

Genetic stability versus somaclonal variation in tissue culture regenerated olive plants (*Olea europea* cv. Kroneiki)

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ABSTRACT

Genetic stability versus somaclonal variation was studied in one-year old tissue culture regenerated olive plants by using SSR and ISSR molecular markers. Fifty olive plants including mother plant were used in this study. NJ tree and NeighborNet analysis grouped the studied plants in three distinct groups, which were supported by K-Means clustering and Evanno test. AMOVA and Gst analyses revealed significant molecular difference among these three genetic groups. STRUCTURE analysis showed genetic variability both within and among these groups. The regenerated plants differed in allelic composition and allele frequency. The somaclonal variants obtained may be used in further hybridization and olive breeding program.

Key words: ISSR, *Olea europea* L., SSR, somaclonal variation

INTRODUCTION

The genus *Olea* belongs to the Oleaceae family, which comprises approximately 30 genera with 600 species. *O. europaea* L. the Mediterranean olive tree, is the only species cultivated for oil extraction and table consumption, which accounts more than 3000 of cultivars. Many of these cultivars might be just different landraces stemmed from the same original genetic stock or different named varieties derived from the same original genetic stock [1].

Cultivated olive is an ancient plant species and is considered as one of the most important tree crop species of the Mediterranean basin, representing not only the 90% of the olive groves of the world but also the 90% of the olive world production [2]. Only Spain, Italy, and Greece produced around 75% of the world's olive oil, and together with Turkey and Tunisia are the five largest producers in the world. The world production and consumption trend of olive oil in the last 30 years have increased significantly and will continue, considering the recent introduction of its cultivation in Japan, USA, Australia, China, South America and South Africa [1].

Micropropagation of woody plants and fruit crops constitutes a major success in the commercial application of in vitro cultures. An important aspect to be considered when deriving perennial plants from micropropagation is the maintenance of genetic integrity with regard to the mother plant. In this regard, somaclonal variation has been reported at different levels (morphological, cytological, cytochemical, biochemical, and molecular) in micropropagated plants. The economic consequence of somaclonal variation among regenerated plants is enormous in fruit crops and woody plants, because they have long life cycles. In consequence, the behavior of micropropagated plants should be assessed after their long juvenile stage in field conditions [3].

Somaclonal variation has been studied extensively in herbaceous plants, whereas few studies have focused on temperate perennial fruit crops. Somaclonal variation is the occurrence of genetic variants among the regenerated plants of a single subculture derived from *in vitro* procedures and developmental genetic changes occur during several sub-culturing among the regenerated plants [4], it is also called tissue or culture-induced variation [5, 6].

Studies on somaclonal variation are important for its control and possible suppression with the aim of producing genetically identical plants, and for its use as a tool to produce genetic variability, which will enable breeders the genetic improvement. Identification of possible somaclonal variants at an early stage of development is very useful for quality control in plant tissue culture, transgenic plant production and in the introduction of variants [7-9].

Many strategies can be used to evaluate plant genetic structure from *in vitro* derived plant clones, including cytogenetic analysis and isoenzyme markers and different DNA molecular markers, but most of them have limitations. Various molecular markers including restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragments length polymorphism (AFLP), inter simple sequence repeats (ISSR) and simple sequence repeats (SSR) [7, 8, 10] have been used in somaclonal and genetic diversity analyses.

The present study considers genetic analysis of one-year old, field grown regenerated plants of olive cultivar cv. Kroneiki for genetic stability versus induced somaclonal variation by using SSR and ISSR molecular markers.

MATERIALS AND METHODS

Propagation of microshoots

Actively growing shoots of olive (cv. Kroneiki) were collected from one-year-old greenhouse grown plants. Leaves were removed and sterilized with bleach (20%) for 5 min, then rinsed three times in sterile distilled water. Apical buds of sterile shoots were removed and shoots were cut into the single nod segments. Uni-nodal segments of sterilized shoots of *Olea europea* L. (cv. Kroneiki) were cultured in DKW [11] medium supplemented with 2-isopentenyl adenine (4 mg l^{-1}). The pH was adjusted to 5.8 before agar addition and autoclaving. Cultures were kept at $23 \pm 2^\circ\text{C}$ and 16h photoperiod. After 60 days, *in vitro* shoots (4 -5 nods) raised from explants, they were used for rooting experiences.

