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ORIGINAL PAPER

Determination of genetic diversity of natural sage populations in Muğla region of Turkey

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Abstract
Turkey, which is a very rich country in terms of medicinal and aromatic plants including sage, is one of the world's leading gene sources. Thyme and sage that are the medicinal and aromatic plants are naturally grown in the Aegean region. In the present study, 16 ISSR molecular markers were used in the determination of genetic correlations among 8 sages picked up from forests and mountains in Muğla province in the Aegean region. In this study, the average polymorphism rate was determined as 69.84%; in addition to 44 polymorphic bands, totally 63 bands were obtained. As a result of unweighted pair group method with arithmetical average (UPGMA) analysis, the most distant sages to each other were the ones belonging to the Bodrum and the Yerkesik locations, and the ones nearest to each other were obtained from Bodrum and Marmaris locations. On the other hand, in terms of the genetic variation, Dalyan, Köyceğiz, Marmaris and Bodrum locations constituted the first group, and Göcek, Dalaman and Fethiye locations constituted the second group, whereas the sages belonging to the Yerkesik location formed a group by itself. This study revealed that there was a significant genetic variation among the populations of sages grown naturally in Muğla region.

Keywords Biodiversity · ISSR · Molecular marker · Population · Sage

Introduction

Turkey has rich flora with 12,000 plant taxon due to the differences in geographical factors or cultivation environments of various plants and contains a large number of medicinal and aromatic plants. Therefore, too many plant species form plant populations and do not exhibit the same characteristics in various regions, as a result of the climate and soil characteristics, the local differences emerge and endemic medicinal and aromatic plants grow.

The Western Mediterranean flora, which is included in Muğla, is in the Mediterranean climate zone and is warm and rainy in the winter and hot and dry in the summer. One of the endemic species in Turkey is the sage that grows naturally in the mountains from İzmir to the shores of Muğla.

Sage is defined as a medicinal herb in Old Latin (Salvare means "cure, save"). This plant, which belongs to the Lamiaceae family, is grown throughout the world and especially in the Mediterranean, Europe and North America. It is commonly called Adaçayı, which means "island tea" in Turkish. Sage is also called Maryam goli in Persian (Altindal and Altindal 2016). It grows well, especially in sandy and loamy soils rich in nutrient. Sage is a bush, grown as single-year or perennial, generally 40–100 cm in length, and has purple-blue-colored cluster-like flowers, and its leaves are gray-green, feathered and pointed. The plants contain both male and female organs or may have also a single female organ. It has two male organs. The varieties of essential oils in plant leaves are at the highest rates before flowering. Therefore, sage leaves are picked up before or at the beginning of flowering in May–June. The leaves collected during the noon hours when active substances reach the peak are left to dry in a shady and narrow area.

The most important ingredient of sage leaves is essential oil (Oleum Salviae) that varies in the rate of 1–2.5% in leaves. Essential oils of sage contain thujone, cineol,

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linalool, borneol, salven, pinene, tannins, tritierpotides, flavones being important in pharmacological terms, estrogen-like substances and resins Triterpenes and flavones are present in the essential oils' composition (Roman et al. 2009). Sage oil is mainly composed of monoterpene ketones α-thujone (23.0%), camphor (21.3%) and β-thujone (13.6%) (Altindal and Altindal 2013). Pizzale et al. (2002) stated that sage includes carnosic acid, carnosol and methyl carnosate components and has strong antioxidant activity according to the species. In addition, the plant contains water, protein, carbohydrates, calcium, iron, magnesium, phosphorus, potassium, sodium and zinc elements as well as vitamins A and C and niacin (vitamin B_3). Sage added to vinegar is reported to be effective against diabetes, stomach aches, flushing, depression (Karamanos 2000), excessive sweating (Mohammad 2011) and hormonal problems (Şensoy 2007).

