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MOLECULAR CHARACTERIZATION OF EIGHT *Origanum* SPECIES GROWN IN THE FLORA OF ANTALYA AT THE SPECIES, LOCATION AND GENOTYPE LEVEL

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Abstract: A total of 63 samples of 8 naturally occurring *Origanum* species within the flora of Antalya were collected from twenty-one different locations during flowering periods. Taxonomic classification studies were made either at the collected sites or brought to experts for morphological characterization. Healthy and young leaves were collected from single plants and were brought to a laboratory on ice and freeze stored until used. Molecular markers generated by simple sequence repeats (SSRs), also known as microsatellites, were used to characterize 63 samples at the species, location, and genotype levels. Due to limited geographic distribution, samples of *Origanum husnucanbaseri* were collected from one location, samples of *O. bilgeri* were collected from 2 locations, while other species were collected from 3 locations. DNA markers generated from 21 primer pairs were analyzed by Nei & Li genetic similarity indices, UPGMA-2000-bootstrap analysis, and principle coordinates analysis. The following conclusions were made: (i) among the 8 species of *Origanum* naturally occurring in flora of Antalya, the section Majorana contained interspecies hybridization, (ii) the use of markers generated using 21 SSR primer pairs, grouped the species as per their sections and from primitive to advance evolution time, (iii) level of genetic diversity varied between species and locations, (iv) species-specific, location-specific and genotype specific markers were identified some of which are diagnostic type markers. Data and findings of this study strengthen the molecular and taxonomic research in *Origanum* L.

Keywords: Genotype-specific markers, Microsatellites, Spatial relationships, Species-specific markers

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1. Introduction

A country's biological richness is directly proportional to its diversity of natural species. Anatolia (Minor Asia) possesses such rich biological assets that it can be described as a "nature museum" in terms of the diversity of living species and genetic characteristics. There are two primary reasons for this. One is that Anatolia, due to its geographical location, lies in the transition zone between three different continents (Europe, Asia, and Africa). The second is that it possesses extremely diverse landforms and, consequently, diverse local climates (ecosystems) within very short distances. The genus *Origanum* (family Lamiaceae) is one of the most attractive plant species for their use in dietary properties of the spice and the various ways of adding them to foodstuff, beverages, and along with medicinal properties (Alekseeva et al., 2021; Sharifi-Rad et al., 2021; Karagöz et al., 2022; Sun et al., 2023). However, the taxonomy of the genus *Origanum* is rather complicated and a current issue of debate since most of the classification studies were based on a large morphological and chemical diversity, resulting in the distinction of 49 taxa and 42 species (Ince et al., 2014; Anonymous, 2025). Based on the taxonomic revision of Ietswaart, there exist ten sections: (i) Amaracus Bentham, (ii) Anatolicon Bentham, (iii) Brevifilamentum Ietswaart, (iv) Longitubus Ietswaart, (v) Chilocalyx Ietswaart, (vi) Majorana Bentham, (vii) Campanula ticalyx Ietswaart, (viii) Elongatispica Ietswaart, (ix) *Origanum* Ietswaart, and (x) Prolaticorolla Ietswaart. Since Ietswaart's publication, more species and hybrids have been recognized, raising the number of species, subspecies, and hybrids. Today the number of *Origanum* species and hybrids is 54, 25 of which originated or are widely grown in Türkiye. Among 25 species of Turkish *Origanum*, three are endemic to the flora of Antalya. These species are *Origanum bilgeri* P. H. Davis, *O. husnucanbaseri* Duman, Z. Aytac et A. Duran, and *Origanum solymicum* P. H. Davis (Elmasulu, 2010; Anonymous, 2025).

It is known that more than 300 scientific names have been given during the last 170 years, and there are more than 70 presently recognized *Origanum* species, subspecies, and hybrids (Gounaris et al., 2002; Sharifi-Rad et al., 2021; Alekseeva et al., 2023a). One of the popular names for oregano is "pizza grass," since it is widely used as a spice in the continents of Europe and the Americas to enhance the flavor of foods (Sun et al., 2023). Utilization of oregano varies from region to region, but most utilization is based on aromatic and medicinal purposes. The whole plant can be used as a raw material for the extraction of essential oil, which is added to perfumes for bathing and sauna (Sun et al., 2023). Several species within the genus have antibacterial, antioxidant, antiviral, anti-inflammatory, analgesic, and immune regulatory properties that have been used in traditional medicine to treat sunstroke, fever, acute gastroenteritis, dysentery, and other diseases (Kosakowska et al., 2021). Increasing customer demands for natural products enhanced the demands for essential oils of oregano. Now oregano essential oil has also been recognized worldwide as a natural feed additive and has certain potential for the preservation of chicken, fish, and rabbit (Sun et al., 2023).

