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Molecular Genetic Diversity of 12 *Origanum vulgare* subsp. *hirtum* Genotypes: EST-SSR Marker Analyses

Uğur TAN [1*], Olcay ARABACI [1]
[1] Aydın Adnan Menderes University, Faculty of Agriculture, Department of Field Crops, Aydın
[*]Corresponding author: ugur.tan@adu.edu.tr

Abstract
Conducting a genetic diversity study on *Origanum vulgare* using genetic markers is important to investigate the genetic variability and evaluation of the population structure of the species. Genetic diversity of *Origanum vulgare* has been determined with various genetic markers among different subspecies. Studies on the genetic diversity of its one of the most well known and widely grown subspecies, *Origanum vulgare* subsp. *hirtum*, have revealed high intraspecific genetic variability among different individuals within the species. In *Origanum vulgare* subsp. *hirtum*, employement of a molecular marker, Expressed Sequence Tagged – Simple Sequence Repeat (EST-SSR), allow the identification of distinct genetic profiles that can assist in cultivar identification and the comprehension of the genetic relationships within species. The primary aim of this study was to investigate the genetic diversity and population structure of 12 *Origanum vulgare* subsp. *hirtum* (Istanbul oregano) genotypes using nine EST-SSR markers. These EST-SSR markers (OR09, OR10, OR12, OR13, OR14, OR27, OR32, OR40 and OR44) were resulted total nine different alleles within all populations. OR9 primer have shown one unique alleles, reflecting genetic distinctiveness within the population. A total number of three clusters were determined in dendongram analysis (Neighbor Joining). The *Origanum vulgare* subsp. *hirtum* individuals investigated in this study exhibited low genetic diversity, with low to medium genetic variation both within and among the five populations (A, B, C, D and E). This limited diversity is might be due to the isolation within and between populations and also small population size. Despite this, certain EST-SSR markers, specifically OR13 and OR40, have proven to be valuable markers for assessing genetic diversity. These markers can play a crucial role in further breeding programs for identifying new varieties within *Origanum vulgare* subsp. *hirtum* populations.

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1. Introduction

Conducting a genetic diversity study on *Origanum vulgare* using genetic markers is important to investigate the genetic variability and population structure of this plant species. By employing EST-SSR markers to identify distinct genetic profiles that can assist in cultivar identification, and comprehend the genetic relationships among different subspecies or populations of *Origanum vulgare* (Mechergui et al., 2017; Alekseeva et al., 2023).

Origanum vulgare subsp. *hirtum*, a subspecies of *Origanum vulgare* L., belonging to the one of the most important medicinal and aromatic plant family, Lamiaceae is renowned for its uses in both culinary and medicinal applications. This subspecies exhibits significant morphological and intraspecific variability, distinguishing it from other subspecies of *Origanum vulgare*. The highly qualified and rich essential oil composition *Origanum vulgare* subsp. *hirtum* makes it an attractive oregano species to be studied for medicinal and industrial uses (Koukoulitsa et al., 2006; Martino et al., 2009; Kocabaş Oğuz, 2021).

Genetic diversity of *Origanum vulgare* subsp. *hirtum* can be determined with molecular markers among subspecies (Mertzanidis et al., 2022). Studies on the genetic diversity of *Origanum vulgare* subsp. *hirtum* have revealed high intraspecific genetic variability among different subspecies. This highlights the complexity and diversity within the genetic structure of the plant (Alekseeva et al., 2021).

Various markers such as AFLP, RAPD, ISSR, ITS, EST-SSR, SCAR, SCoT, and TU-DAMD have been employed in genetic diversity analyses of oregano species. Notably, Ayanoglu et al. (2006) used AFLP markers to study Turkish *Origanum* specimens, revealing significant genetic diversity without a direct correlation to collection areas, suggesting environmental adaptation and mating behaviors as influencing factors. Van Looy et al. (2009) explored genetic dissimilarities in relation to flooding events, while Zaghloul et al. (2014) found 57 % genetic polymorphism in O. *syriacum* subsp. *sinaicum*. Azizi et al. (2016) observed 75 % allelic polymorphism in O. *vulgare*, and El-Demerdash et al. (2019) reported 53 % polymorphism in cultivated O. *vulgare* and wild O. *syriacum*. The dissertation introduces SRAP markers for *Origanum* species for the first time.