In Turkey, *Salvia* L. has 97 species, 4 subspecies and a total of 109 taxa belonging to 8 varieties. Fifty-one of these species are endemic. Fifty-eight of the 97 species grown in Turkey (59.7%) are in Iran-Turan region, 27 (27.8%) in the Mediterranean, 5 (5.0%) in European Siberian phytogeographical region, while 7 (7.0%) are spread in more than one phytogeographical region. Anatolia Sage (*Salvia fruticosa* Mill.) populations, which show the natural spread in Muğla province, has been widely used in public medicine for many years (Karık 2015). *Salvia* L. species, which are used as gas extractor, sedative, carminative, diuretic, stomach healer, sweat preventer, external wound healer and antiseptic in public medicine, have a wide range of biological effects such as antibacterial, antifungal, antiviral, antiseptic, analgesic, antioxidant, astringent, antispasmodic, hallucinogenic, central nervous system depressant, antisudorific, antidiabetic, anticancer, tuberculostatic, cardiovascular therapeutic and insecticide (Lu and Yeap 2002).

Despite many studies on *Salvia officinalis*, there is very little information about the endemic sage of Turkey. *Salvia verbenaca* ($2n = 14–64$) is the species grown almost everywhere in Europe and in Turkey. *S. hasankeyfensis*, a new species native to Turkey (Dirmenci, Celep & Ö Güner), has recently been added to the Verbenaca group as a species grown at a height of 650–700 m in the hills and surrounding of Hasankeyf (Batman, southeastern Turkey) (Mahdjoub et al. 2018).

As in all over the world, the natural flora plants have been used in the food, insecticide and cosmetics industry through the human history and have become a traditional activity. However, with the modernization, the richness of the plant could not be sufficiently and regularly benefited. Therefore, endemic medicinal–aromatic plant species have been lost. Twenty-five percent of the endemic plant species in Turkey is about to be depleted due to unconscious use. This genetic diversity of Turkey which is among the important countries in terms of biological diversity in the world should be preserved sufficiently and transferred to the future generations. The construction of buildings in Muğla flora, where these species are spreading, is irregular, and intensive animal grazing reduces the population of these species, causing the continuity of species to be compromised over time. Fragmentation or disappearance of natural populations can lead to gene flow among populations with certain genetic characteristics (Ouborg et al. 2006), causing the lack of genes in populations due to genetic drift, thus increasing the genetic differentiation between populations and genetic structures (Hatmaker et al. 2018).

There is much talk about the disappearance of plant species as a result of erosion in natural plantations where genetic variability is wide, but the number of visible measures is still limited. The decrease in genetic diversity, identification and evaluation of the genetic erosion is the first priority. By improving scientific and technical possibilities, genetic resources of plants can increase (Kahraman et al. 2012). For this reason, the results of genetic erosion must be determined quickly, and genetic resources must be protected. To determine endemic and rare medicinal–aromatic plant species, scientific research should be carried out and studies to determine antioxidant properties of medicinal–aromatic plants and to learn phytochemical structures should be given priority (Celep et al. 2010).

For the protection of genetic diversity in the early nineteenth century, the plant genetic resources were identified and kept in many countries that were aware of the danger. For example, in 1898, hundreds of thousands of specimens were collected by "the seed and plant introduction unit," which was established in accordance with the USDA (United States Department of Agriculture) (Hymowitz 1984).

In molecular studies, genetic markers with repeatable and easily interpreted properties are needed. The genetic marker systems chosen for the fixed purpose should include genomic analysis to determine variations (polymorphism) and have the ability to detect variations. Today, molecular methods are used extensively in plant genome analysis. The most important of these are the markers based on DNA which have made great progress in recent years. Many molecular techniques based on DNA have been developed with this method, and the characteristics of the ideal DNA marker have been determined with these methods. DNA markers that are easy to find, easy to try, and fast, highly polymorphic, replicable in genomes, unaffected by environmental conditions, easy to exchange data between different

laboratories, and with codominant inheritance have been developed up to now (Sharma and Rao 1989).

In studies, morphological and biochemical traits, molecular markers have been used for the identification of genetic relationships (Sarıkamış et al. 2010). Since morphological markers are affected or limited by environmental conditions and biochemical markers have low polymorphic levels, their use in characterization studies is limited. Selection of DNA marker system in the characterization of plant genetic resources varies according to purpose, structure of population, type of plant studied, time required for analysis and cost. The appropriate molecular DNA techniques for exposing genetic variation in plant species are SCoT markers (Guo et al. 2012) in terms of polymorphism, SSR, and AFLP markers techniques in terms of cost, RAPD and ISSR techniques in terms of repeatability, RFLP, SSR, ISSR ve AFLP DNA techniques, which have been reported to be advantageous (Powell et al. 1996). The ISSR markers, like RAPD markers, are also quick to use, easy to implement, but their primers contain more nucleotides. Therefore, their primers are more reliable for being longer (Bornet and Branchard 2001). The ISSR markers give many polymorphic bands and have been reported to be useful method in the genetic diversity of plants, phylogenetic studies, genomic maps, breeding and evolution studies (Sarwat 2018; Ekincialp and Şensoy 2018).