Ex vitro rooting and acclimatization

In vitro shoots that were grown on DKW with hormone medium were treated by dipping the base of microshoots in the IBA (500 mg l^{-1}) for 15 min and then the explants were directly transferred to the jiffy pots. Shoots were kept in a transparent box. Thirty days after treatments length and number of roots were measured.

Genetic variability analysis

Five randomly selected leaves of 49 shoots (No. 1 to 49) as well as mother plant (No.50) were used for DNA extraction and molecular analysis. Genomic DNA was extracted using CTAB activated charcoal protocol [12]. The quality of extracted DNA was examined by running on 0.8% agarose gel.

Ten ISSR primers; (CA)7GT, (AGC)5GG, (GA)9A, (GT)7CA, (CA)7AT, (GA)9C, UBC807, UBC810, UBC811 and UBC834 commercialized by UBC (the University of British Columbia) and Five SSR primers, OeUA-DCA-3, OeUA-DCA-9, OeUA-DCA-11, UDO99-011, and UDO99-43 [13, 14] were used.

SSR and ISSR PCR reactions were performed in a 25 μl volume containing 10 mM Tris-HCl buffer at pH 8; 50 mM KCl; 1.5 mM MgCl_2 ; 0.2 mM of each dNTP (Bioron, Germany); 20 ng genomic DNA and 1 U of *Taq* DNA polymerase (Bioron, Germany); 0.2 μM of a single primer for ISSR and 0.2 μM of forward and reverse primers for SSR reactions.

The amplifications' reactions for ISSR- PCR were performed in Techne thermocycler (Germany) with the following program: 5 min initial denaturation step 94°C , 35 cycles of 30 s at 94°C ; 1 min at 50°C and 1min at 72°C . The reaction was completed by final extension step of 7 Min at 72°C . The SSR-PCR reaction was carried out with a denaturation at 94°C for 5 min, 35 cycles of 94°C for 20 s, the annealing temperature 50°C for 30 s and 72°C for 30 s and final extension at 72°C for 7 min.

The amplification products were visualized by running on 2.5% agarose gel, followed by the ethidium bromide staining. The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

Data analyses

ISSR and SSR bands obtained were treated as binary characters and coded accordingly (presence = 1, absence = 0). Nei's genetic distance was determined among the studied populations and used for clustering [15, 16]. For grouping of the plant specimens, Neighbor Joining (NJ) clustering and NeighborNet methods were performed after 100 times bootstrapping [15]. Similarly, principal co-ordinate analysis (PCoA), and multidimensional scaling (MDS) [17] were performed. We used PAST ver. 2.17 [18], DARwin ver. 5 (2012) and Splits Tree4 ver. 4 (2013) programs were used for these analyses.

Two methods were used to determine population stratification and genetic fragmentation. First we performed Evanno method for identifying the proper number of K [19]. For this we did model-based clustering by using STRUCTURE ver. 2.3 [20]. The Markov chain Monte Carlo simulation was run 20 times for each value of K (2–6) for 106 iterations, after a burn-in period of 10^5 . All other parameters were set at their default values. Data were scored with dominant markers and analysis following the method suggested by Falush *et al.* [20]. STRUCTURE Harvester web site [21] was used to perform Evanno test based on ΔK value.

Second, we performed K-Means clustering as done in GenoDive ver. 2. (2013). In the K-means clustering, the optimal clustering is the one with the smallest amount of variation within clusters. This is calculated by using the within-clusters sum of squares. The minimization of the within-groups sum of squares that is used in K-Means clustering is, in the context of a hierarchical AMOVA, equivalent to minimizing the among-populations-within-groups sum of squares, SSDAP/WG [22].

We used two summary statistics to present K-Means clustering, 1- pseudo-F [23] and 2- Bayesian Information Criterion (BIC) [24]. The clustering with the highest value for pseudo-F is regarded to provide the best fit, while clustering with the lowest value for BIC is regarded to provide the best fit [22].

Significant genetic difference among the genetic groups obtained after K-Means clustering and Evanno test, was determined by different methods: 1- AMOVA (Analysis of molecular variance) test (with 1000 permutations) as performed in GenAlex 6.4 [25], and 2- Nei's G_{ST} analysis of GenoDive ver.2 (2013) which was originally written by Meirmans and Van Tienderen [26]. 3- G'_{ST} est = standardized measure of genetic differentiation [27], and D_{est} = Jost measure of differentiation [28].