Understanding the complex genetic diversity of *Origanum vulgare* subsp. *hirtum* through EST-SSR markers can also contribute to the sustainable management and utilization of this plant species in various fields, including agriculture, medicine, and conservation. The primary aim of this study is to investigate the genetic diversity and population structure of *Origanum vulgare* Subsp. *hirtum* (Istanbul oregano) using EST-SSR markers.

2. Matrerials and Metods

2.1. Plant material

The study material was selected from the A and B clones that were created in the previous breeding study under the TUBITAK project number 113O285, which involved the selection of high-yield and high-quality clones from five populations (A, B, C, D, and E) of *Origanum vulgare* subsp. *hirtum*. The initial specimens of the *Origanum vulgare* subsp. *hirtum* clone populations (Arabacı et al., 2016) were collected from Ida Mountains (Kazdağları) where these species naturally and widely distributed (Yenice region) Table 1.

Table 1. Origin of Istanbul oregano (*Origanum vulgare* subsp. *hirtum*) genotypes

Population	Coordinates(x, y)	Altitude (m)	Directions
A	542066, 4422267	586	South
B	545202, 4423405	599	Southeast
C	546586, 4426023	627	South
D	524032, 4394952	359	Stream bed
E	525416, 4394939	381	North

2.2. Molecular genetics study

2.2.1. DNA isolation

The modified DNA isolation method of Doyle and Doyle (1990) was used in plant samples. Fresh leaves of *Origanum vulgare* subsp. *hirtum* were collected from the field and their genomic DNA were isolated in August 2019. The leaf samples were placed in eppendorf tubes and the samples weretreated with liquid nitrogen. Metal beads were added and leaves were crushed in a homogenizer. This procedure was followed by the addition of warm CTAB (70 ºC) to the samples and followed by short vortex treatment. Samples with warm CTAB were placed into incubator for 90 minutes (min). The samples were treated with chloroform:isoamyl alcohol (24:1) and centrifugated at 1300 rpm for 20 min. Isopropane was added to the centrifuged samples and left (+4 ºC) overnight. Further, the samples were centrifuged again at 1300 rpm for 20 min, DNA was isolated, isopropane was removed and ammonium acetate was added for final precipitation. After the final cleaning, the DNA samples were stored at -20 °C in distilled water. Nanodrop tests were performed to determine the purity of genomic DNA.

2.2.2. Molecular marker analyses

In the study, nine EST-SSR markers of Novak et al. (2008) that gave amplification in *Origanum* species were used in the study (Table 2).

Table 2. EST-SSR primers and their sequence information used in the study

Primer Name	Sequence (5′ to 3′)	Repeat Pattern
OR09	F: TTGAAGCATTGTTGGAGGTAGATG R: TCCCAACTAGGGAGAAATGTGC	(TTTTTC)4(T)5(TTTTTC)1
OR10	F: TTTGCTCCGACATCTTCAACC R: AGCCTGCTGTGTTTGGATCAG	(ACC)1ATC(ACC)4
OR12	F: GCCCCTGCAGTGACTCCTAC R: AAAAAGGCTTCGGACTCGATC	(AG)7G(AG)3
OR13	F: GAGAGAATCCAAGCCTCCGC R: TGAAGGAGTCCGATGTTGACG	(AAC)7AGC(AAC)1
OR14	F: TGTTTGGTGGAAACCGATCC R: AGACGACGAGCTCCAATAACG	(GAT)8
OR27	F: TCAGAAACAATGAAGGCCGC R: CCGTACAGGTCAAACACCGG	(CCT)6
OR32	F: TCTTGCCAATTTATGCGTGTTC R: GAAACAAGCATCTTTTCCTGAATTC	(AG)6GG(AG)2GA(AG)5GG(AG)1
OR40	F: GCCCAAGGACATCCAACTTG R: CAACTGAACACCTCCCACAATG	(GGT)4GTT(GGT)1
OR44	F: TCAAGGGTAGAGCTGCTGCAG R: GCTTTACGGAGGAAGAATGGG	(GAT)3GAA(GAT)4

Polymerase Chain Reactions (PCRs) were performed with the preparation of MasterMix (10 µl 10x Premix, 0.75 µl forward and reverse primers, 6.5 µl H_2O for each tube) and a total of 20 µl PCR was performed for each sample using 2 µl of DNA. PCR conditions were as follows; initial cycle of 95 ºC – 5 mins, followed by the core cycle of 95ºC – 30 seconds (s), 59 ºC – 30s, 72 ºC – 1 min: (34x cycle), followed by the final elongation step of at 72 ºC for 5 mins. The appropriate Ta (ºC) (primer melting temperature) temperatures were set for each primer according to their Tm (ºC) values. After conducting PCR reaction, specimens were run on 1.6 % agarose gel in 120 Volts and visualized under UV light.