The genetic variability among 6 Salvia species gathered from different parts of the Iran Alborz mountain region was investigated through inter-simple sequence repeats (ISSR) molecular markers (Safaei et al. 2016).

To compare genetic and metabolic diversity in *S. officinalis*, the genetic difference among 7 different sage populations grown in Greece was also investigated. The significant genetic differences among 7 sage populations grouped in three main clusters according to the UPGMA ISSR data-based dendrogram and principal coordinate analysis were revealed by using inter-simple sequence repeats (ISSR) Analysis (Sarrou et al. 2017).

ISSR marker system is dominant marker, one of the advantages of which is its availability for primer design with no need for sequence knowledge (Çolak and Alan 2017), and it is one of the simplest and widely used techniques (Vijayan 2005). The ISSR and start codon targeted (SCoT) markers were used to determine the genetic variation of the 9 sage species collected from different geographical regions of Iran. The study illustrated the high genetic diversity; in addition, it was shown that there was a similar polymorphism distribution between ISSR and SCoT techniques and that SCoT markers could be used as reliable and informative techniques for the evaluation of genetic diversity and the relationships among Salvia species (Etminan et al. 2018).

In this study, 8 naturally grown sages in Muğla were picked up and genetic diversity among the sage populations was investigated by ISSR molecular marker technique.

Materials and methods

In the study, genetic variability was determined by gathering the sage that grows in natural conditions in the coastal areas and nearby provinces of Muğla city. In the study, sage plants collected from 8 locations (Table 1) were used.

Table 1 Locations where the sage populations were picked up

Location	Altitude (m)	Average precipitation (mm)	Average temperature (°C)
Fethiye	319	983	17.7
Göcek	345	998	17.9
Dalaman	5	1018	18.1
Köyceğiz	50	1032	18.3
Yerkesik	654	1088	15.3
Dalyan	5	1023	18.3
Marmaris	109	928	18.8
Bodrum	8	765	18.9

Table 2 Nucleotide sequences and Tm degrees of the ISSR primers

No.	Primer	Nucleotide sequence (5^{I}–3^{I})	Tm degree (°C)
1	LOL-3	$(CA)_6AC$	41.0
2	LOL-4	$(CA)_6GT$	41.0
3	LOL-5	$(GA)_6GG$	44.0
4	LOL-8	$(GT)_6CC$	44.0
5	LOL-9	$(CAC)_3GC$	38.0
6	LOL-10	$(GAG)_3GC$	38.0
7	PHV-4	$GGC(GT)_8$	59.0
8	PHV-5	$ACG(CA)_8$	57.0
9	PHV-6	$CCA(CT)_8$	57.0
10	UBC-807	$(AG)_8T$	50.0
11	UBC-810	$(GA)_8T$	50.0
12	UBC-811	$(GA)_8C$	52.0
13	UBC-812	$(GA)_8A$	50.0
14	UBC-814	$(CT)_8A$	54.0
15	UBC-826	$(AC)_8C$	52.0
16	UBC-854	$(CT)_8RG$	55.0

Fethiye district where the sage is gathered is located on the west of the Mediterranean region and east of Muğla province. In Fethiye where the characteristic Mediterranean climate is seen, the summers are hot and dry and the winters are warm and rainy. Sage was picked up from the 319-m altitude locations of Fethiye Ölüdeniz region. The altitude of Göcek, which is one of the other locations, is 345, located to the west of the Mediterranean region and to the east of Muğla province, and is surrounded by the Fethiye on the east, the Mediterranean on the west, Dalaman province on the northwest, and Denizli province on the north. The characteristic Mediterranean climate is seen in Göcek, its summers are hot and dry, and the winters are warm and rainy. Another location is Dalaman district that is located between Muğla province Köyceğiz, Ortaca and Fethiye districts and Denizli province Çameli district. The average annual temperature of Dalaman district is 18.1 °C, annual average rainfall amount is 1018 mm, and the altitude is 5 m. Köycegiz district is located where the Mediterranean and Aegean regions meet and has 50-m altitude. The Mediterranean and continental climates are seen in the coastal and mountainous regions of Köycegiz, respectively. The annual average temperature of the district is 18.3 °C, and annual average rainfall is 1032 mm. The distance of Yerkesik district is approximately 10 km to the city center of Muğla with altitude 654 m. A warm and temperate climate exists in the region. In winter, there is much more rainfall than in the summer months. The average annual temperature is 15.3 °C, and the average annual rainfall is 1088 mm. Marmaris and Bodrum districts and Dalyan, which is the town of Ortaca district, have a warm and mild climate, with more rainfall in the winter than in the summer months. The altitude of these three locations is 109, 8 and 5 m, respectively. The annual rainfall in Marmaris and Bodrum (928 and 765 mm, respectively) is less than other districts (Table 1) (Anonymous 2018).