RESULTS

All primers produced reproducible bands and finally a combined data matrix of 86 X 50 was formed for further analysis. NJ tree and NeighborNet network produced similar results (Figures 1 and 2).

In NJ tree 3 major clusters were formed. Thirteen olive plants formed the first major cluster. These plants were scattered in 2 sub-clusters. Twenty olive plants formed the second major cluster and showed a higher degree of genetic similarity to the plants of the first cluster. These trees were scattered in 3 sub-clusters.

Seventeen olive plants comprised the third major cluster. They were placed with some distance from the other olive plants due to genetic difference. The mother plant (plant No. 50) was placed along with plants number 46 and 49 in a separate sub-cluster.

Scattering of these olive trees in different subclusters, indicated their genetic difference which was also supported by NeighborNet network (Figure 2). In this network, plant numbers 47 and 48 differed genetically from the other plants as they had a longer length branch in the network obtained. The mother plant (No. 50) was placed close to plant No. 46. The plants that were placed in the second major cluster of NJ tree (plant numbers 1-20, and 23-29) formed a distinct group in the NeighborNet plot and were placed far from the other two groups. However, these plants also showed genetic variability among each other and supported 3 sub-groups identified by NJ tree in this major cluster.

Figure 1. NJ tree of olive plants. No : 1-49 regenerated shoots, No 50: mother plant

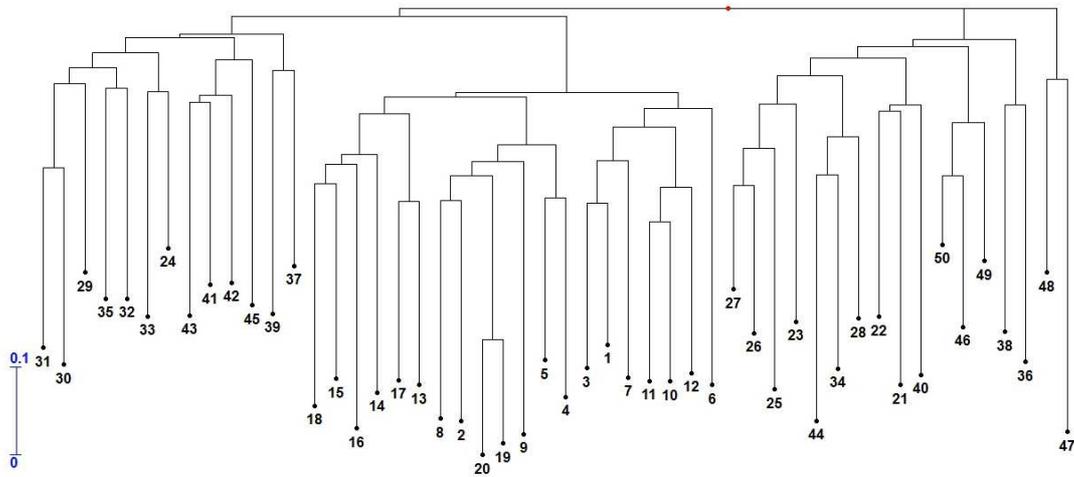
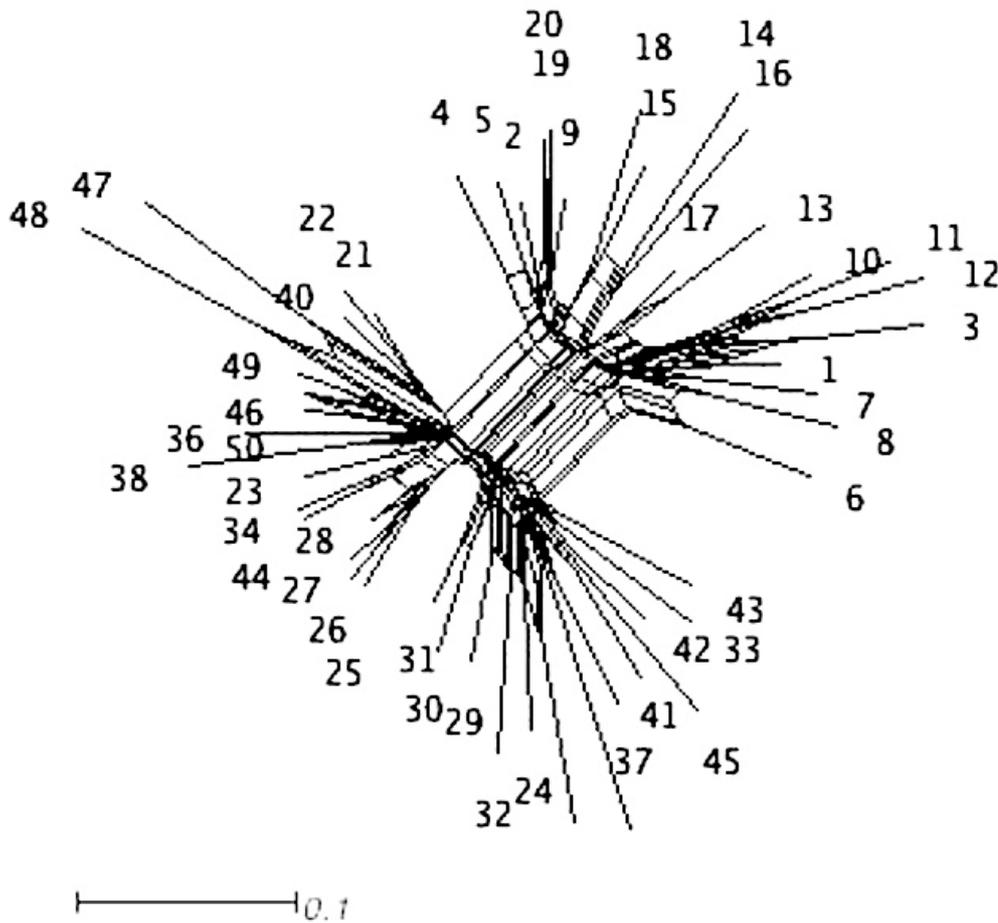


