the other hand, the combined dendrograms indicated that clones Y1, 2 and 3 (*Cluster III*) and Y4 and 5 (*Cluster IV*) of cv. 'Yaglik' were in different groups. This was not surprising as there were many polimorfic bands in PCR gel analysis (Fig. 5B).

Retrotransposon-based marker techniques have been extensively used to determinate genetic relationships between numerous plant species and cultivars (Queen *et al*., 2004; Branco *et al*., 2007; Agarwal *et al*., 2008; Vukich *et al*., 2009; D'Onofrio *et al*., 2010). However, there have been a few reports on olive cultivar identification via retrotransposon-based marker techniques and one of them used SCAR marker (Hernández *et al*., 2001). Accordingly, the present report will be the first comprehensive study on molecular characterization of olive cultivars using both IRAP and REMAP molecular markers. All primers gave obvious amplification patterns. The co-dominant nature of these markers detected to higher levels of expected heterozygosity.

Although the two marker systems produced different cluster numbers in all cultivars according to the dendrogram analyses, high compatibility was obtained from both and their polymorphism rate was very similar (96.82% for IRAP and 97.52% for REMAP). The high level of polymorphism was detected with B1-5, Balıkesir cv. 'Edincik' by both the two marker systems; indeed this cultivar was very distant from the others and was grouped into different cluster (it

was seen in "*Cluster I*" for three dendrograms). This cultivar is very different from the others also for the morphological characteristics; it has relatively bigger fruits, low oil and high water content (Isik *et al.,* 2011).

The dendrogram analyses almost fully matched with same clones, however there was some evidence for clustering of clones derived from different branches. Clone (cv. 'Yaglik') Y1-3 and Y4-5 were in different groups and their similarity ranges were between 0.419 and 0.480. These differences could be the result of cross-pollination with local populations (Contento *et al*., 2002), somatic mutations (Belaj *et al*., 2004), and sometimes could also be due to the presence of a high level of homonymy in the collection. This is a significant problem and is a great risk for olive producers, as the renewal of certified orchards should be based on certified plants (Gemas *et al*., 2004; Martins-Lopes *et al*., 2007; Hannachi *et al*., 2008). However, classical olive certification system is based on morphological and agronomic procedures, which are affected by the environmental conditions, and mislabeling accessions can negatively affect certification of olive products (Hannachi *et al*., 2008). Molecular marker systems are of great importance to overcome such problem, and is necessary to determine the polymorphism level of olive cultivars and homonymy and synonym problems in olive germplasm. High values of observed heterozygosity were recorded for all the IRAP and REMAP markers investigated.

Determination of genetic relationships among cultivars eases efficient sampling, operating and using of germplasm resources. In the present study, IRAP and REMAP analysis displayed a high level of genetic variability among olive cultivars, indicating a potential resource for the use of this germplasm in clonal selection programs.

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Conflict of interest

The authors declare that they have no conflict of interest.

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