To perform ISSR-PCR analysis on these species, 20 ng DNA, 50 mM 10X buffer, 25 mM $MgCl_2$, 1.25 mM dNTP, 20 µM primer and 5 U/µl Taq DNA polymerase were used as stocks for a total of 25 µl of the optimal PCR mix in each tube. In addition, 14 of 16 primers (Table 2) (Altındal 2014) which produce polymorphic bands, belonging to LOL, PHV and UBC sets, were used through the ISSR-PCR method. The Bio-Rad Cycler thermal PCR device was used for PCR, and the program carried out as 1 cycle at 3 min at 94 °C, 30 s at 94 °C, 45 s at 38–59 °C (Table 2) and then 35 cycles at 1 min at 72 °C, followed by 10 min at 72 °C, and ended at 4 °C. After the PCR process, the replicated DNA was run in 2% agarose gel (containing ethidium bromide) in electrophoresis using 1 × TBE buffer at 120 V for 120 min. Then, the amplified bands were displayed under UV light and photographed. The ISSR-PCR was repeated three times.

Table 3 Band characteristics of the ISSR primers

Primer	Total band number	Polymorphic band number	Polymorphism ratio (%)
LOL-3	8	2	25.00
LOL-4	16	4	25.00
LOL-5	5	1	20.00
LOL-9	13	4	30.77
LOL-10	5	1	20.00
PHV-5	11	3	27.27
PHV-6	15	3	20.00
UBC-807	10	2	20.00
UBC-810	10	4	40.00
UBC-811	8	2	25.00
UBC-812	16	5	31.25
UBC-814	4	3	75.00
UBC-826	4	2	25.00
UBC-854	3	1	33.33
Total	128	41	28.91

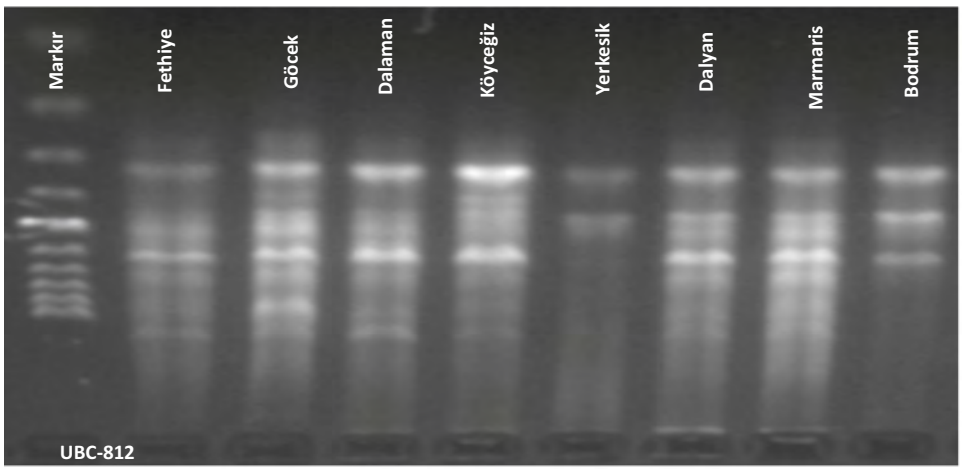

Fig. 1 Gel image obtained by UBC-812 primer

Table 4 Similarity matrix values of sage populations

	Fethiye	Göcek	Dalaman	Köyceğiz	Yerkesik	Dalyan	Marmaris	Bodrum
Fethiye	1							
Göcek	0.51	1						
Dalaman	0.41	0.51	1					
Köyceğiz	0.33	0.48	0.43	1				
Yerkesik	0.26	0.20	0.23	0.31	1			
Dalyan	0.47	0.25	0.39	0.40	0.34	1		
Marmaris	0.32	0.17	0.30	0.42	0.32	0.46	1	
Bodrum	0.29	0.27	0.41	0.54	**0.10**	0.30	**0.60**	1