Figure 2. NeighborNet diagram of olive shoots
Numbers are according to figure 1

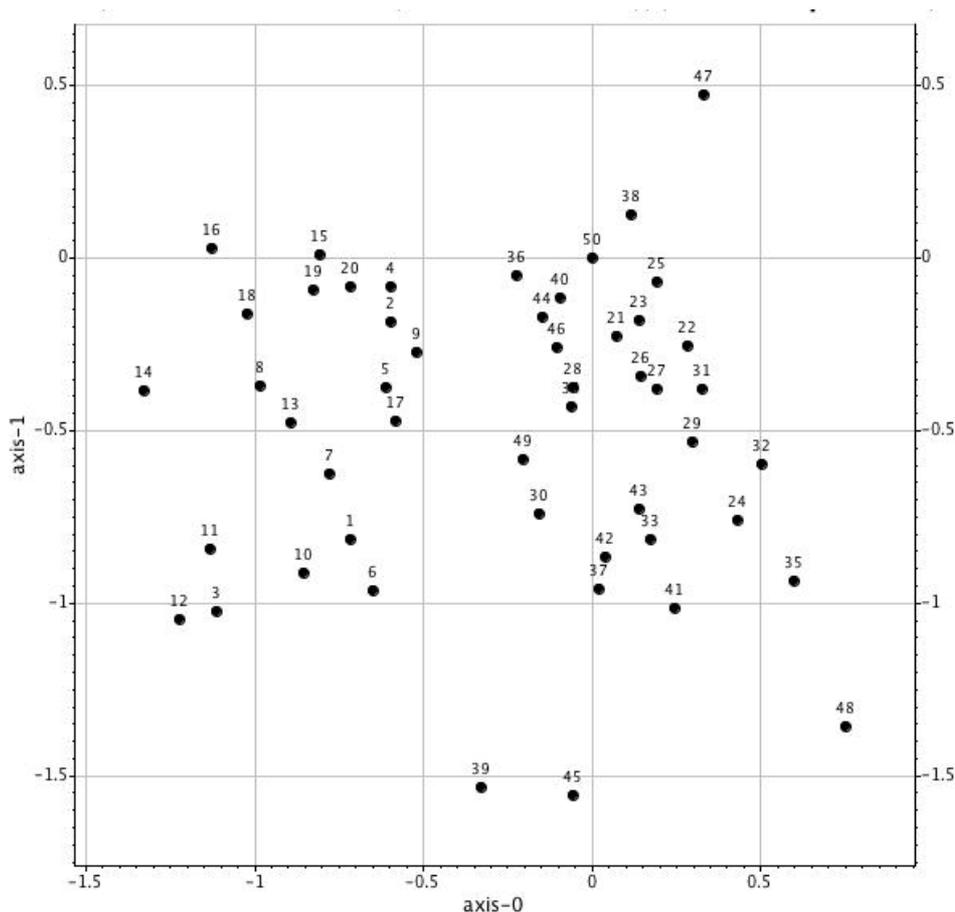


Due to close genetic affinity of the regenerated olive plants and close proximity revealed by NJ and NeighborNet

trees/diagrams, we used non-metric MDS analysis which tries to find similarities among the studied objects by using Proximities.