Redundancy in different names given to *Origanum* is probably due to the extent of morphological variation in the genus. Species identification in the genus *Origanum* is based on morphological (Figure 1) and chemical characteristics (Elmasulu, 2010; Ince et al., 2014; Contreras et al., 2021). With respect to the essential oils, several chemotypes were found in natural populations of *Origanum* (Sun et al., 2023). However, the use of essential oil characteristics in species identification and chemosystematics has significant limitations, such as they have a low number of polymorphisms and lower numbers of markers. Furthermore, chemical markers are under the influence of environmental factors including the soil type and topography and stage of plant development (Novak et al., 2008; Contreras et al., 2021; Alekseeva et al., 2023a; Sun et al., 2023).

In the last four decades, continuous advances in molecular biology and related sciences have dramatically improved the development of molecular markers and offered a set of new techniques useful for systematic, phylogenetic, and identification studies. Today the use of next-generation DNA sequencing techniques enhanced the speed of development of molecular markers such as simple sequence repeats (SSRs) and single nucleotide polymorphisms (SNPs) (Alekseeva et al., 2023b). Due to its codominant inheritance and multi-allelic properties, SSRs seem to be more powerful methods for plant breeding (Ince et al., 2014).

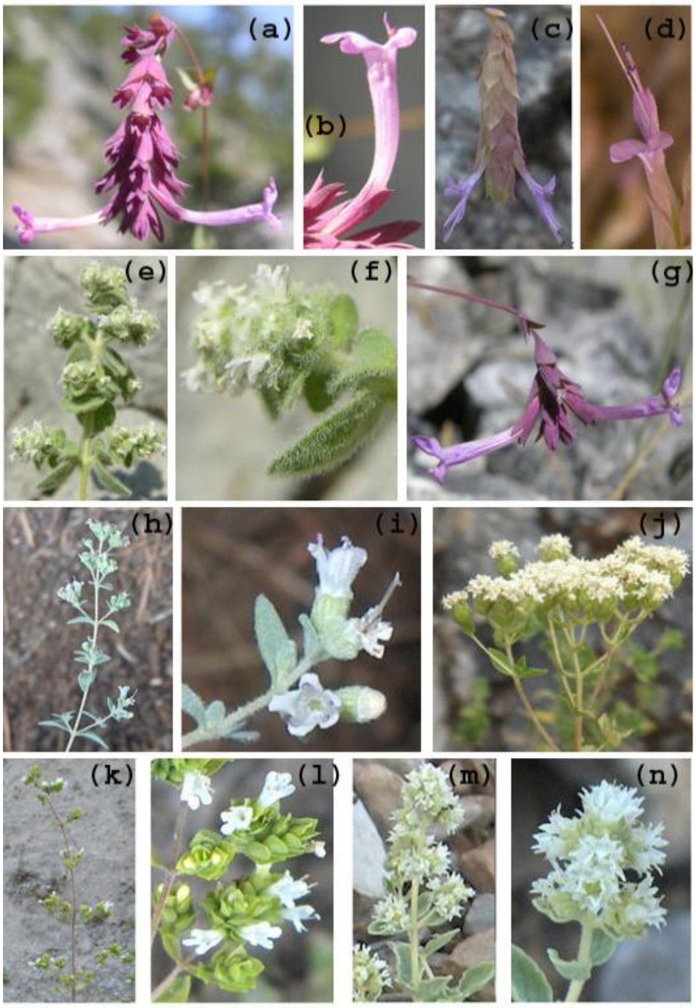

Figure 1. Flowers of *Origanum* samples are used in this study. (a) and (b): *O. saccatum*, (c) and (d): *O. solymicum*, (e) and (f): *O. bilgeri*, (g): *O. husnucanbaseri*, (h) and (i): *O. minutiflorum*, (j): *O. onites*, (k) and (l): *O. vulgare* subsp. *hirtum*, and (m) and (n) *O. majorana*.

Simple sequence repeats, also known as microsatellites, have been used in genetic studies of many organisms, including many agronomically important plant species. The utilization of SSRs includes fingerprinting, genetic relationship, mapping, gene pyramiding, marker-assisted selection, conservation, and management of genetic resources (Ince et al., 2014; Sun et al., 2023). However, the use of SSRs in the genus *Origanum* is low in comparison to other medicinal and aromatic plant species. One of the first works of SSR development and utilization in *Origanum* was Novak et al. (2008), followed by Ince et al. (2014), Sun et al. (2023), and Alekseeva et al. (2023b).

In this study, nucleotide sequence information of 50 primer pairs, which were kindly provided by Dr. Johannes Novak, who published 13 *Origanum* specific primer pairs in 2007, were commercially synthesized and were initially screened using bulked *Origanum* DNA samples. Results revealed that among the primer screened 21 were found to be suitable to use in diagnostic markers for species, location, and genotype identification studies of *Organum* species naturally grown in the flora of Antalya. Here, it was reported that the section Majorana contained interspecies hybridization, the use of markers generated using 21 SSR primer pairs, grouped the species as per their sections and from primitive to advance evolution time. The use of these marker allows for the preservation of large populations of *Origanum*, maintaining the natural balance of the ecosystem.