2.3. Statistic analyses

The agarose gel images were dominantly scored to be used for assessing the genetic diversity of populations according to the presence “1” or absence “0” of each band for each EST-SSR marker. Genetic Diversity (GD) values were calculated with R program. Neighbor Joining (NJ) dendrogram was built with the allelic data related to nine EST-SSR

markers for the specimens using DARwin 6.0.8 software (Perrier and Jacquemoud, 2006). The structure of the population was evaluated with the Bayesian inference via STRUCTURE 2.3.4 software (Pritchard et al., 2000). The sub populations (SPs) (*K*) were tested with a range of *Ks* from 1 to 10. Each iteration was made 10 times for each *K*.

3. Results and Discussion

3.1. Marker diversity

The genetic diversity among OVH (*Origanum vulgare* subsp. *hirtum*) individuals were evaluated with 11 different parameters (NA, PA, UA, Ne, He, Ho, I, DP, MI, PIC, P) (Table 3). The number of alleles per marker was the highest for OR13 primer with four alleles; while OR12, OR14 and OR32 has the lowest number of alleles. Effective population size (Ne) changed between 1.0-2.06 and highest values obtained from OR13 primer. Effective population size (*Ne*), is a measure of allele frequency, indicating how evenly the alleles are distributed in the population. It is a useful measure for understanding allelic pattern and genetic variability within populations (Alekseeva et al., 2023).

Table 3. Genetic diversity parameters on studied 12 genotypes of *Origanum vulgare* ssp. *hirtum* based on data from 9 EST-SSR markers

Primer	NA	PA	UA	Ne	He	Ho	I	DP	MI	PIC	P(%)
OR9	3	2	1	1.52	0.34	0.08	0.62	0.34	0.01	0.31	66
OR10	2	2	0	1.39	0.28	0.00	0.45	0.28	0.00	0.24	100
OR12	1	1	0	1.00	0.00	0.00	0.00	0.00	0.00	0.00	100
OR13	4	2	0	2.06	0.51	0.33	0.98	0.51	0.08	0.48	50
OR14	1	0	0	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0
OR27	2	2	0	1.80	0.44	0.33	0.64	0.44	0.05	0.35	100
OR32	1	1	0	1.00	0.00	0.00	0.00	0.00	0.00	0.00	100
OR40	2	2	0	1.95	0.49	0.83	0.68	0.49	0.15	0.37	100
OR44	2	1	0	1.18	0.15	0.00	0.29	0.15	0.00	0.14	50

He: Expected Heterozygosity, **PIC:** Polymorphic Information Content, **Ne:** Effective Number of Alleles , **I:** Shannon Index , **DP:** Discriminatory Power , **MI:** Marker Index , **Ho:** Observed Heterozygosity , **PA:** Polymorphic Alleles (%) , **TA:** Total Allel, **PB:** Polymorfic allele , **UA:** Uniuqe allele

Primers such as OR13 and OR40, exhibited high PIC (Polymorphism Information Content) values and primer OR 9 shows unique allelel. High PIC values indicate a primer's effectiveness in detecting polymorphisms, which is critical for assessing genetic diversity (Lemos et al., 2019).

In many cases, the observed heterozygosity (Ho) is lower than the expected heterozygosity (He) for most primers. This discrepancy may be attributed to factors like inbreeding or genetic drift, indicating that there are fewer heterozygous individuals than expected (Waller, 2021).

This could be due to several factors such as inbreeding. Inbreeding increases the proportion of homozygous individuals in the population because related individuals are more likely to mate and pass on the same alleles. Another reason can be genetic drift, In small populations. A random changes in allele frequencies can lead to an increase in homozygosity and a decrease in heterozygosity (Oakley et. al. 2019; Gao and Gao, 2016).

A higher *Ne* value suggests more equal distribution of alleles, which reflects higher genetic diversity (Torres et. al., 2003). In our study, OR12, OR14, and OR32 EST-SSR primers exhibited the low Ne values, indicating the uneven distribution of alleles with one dominant allele. In contrast, the OR13 primer shows an even distribution of alleles, which reflects greater genetic diversity.