The data for MDS analyses are called proximities. Proximities indicate the overall similarity or dissimilarity of the objects under investigation. An MDS program looks for a spatial configuration of the objects, so that the distances between the objects match their proximities as closely as possible [29]. The MDS plot is presented in Figure 3. It showed details of genetic similarity versus differences of the studied olive plants. This plot revealed genetic difference between plant No. 47 and 48. It showed also that plant numbers 39 and 45 are genetically different from the others, as they are placed close to each other and far from the other studied plants.

Figure 3. MDS plot of olive plants
Numbers are according to figure 1



The MDS plot clearly showed two subgroups among the plant numbers 1-20 that were placed close to each other in both NJ and NeighborNet diagram. Therefore we have a good level of genetic variability among the members of this cluster. This plot also revealed the presence genetic variability among plants placed in the other two groups formed by NJ and network trees. Therefore, more detailed information about genetic differences of the studied plants is provided by MDS plot.

Both Evanno test (Figure 4) and K-Means clustering (Table 1) revealed that the studied plants are better grouped in 2 or 3 genetic groups. It produced the highest value of pseudo-F (9.199) for $k = 2$, while produced the lowest value of Bayesian Information Criterion (307.493) for $k = 3$.

Figure 4. Evanno test plot

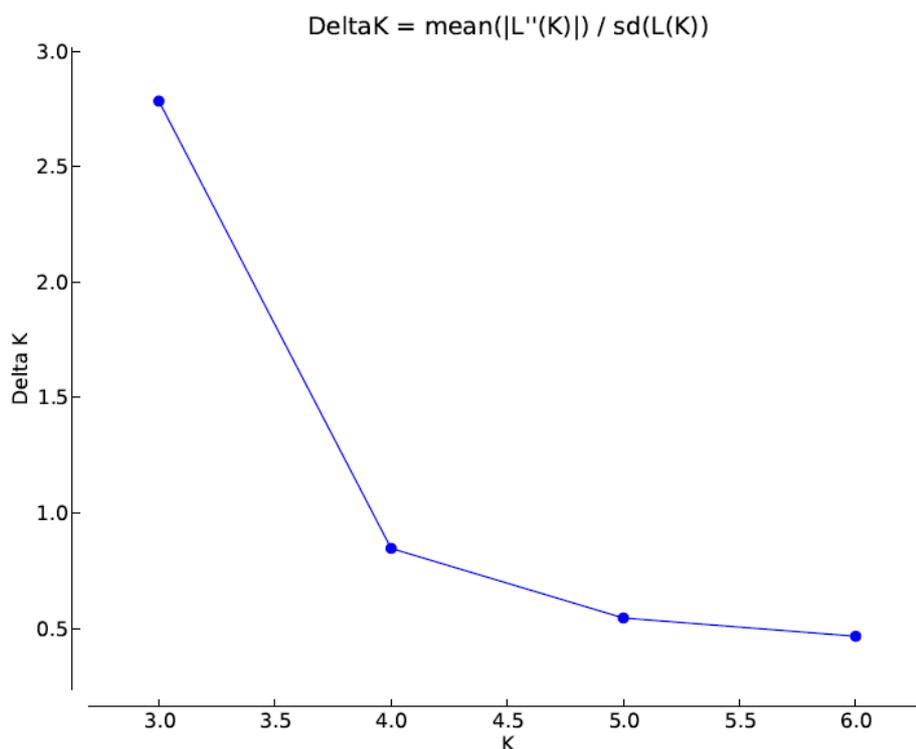


Table 1. K-Means clustering result for k = 2- 5

k	SSD(T)	SSD(AC)	SSD(WC)	r-squared	pseudo-F	BIC	Rho
2*	479.14	77.057	402.083	0.161	9.199	307.657	0.255
3&	479.14	108.534	370.606	0.227	6.882	307.493	0.267
4	479.14	131.177	347.963	0.274	5.78	308.253	0.285
5	479.14	151.353	327.787	0.316	5.195	309.178	0.298