2. Materials and Methods

2.1. Plant Materials

A total of 63 individual plants of Antalya flora were collected from their natural growing locations. Plant samples, locations where they were collected, and altitude above sea level (m), along with sectional information were depicted in Table 1. As shown in Table 1, samples of *O. husnucanbaseri* were collected from Alanya since it originated from Alanya and no other growing locations are known. Samples of *O. bilgeri* were collected from Akseki and Gündoğmuş while other samples were collected from three different locations. Samples were collected during flowering, and in some cases, the same locations were visited more than once to capture the flowering time. The total number of locations was 21, covering a large portion of Antalya. Twenty-one locations were visited during flowering periods, and taxonomic classification studies were made either at the locations or brought to taxonomy experts for morphological characterization. Healthy and young leaves were collected from single plants and brought to a laboratory on ice and freeze stored until used. It was ensured that harvested plants had enough leaves and flowers to survive after collection.

Table 1. Information regarding locations, species, and sections of *Origanum* used in this study

Location No	Section	Species	Location	Altitude (m)
1	Amaracus (Gleditsch) Bentham	*O. saccatum* Davis	Gündoğmuş	1008
2		*O. saccatum* Davis	Alanya	1173
3		*O. saccatum* Davis	Gazipaşa	281
4		*O. solymicum* Davis	Kemer	51
5		*O. solymicum* Davis	Kemer	471
6		*O. solymicum* Davis	Kemer	1149
7	Brevifilamentum Ietswaart	*O. husnucanbaseri* H. Duman, Z. Aytac et A. Duran	Alanya	1359
8	Chilocalyx (Briq.) Ietswaart	*O. bilgeri* Davis	Gündoğmuş	1545
9		*O. bilgeri* Davis	Akseki	1115
10		*O. minutiflorum* Schwartz et Davis	Bozburun	1332
11		*O. minutiflorum* Schwartz et Davis	Sakarpınar	1788
12		*O. minutiflorum* Schwartz et Davis	Kemer	1339
13	Majorana (Miller) Benth	*O. majorana* L.	Akseki	582
14		*O. majorana* L.	Gazipaşa	1372
15		*O. majorana* L.	Alanya	1041
16		*O. onites* L.	Elmalı	1037
17		*O. onites* L.	Faselis	-7
18		*O. onites* L.	Kumluca	521
19	*Origanum*	*O. vulgare* L. subsp. *hirtum* (Link) Ietswaart	Gazipaşa	1468
20		*O. vulgare* L. subsp. *hirtum* (Link) Ietswaart	Kemer	1053
21		*O. vulgare* L. subsp. *hirtum* (Link) Ietswaart	Alanya	1317

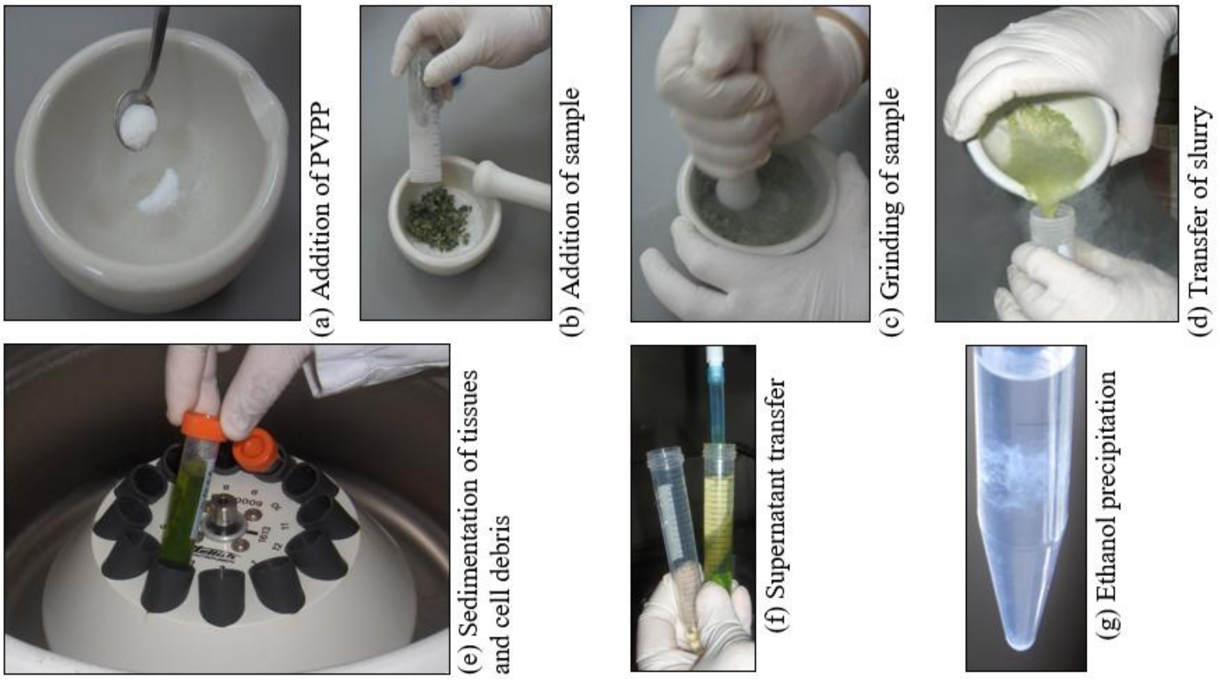
(a) Addition of PVPP
(b) Addition of sample
(c) Grinding of sample
(d) Transfer of slurry
(e) Sedimentation of tissues and cell debris
(f) Supernatant transfer
(g) Ethanol precipitation

Figure 2. Grinding of *Origanum* sample in the presence of PVPP and liquid nitrogen.