The lowest I (Shannon's Information Index) was determined from OR12, OR14, OR32. Highest I (0.983) were determined from OR13. This indicates high diversity and evenness, with a wide variety of alleles present in fairly equal proportions. In *Origanum vulgare*, Shannon's Information Index was employed to

evaluate genetic diversity. A study found that certain SSR markers showed higher Shannon's Index values, indicating significant genetic variation and even distribution of alleles within populations (Alekseeva et al., 2021).

Primers OR12, OR14, and OR32 showed the lowest DP values (discriminatory power), indicating they are not effective at distinguishing between genotypes due to low or no genetic variability. In contrast, the OR13 primer demonstrated the highest DP value (0.514). This primer is highly effective at distinguishing between different genotypes, indicating its utility in genetic studies. According to Azizi et al. (2009) discriminatory power (DP) was assessed in thier study using genetic markers for *Origanum vulgare* and this markers demonstrated varying degrees of effectiveness in distinguishing between subspecies, with certain markers showing high DP values, thus proving their utility in genetic differentiation.

The lowest MI (Marker Index), were found at OR10, OR12, OR14, OR32, OR44. These primers have low informativeness for genetic studies. Highest MI (0.149) OR40. This indicates that OR40 is a relatively informative marker for genetic variability.

OR13 stands out across several metrics (Ne, I, DP, MI), it is highly informative, diverse, and effective at distinguishing between genotypes. OR14 consistently shows the lowest values across these metrics, indicating it has very limited utility in genetic studies due to low variability and informativeness. OR40 also shows high values in several metrics, particularly in Ho (observed heterozygosity) and MI, making it a valuable marker for genetic diversity studies.

3.2. Dendrogram profile of *O. vulgare* subsp. *hirtum*

According to UPGMA dendrogram (Figure 1), there were found 3 clusters. Cluster 1: C425, C290, C548 and E11 form a tight cluster with a high bootstrap value of 56. This means they share a significant amount of genetic similarity. Cluster 2: D92 and D99 are grouped together (bootstrap value of 51). Cluster 3: E302, B575, and A320 form another subgroup with a bootstrap value of 44. B201 and E400 form a distinct group with a high bootstrap value of 91, distinct difference from the rest of the individuals. A210 appears to be a significant outlier, branching off early in the dendrogram with a bootstrap value of 38, it is quite genetically distinct from the other clusters.

Even if same letters (A, B, C, D and E) are same population, there are distinction within same population only C population and D population are within same group. It is worth the use more genetic markers and genotypes to determine similarity within and between populations (Figure 1.). The dissimilarity indices were calculated among individuals and resulted in a mean dissimilarity of 0.15 with values ranging from 0 to 0.38.

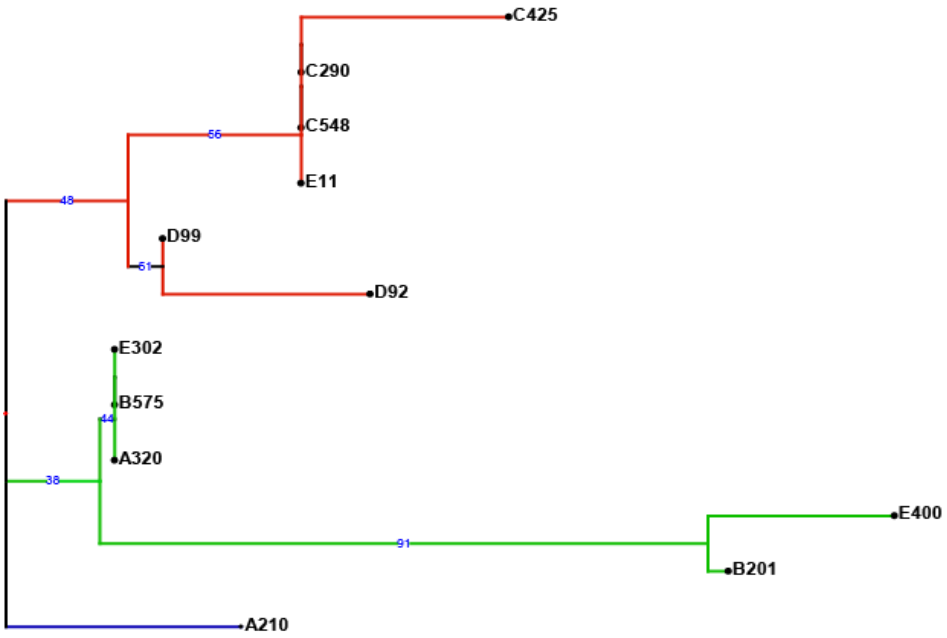

Figure 1. The UPGMA dendrogram, computed using genetic distance matrix based on EST-SSR markers