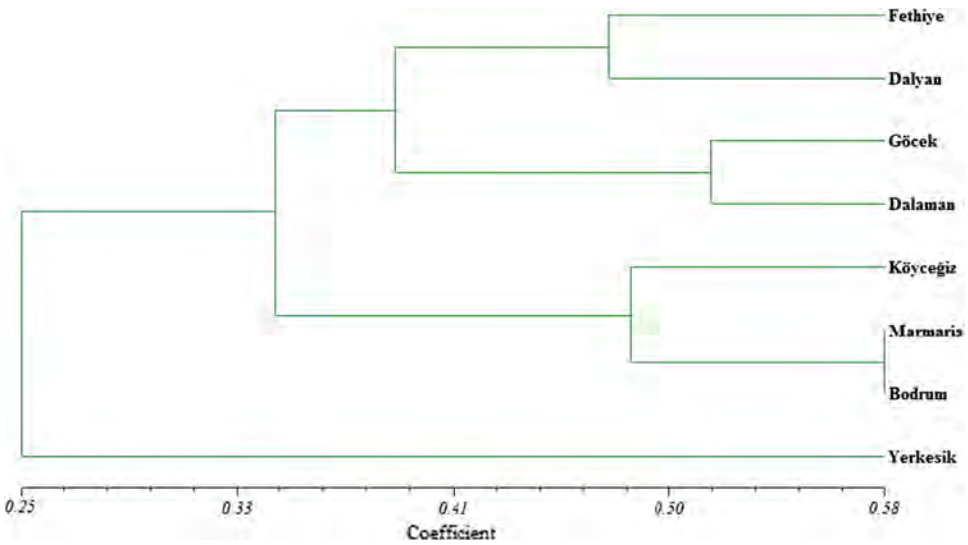

Fig. 2 Dendrogram showing the genetic relationship among sage populations

The bands that are visible and easily counted on the gel are recorded as existent or not existent (1/0).The generated data matrix was examined using the Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc ver. 2.2) (Rohlf 1991) software. In addition, principal component analysis (PCA) was performed to investigate the genetic variation among sage populations.

Results and discussion

Fourteen of the primers used in the study gave reproducible and reliable polymorphic ISSR-PCR products. The band characteristics of these primers showing amplification are given in Table 3. The maximum band-producing primers are LOL-4 and UBC-812 primer (Fig. 1), with 4 and 5 polymorphic bands, respectively. UBC-854 primer gave the least with 3 bands (Table 3).

According to Table 3, a total of 128 bands were obtained from 14 primers showing amplification in the sage populations. Forty-one of these amplified bands displayed polymorphic bands. Total polymorphism rate was 28.91%.

In a similar study, ISSR and RAPD molecular techniques were used to evaluate the genetic correlations among 21 ecotypes of 8 *Salvia* species. As a result, they determined that genomic DNA amplification produced 280 bands, 91% of which was polymorphic (Yousefiazarkhanian et al. 2016). Safaei et al. (2016) reported that the highest polymorphism rate (57.14%) was found in *S. limbata* sage and the lowest polymorphism rate (28.5%) was found in *S. reuterana*. In another study conducted with ISSR method, the polymorphic rate of 9 sage species was determined to be 0.38% (Etminan et al. 2018).

The similarity ratios of PCR products obtained by polymorphic primers are given in Table 4. The highest similarity rate (60%) was obtained between Marmaris and Bodrum sages, while the lowest one (10%) was found between Yerkesik and Bodrum sages. According to the obtained data, the most distant sages to each other were the Yerkesik and Bodrum sages and the nearest ones were the Marmaris and Bodrum sages. We examined the location information where