2.2. Genomic DNA Extraction

All reagents used in this study were high molecular biology grades purchased from Amresco Inc., (Solon, Ohio, USA) and Invitrogen Corp., (Carlsbad, CA, USA). *Origanum* leaf tissues (3-5 g) were powdered in liquid nitrogen in the presence of 100 mg insoluble polyvinyl pyrrolidone (PVPP, Sigma) and were transferred into 30 mL or 50 mL Falcon tubes (Figure 1). About 1 g of powdered tissue was transferred to a 15 mL centrifuge tube and left on ice for 10 minutes or until completely thawed. About 5 mL of isolation solution (Doyle and Doyle, 1987) and 50 µL of β-mercaptoethanol (BME) were added and vigorously vortexed for 1-2 minutes before transferring to a 65 °C water bath for 2 hours, vortexing vigorously in 20 minutes intervals. After incubation, 5 mL chloroform isoamyl alcohol (CIA) were added to each tube, mixed well and incubated for 10 minutes at room temperature, centrifuged at 13770 xg for 10 minutes, and supernatants were transferred into clean tubes (Figure 2). An equal volume of isopropyl alcohol (stored in a freezer at -20 °C) was added to the supernatant, mixed by inversion until the DNA fibers appeared, and centrifuged at 13770 xg for 10 minutes. The pellets were dissolved in 5 mL isolation buffer with 50 µL of BME, and incubation and centrifugation steps were repeated as before. Pellets were dissolved in 0.5 ml TE (10 mM Tris and 1 mM EDTA, pH: 7.5) and 5 µL RNase (DNase free).

Solution was mixed well by inverting the tubes several times before incubation at 37 °C for 30 minutes. After incubation, 50 µL of 3 M sodium acetate (pH 5.2) and 1 mL of 100% ethanol were added, mixed by inversion several times, and centrifuged at 13770 xg for 5 minutes. After centrifugation, the alcohol was poured off, and the tube was left to dry until the alcohol completely evaporated. After drying, the DNA in the tube was first washed with 0.7 mL of 70% cold (-20 °C) ethanol and centrifuged at 20370 xg for 5 minutes. The alcohol was decanted, and a second 0.7 mL of 96% cold (-20 °C) ethanol was added and centrifuged at 20370 x g for 5 minutes. The alcohol was decanted, and the tube was left to dry. After the alcohol had completely evaporated, the DNA was dissolved in 200-300 µL of TE (pH 8.0), and the obtained DNA samples were stored at –20 °C.

2.4. Determining DNA Quality and Quantity

Controlling the quality and quantity of DNA from isolated samples is essential for optimizing and standardizing the study procedures. Both agarose gel electrophoresis and spectrophotometric analysis methods were used for this purpose. Agarose gel electrophoresis was used to confirm the presence and the integrity of genomic DNA molecules, purity (RNA contamination and presence of polysaccharides), and the presence of high molecular weight DNA. A 1% agarose gel with 1x TBE solution (89 mM Tris, 89 mM Borate, 2 mM EDTA, pH 8.3) was prepared, and electrophoresis was used to visualize DNA in the presence of ethidium bromide.

In the spectrophotometric method, 20 µL of the sample DNA was added to 480 µL of distilled water (25-fold dilution). The quality and quantity of DNA samples were calculated using absorbance values at wavelengths of 260 nm (A260), 280 nm (A280), and 230 nm (A230) using a spectrophotometer (Ince et al., 2014). The DNA amount (µg/mL) = A260 x 50 x dilution value. In the formula, 50 represents the existence coefficient of dsDNA.

2.5. Touchdown Polymerase Chain Reactions (Td-PCRs)

Td-PCRs were carried out in 25 µL reaction volumes containing 60 ± 5 nanograms of *Origanum* genomic DNA sample as the template, 0.5 µM of each primer pair (Table

2), 80 mM TRIS–HCl (pH 8.8), 19 mM $(NH_4)_2SO_4$, 0.009% Tween-20 (w/v), 0.28 mM of each dNTP, 3 mM $MgCl_2$, and 1 unit of Taq DNA polymerase (Invitrogen Corp. Carlsbad, CA, USA).