Structure analysis was performed to obtain information about the population structure of the genotypes used in the study. *K* values and Delta *K* (Δ*K*) values of 12 genotypes of Istanbul oregano (*Origanum vulgare* subsp. *hirtum*) are given in Figure 2 and structure analysis results are given in Figure 2. The band results obtained with 9 EST-SSR primers in 12 Istanbul oregano genotypes used in the structure program to determine the population structure are given below. For all *K* values, the most probable value was accepted as the Δ*K* value and this value was determined as 3. It is seen that 12 genotypes of Istanbul oregano consist of 3 gene pools according to 9 EST-SSR primers.

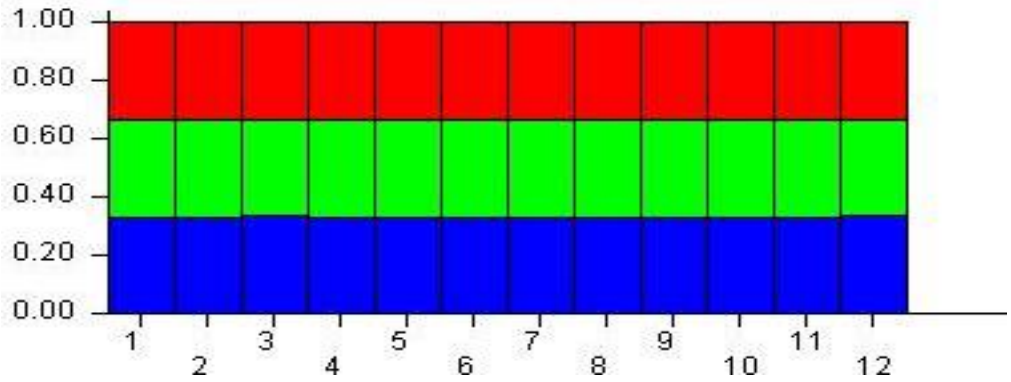

Figure 2. The structure of 12 genotypes of Istanbul oregano (*Origanum vulgare* subsp. *hirtum*), *K*=3.

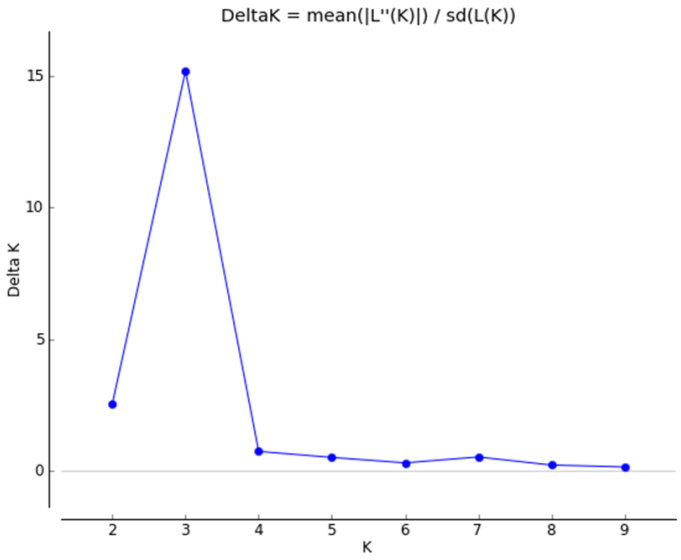

Figure 3. K values and Delta K

4. Conclusions

The *Origanum vulgare* subsp. *hirtum* plants investigated in this study exhibit low genetic diversity, with low to medium genetic variation both within and between populations. This limited diversity is likely due to the isolation and small size of the populations studied. Despite this, certain EST-SSR markers, specifically OR13 and OR40, have proven to be valuable markers for assessing genetic diversity. These markers can play a crucial role in breeding programs by identifying new varieties within *Origanum vulgare hirtum* populations.

Declaration of Author Contributions

The authors declare that they have contributed equally to the article.

Declaration of Conflicts of Interest

All authors declare that there is no conflict of interest related to this article.

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