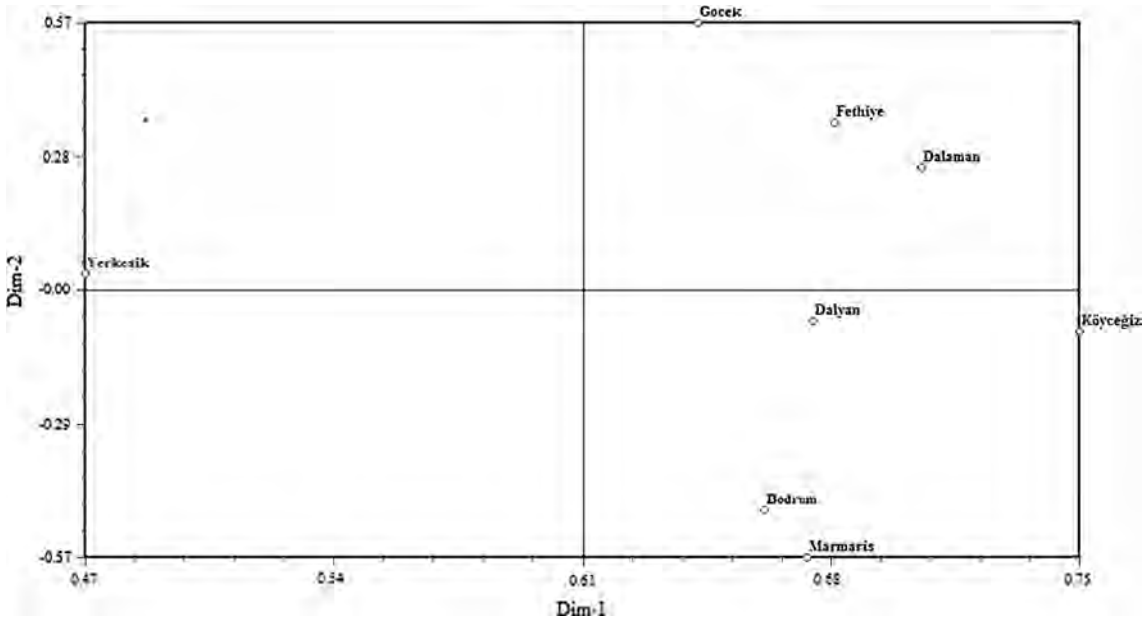

Fig. 3 Principal component analysis for sage populations

sage populations are picked up (Table 1), and because the Yerkesik location has the highest altitude, the average annual precipitation is higher, the average annual temperature is lower, and the geographical location is different; it appears that the sage belonging to this location is not similar to the sage populations growing at other locations. In addition, although the altitude difference between Marmaris and Bodrum is very high, the sage collected from these locations is similar because the average annual rainfall and temperature are same in both districts. This can be explained by the fact that sage populations may be linked to climate and ecological characteristics of the places where they are grown or may emerge independently of these characteristics.

In the present study, 2 main clusters were formed in the dendrogram (Fig. 2) which was created according to the similarity index of 8 sages. In the first main set, while the Yerkesik sage is located alone, the other main set is divided into two subsets. Bodrum sage was similar to Köycegiz sage in the rate of 60% and to Marmaris sage in the rate of 42%. The other subset is also divided into two subsets. The similarity between Göcek and Dalaman sages is 51%, and that between Fethiye and Dalyan sages is 47%.

In this study, principal component analysis (PCA) was performed to determine the genetic variation among sage populations (Fig. 3). According to the results, in terms of genetic variation, the sages belonging to Marmaris, Bodrum, Dalyan and Köyceğiz locations were in the same group. Sages, which are provided from Fethiye, Dalaman and Göcek, are included in the same group, and Yerkesik sage is classified in the separate group.

The sages took place in 3 groups (except Dalyan) similarly to the dendrogram obtained from the genetic similarity equation. Although Dalyan, Göcek, Fethiye and Dalaman are placed in the same group in the dendrogram, the sage belonging to the Dalyan location was included in the different group by the principal component analysis (PCA). This can be explained by the fact that the location where the sage grows naturally has different ecological conditions or different genetic characteristics.

Conclusion

In the research area, the climate characteristics are rainy and temperate and at the same time, the ecological structure has suitable conditions, which causes different plant vegetation to develop and thus plant diversity is increasing. A detailed inventory of the biological diversity and genetic resources of Muğla province, morphological examination of its genetic resources, molecular characterization, identification and

conservation should be realized. In addition, some legal and technical measures should be taken, gene banks should be established, the benefits of biological wealth should be taught with educations, and public awareness should be made on this issue.

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