The Td-PCR amplification profile consisted of an initial denaturation at 94°C for 3 minutes, ten cycles with denaturation at 94°C for 30 seconds, annealing at 66 °C, 60 °C, or 55 °C (during initial screening studies) for 30 seconds in the first cycle, diminishing by 0.5 °C each cycle, and extension reactions at 72°C for 1 minute using a Veriti 96-well thermal cycler (Applied Biosystems, Foster City, CA, USA). An additional 30 more PCR cycles were run using the same cycling parameters mentioned above with constant annealing at 61 °C, 55 °C, or 50 °C (during initial screening studies). Denaturation and extension conditions were the same as indicated above. The amplifications finished with final extension reactions at 72 °C in 10 minutes.

Table 2. Forward and reverse sequences of SSR primer pairs provided by Johannes Novak

Primer ID	5'→3' forward and reverse sequences
OR-SSR201-F	TTCTTTTCATCAACCAACCTAGCTAC
OR-SSR201-R	GCCATGAGAAAAGCAGATAGGAG
OR-SSR202-F	AAGTGAAGTAACGCTTCCATGAGAG
OR-SSR202-R	CCCGAGTACAAAAGAGCTACAGATG
OR-SSR209-F	TTGAAGCATTGTTGGAGGTAGATG
OR-SSR209-R	TCCCAACTAGGGAGAAATGTGC
OR-SSR210-F	TTTGCTCCGACATCTTCAACC
OR-SSR210-R	AGCCTGCTGTGTTTGGATCAG
OR-SSR212-F	GCCCCTGCAGTGACTCCTAC
OR-SSR212-R	AAAAAGGCTTCGGACTCGATC
OR-SSR213-F	GAGAGAATCCAAGCCTCCGC
OR-SSR213-R	TGAAGGAGTCCGATGTTGACG
OR-SSR217-F	TTTGCGCAGATCTCAAGTGC
OR-SSR217-R	AAGCGGTGACTGACGGAGAC
OR-SSR218-F	GGATGATGCTGAGTTGGTGATAAG
OR-SSR218-R	CCTGACACGCCACAAAAGTG
OR-SSR219-F	GGGCATTAAAGCTAAGGAGCG
OR-SSR219-R	CAGCCGATCACCTGTCCTTC
OR-SSR221-F	CACACGCACTGGTGAGGTG
OR-SSR221-R	TTCCCGCAGATCTCCAGAAC
OR-SSR227F	TCAGAAACAATGAAGGCCGC
OR-SSR227R	CCGTACAGGTCAAACACCGG
OR-SSR231F	ACCCCCTGTATACGTTGGACC
OR-SSR231R	GCATCAAAAAGGGTTCGGAC
OR-SSR238F	AGCCAAACTCGCTGCTTCTG
OR-SSR238R	CAAGGTAAAAAGGGTAATAGACGTGG
OR-SSR240F	GCCCAAGGACATCCAACTTG
OR-SSR240R	CAACTGAACACCTCCCACAATG
OR-SSR244F	TCAAGGGTAGAGCTGCTGCAG
OR-SSR244R	GCTTTACGGAGGAAGAATGGG
OR-SSR252F	ATGCCCAGGGACATCCAAC
OR-SSR252R	ACTGAACACCTCCCCACAATG
OR-SSR266F	AAGATCGAAGGCATCGATCG
OR-SSR266R	GGTGAAAAATGAATACAGTGGGC
OR-SSR267F	TCTTCATCAAGTTCAATAATGCTGTG
OR-SSR267R	GATTCAGATAGTTGCATCGAGGTTAG
OR-SSR270F	TCCCATCATTTTCCTCCGTC
OR-SSR270R	CCCCACTACAGCAGAAACCG
OR-SSR277F	TGAAGTCAGTTTGGATGATGGTG
OR-SSR277R	GTCACGTATGGAATGCACGG
OR-SSR281F	GAAGTTCGCCGAGGCTCTC
OR-SSR281R	CAAAGCACAAGAAAATACAATAGCAC

2.6. Agarose Gel Electrophoresis

Five microliters of DNA loading buffer (prepared with 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, and 40% (w/v) sucrose in water) were added to each 25 µL PCR amplified products, and 8–10 µL of these mixtures were loaded in 4% (w/v) high-resolution Serva agarose gels (SERVA Electrophoresis GmbH, Heidelberg, Germany) containing 0.5 µg/mL ethidium bromide. SSR markers were electrophoresed at 5 V cm-1 at constant voltage for 8–12 h in the presence of 1x TRIS–Borate ethylenediamine tetra acetic acid (EDTA) buffer [89 mM TRIS, 89 mM Borate, 2 mM EDTA, pH 8.3] and photographed on an ultraviolet transilluminator for analysis (Ince et al., 2014).

2.7. Data Analysis

Amplified products of 63 *Origanum* samples were scored as presence (1) or absence (0), and scores were used in the calculation of Nei and Li's genetic similarity indices. Nei and Li's genetic similarity indices (GSIs) among different *Origanum* species were calculated using the formula: GSIXY: 2a/(a + b) + (a + c), where X and Y are the numbers of SSR markers in individuals X and Y, respectively, a is the number of markers shared between individuals (species) X and Y, b is the number of markers present in individual (species) X but absent in individual Y, and c is the number of markers absent in individual X but present in individual Y.