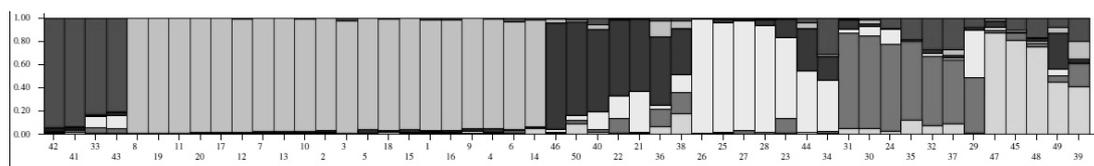
* Best clustering according to Calinski & Harabasz' pseudo-F: k = 2
& Best clustering according to Bayesian Information Criterion: k = 3

In the next step, based on groupings obtained from previous analyses, we performed AMOVA and G_{st} analyses to indicate significant molecular difference among the plants of these groups. The results revealed a significant difference among the 3 groups ($p = 0.01$). It also showed that about 78% of total genetic variability occurred among 3 groups, while about 21% of genetic variability occurred within the studied groups. This result showed a good degree of genetic diversity among olive plants in general. Similarly, G_{st} analysis, produced G_{st} value = 0.21 (fixation index), $G'_{st} = 0.21$ (Hedrick, standardized fixation index) and $D_{est} = 0.06$ (Jost, differentiation index) that were all significant ($p = 0.01$) among the studied plant groups. All these results indicated genetic differentiation among the groups identified by NJ and network analyses.

Bayesian model based STRUCTURE plot obtained (Figure 5) revealed allelic similarities versus differences among the studied plants. It grouped olive plants in 6 allelic composition groups but also revealed within group allelic variability. For example, it showed close affinity between plant numbers 33 and 34, that were also grouped together in NJ and network trees, but it also showed that these plants differ in their allelic composition (differently colored segments) and allele frequency (different proportion of each colored segment). Plant numbers 1-20 contained allelic forms (green colored segments) that differed from the other studied olive regenerated plants.

An interesting result was obtained when we looked at mother plant (No. 50). It showed the presence of all allelic forms present in the other regenerated plants, but mainly contained blue colored segment. Plants that showed genetic similarity to the mother plant were plant numbers 21, 22, 36, 38, 40, and 46 (they also contained a high level of blue colored segment). However, they had other allelic forms too (other colored segments). It is also interesting to note that some of the regenerated plants had lost this blue colored segment (allelic forms) possibly due to somaclonal variation.

Figure 5. STRUCTURE plot of olive data
Numbers are according to figure 1



DISCUSSION

The present study showed the occurrence of high genetic variability within tissue culture regenerated olive plants. These changes often arise as a manifestation of epigenetic influence or changes in the genome of differentiating vegetative cells and are expected to generate stable plants carrying interesting heritable traits [8].

Four important variables influence the induced variability discussed, these are: Genotype, explant origin, cultivation period and the cultural condition in which the culture is made [30].

Variations induced may be manifested as cytological abnormalities, frequent qualitative and quantitative phenotypic mutation, sequence change, gene activation and silencing [9, 31]. In our study, we investigated SSR and ISSR variability that represents change on sequence repeats. STRUCTURE analysis clearly revealed changes in the type of sequences and their frequency among the studied plants. Absence of sequences that were present in the mother plants in some of the regenerated plants indicates loss of these sequences due to somaclonal variation. Different genomes respond differently to the stress-induced variation, which indicate that somaclonal variation also has genotypic components [3]. The differences in genetic stability are related to differences in genetic make-up, because some components of the plant genome may become unstable during the culture process, for example the repetitive DNA sequences, which can differ in quality and quantity between plant species [3]. Both SSR and ISSR primers used in the present study deal with repetitive sequences and therefore genetic variations observed may be related to such changes in the cultivars' genome. However, other genomic cryptic changes, such as point mutations, transpositional events, such as the activation of transposable elements, putative silencing of genes and a high frequency of methylation pattern variation among single-copy sequences, play a role in somaclonal variation as well [3].

CONCLUSION

The present investigation shows that regenerated olive plants are not completely true to type of the mother plant used and we have extensive genetic difference among them. These genetically different olive plants can be used in further hybridization and breeding program in olive.

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