Multivariate clustering analysis and principal coordinates analysis (PCoA) were used in data analysis. PCoA is an ordinate method that uses a distance matrix rather than values (Manly, 1994). Unweighted pair group method with arithmetic mean (UPGMA), Bootstrap analysis, and principal coordinates analysis (PCoA) based on the Nei & Li matrix were used to demonstrate the relationships between samples using MVSP software version 3.13. The accuracy of genetic relationships between samples was calculated using bootstrap analysis with 2000 replicates (Ince et al., 2009).

Furthermore, genetic variation within species and within locations was determined using the average genetic similarity index (GSI) according to Ince et al. (2009) using the formula GSI = ∑GSI/n. In the formula, n represents the total number of GSI values. Determination of species-specific SSR markers in the present study was based on the following criterion (Karaca et al., 2008; Ince and Karaca, 2012): a species-specific marker was defined as a distinct and reproducible SSR markers present in all the individuals of one species but absent in all the other individuals of the seven species used in this study.

3. Results and Discussion

A total of 50 SSR primer pairs kindly provided by Dr. J. Novak were first analyzed using in silico approaches and PCR screening studies. Twenty-nine of these primer pairs were not selected due to sequence similarity or great differences between annealing temperatures within the primer pair, nonspecific amplification and PCR fail. The remaining 21 *Origanum* L.-specific (OR-SSR) primer pairs were found suitable, and they were commercially synthesized. Each OR-SSR primer pair was further tested in PCR studies using a randomly selected *Origanum* genotype and annealing temperatures. Although some primer pairs produced nonspecific amplified products along with SSR patterns, they were suitable for genetic studies of 8 *Origanum* species naturally grown in the flora of Antalya (Table 1).

In this study, DNA analysis was performed on *Origanum* L. taxa at three different levels using SSR markers. Analyses were conducted by combining DNA samples from taxa within the same species (by species level), combining DNA from genotypes collected from the same location (by location level), and finally, analyzing each genotype separately (by genotype level). Results clearly showed that *Origanum* species had higher levels of genetic variations, as shown in representative agarose gel photographs (Figure 3). Based on the criteria to define species- specific markers (Karaca et al., 2008; Ince and Karaca, 2012), 14 putative species-specific SSR primer pairs from 21 SSR primer pairs were identified. Further studies were performed using these putative species-specific SSR primer pairs that were used in 63 *Origanum* samples to identify species-specific, location specific and genotype specific markers (diagnostic markers).

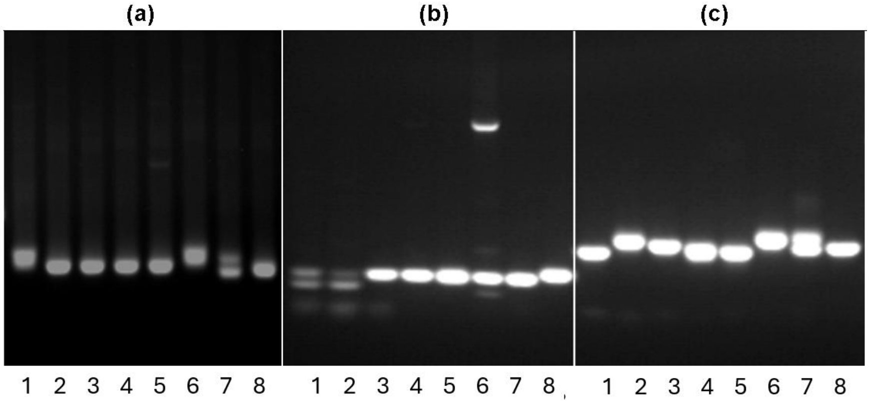

Figure 3. Representative analysis at a special level. OR-SSR240 (panel a), OR-SSR201 (panel b), and OR-SSR209 (panel c) primer pairs amplified products in *Origanum* species. Lane 1: *O. saccatum*, 2: *O. solymicum*, 3: *O. husnucanbaseri*, 4: *O. bilgeri*, 5: *O. minutiflorum*, 6: *O. majorana*, 7: *O. onites*, and 8: *O. vulgare* L. subsp. *hirtum.*

Table 3. Interspecies genetic similarity indices* (Nei and Li)

Species	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
O. saccatum (1)	1.000							
O. solymicum (2)	0.519 ± 0.018	1.000						
O. husnucanbaseri (3)	0.430 ± 0.007	0.435 ± 0.009	1.000					
O. bilgeri (4)	0.497 ± 0.022	0.473 ± 0.020	0.472 ± 0.006	1.000				
O. minutiflorum (5)	0.460 ± 0.018	0.463 ± 0.013	0.480 ± 0.012	0.675 ± 0.015	1.000			
O. majorana (6)	0.379 ± 0.026	0.378 ± 0.014	0.394 ± 0.009	0.503 ± 0.018	0.567 ± 0.018	1.000		
O. onites (7)	0.436 ± 0.015	0.382 ± 0.018	0.433 ± 0.008	0.527 ± 0.017	0.517 ± 0.022	0.556 ± 0.021	1.000	
O. vulgare subsp. *Hirtum* (8)	0.345 ± 0.014	0.345 ± 0.014	0.357 ± 0.013	0.441 ± 0.016	0.481 ± 0.016	0.479 ± 0.016	0.531 ± 0.019	1.000

*: standard deviations indicated ± in the table above.

Genetic similarities among 8 *Origanum* species based on Nei and Li indices were studied (Table 3). Based on the SSR markers, *O. bilgeri* and *O. minutiflorum* were most related while *O. vulgare* L. subsp. *hirtum* and *O. saccatum*, and *O. vulgare* L. subsp. *hirtum* and *O. solymicum* were the most unrelated species among 8 *Origanum* species (Table 3), agreeing with morphological differences shown in Figure 1. Overall genetic relationships among the species used in the present study agreed with the Iteswaart classifications, which were established based on morphological markers (Ietswaart et al., 1982).

To investigate genetic variation between locations where samples were collected, location-level SSR analyses were performed. As shown in Figure 4, three samples for each location were amplified using SSR primer pairs that produced putatively species-specific markers. Primer pair OR-SSR209 (Figure 4 panel a), and OR-SSR238 (Figure 4 panel b) showed representative analysis of defining species-specific markers (Karaca et al., 2008; Ince and Karaca, 2012). Primer pair OR-SSR209 produced species-specific marker for *O. saccatum*, and OR-SSR238 produced species-specific marker for *O. majorana*.

A total of 14 SSR primer pairs produced species-specific markers which were defined as markers that definitively distinguished 8 *Origanum* species from each other. Species-specific markers were OR-SSR201 (*O. vulgare* subsp. *hirtum*), OR-SSR202 (*O. saccatum*), OR-SSR209 and OR-SSR212 (*O. onites*), OR-SSR213 (*O. minutiflorum*), OR-SSR218 and OR-SSR219 (*O. onites*), OR-SSR227 (*O. saccatum*), OR-SSR231 (*O. majorana*), OR-SSR231 (*O. solymicum*), OR-SSR238 (*O. majorana*), OR-SSR240 (*O. husnucanbaseri*), OR-SSR244 (*O. vulgare* subsp. *hirtum*), and OR-SSR267 (*O. onites*). It should be noted here these results were based on 4% high-resolution grade agarose gel separation, using a capillary separation system would produce a greater number of species-specific markers among 8 *Origanum* species naturally occurring in the flora of Antalya.

To investigate the level of variation in genetic similarity indices (Nei and Li) within species, the mean, minimum-maximum, and standard deviation (SD) were calculated (Table 4). Results revealed that *O. bilgeri* grown in Gündoğmuş and Akseki had the highest genetic variation within a species among *Origanum* species grown in the flora of Antalya. On the other hand, the lowest genetic variation was observed in *O. husnucanbaseri* collected from Alanya. It was also noted that *O. majorana*, *O. onites*, and *O. vulgare* subsp. *hirtum* had very similar means genetic similarity within their samples.

As shown in a representative image in Figure 5, fourteen OR-SSR primer pairs were screened 63 *Origanum* samples (Figure 5). In general, individuals collected from the same locations or close distances contained the same alleles, while individuals collected from long distances consisted of different alleles (Figure 5). This indicated that high level of gene flow did not occur. It should be noted that among the *Origanum* species used in this study, location-specific markers for six individuals belonging to *O. bilgeri* (endemic to Antalya) could not be identified due to higher intra-specific variations, probably induced by cross-species hybridization. Location specific markers for remaining 7 species were also determined; these markers were from those species-specific markers. Some genotypes produced genotype specific markers as represented in Figure 4c. These types of markers could differentiate a genotype from remaining of 62 different samples of *Origanum* collected from the flora of Antalya.

The estimated phylogenic tree of UPGMA-Bootstrap (one dimension-based method) and PCoA (two dimensions-based method) generally agreed with the Iteswaart classifications (Figure 6 and Figure 7).

However, it was noted that principal coordinates analysis (PCoA) shown in Figure 7, although confirmed the clear separation of *Origanum* samples used in this study, indicated problems in which two samples of *O. onites* from the same location and two samples of *O. majorana* from the same location, had a lack of clear separations (Figure 7). It was speculated that these samples were either natural hybrids, or mistakenly labeled samples, or natural mutants (Lukas et al., 2013). These four samples were not considered in the studies of identification of species-specific, location-specific and genotype-specific marker development.

Table 4. Within species genetic variation in eight *Origanum* L.

Location	Mean	SD	Min.	Max.
O. saccatum: Gündoğmuş, Alanya, Gazipaşa	0.92	0.04	0.85	1.00
O. solymicum: Kemer	0.94	0.02	0.90	1.00
O. husnucanbaseri: Alanya	0.96	0.01	0.95	0.97
O. bilgeri: Gündoğmuş, Akseki	0.89	0.06	0.83	0.98
O. minutiflorum: Bozburun, Sakarpınar, Kemer	0.95	0.02	0.92	0.99
O. majorana: Akseki, Gazipaşa, Alanya	0.93	0.02	0.90	0.99
O. onites: Elmalı, Faselis, Kumluca	0.93	0.03	0.87	0.99
O. vulgare subsp. *hirtum* : Kemer, Gazipaşa, Alanya	0.93	0.03	0.89	1.00

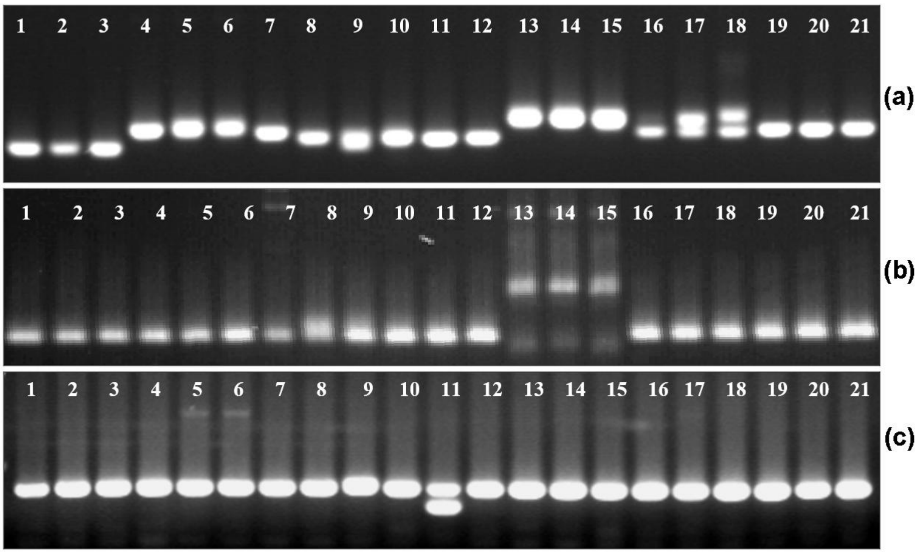

Figure 4. Representative analysis at the local level. OR-SSR209 (panel a), OR-SSR238 (panel b), and OR-SSR270 (panel c) primer pairs amplified products in *Origanum* samples collected from different locations. Lanes 1-3: *O. saccatum* collected from Gündoğmuş, Alanya, and Gazipaşa, 4-6: *O. solymicum* collected from different regions of Kemer, 7: *O. husnucanbaseri* collected from Alanya, 8-9: *O. bilgeri* collected from Gündoğmuş, Akseki, 10-11: *O. minutiflorum* collected from Bosburun, Sakarpınar, Kemer, 13-15: *O. majorana,* collected from Akseki, Gazipaşa, Alanya, 16-18: *O. onites* collected from Elmalı, Faselis, and Kumluca and 19-21: *O. vulgare* L. subsp. *hirtum* collected from Gazipaşa, Kemer, and Alanya.

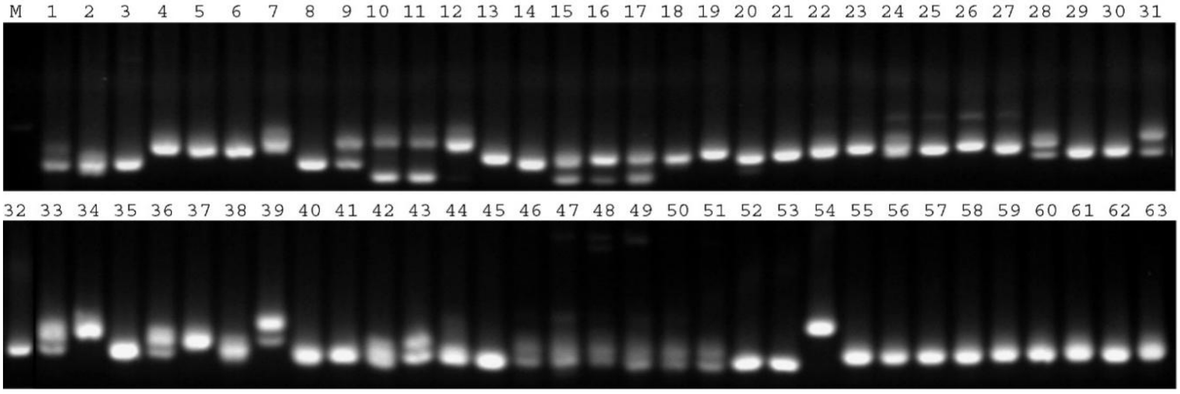

Figure 5. Representative analysis at the genotype level. OR-SSR202 primer pair amplified products. Lanes: M size marker 200 bp, 1-9: *O. saccatum* genotypes, 10-18: *O. solymicum* genotypes 19-21: *O. husnucanbaseri* genotypes, 22-27: *O. bilgeri* genotypes, 28-36: *O. minutiflorum* genotypes, 37-45: *O. majorana* genotypes, 46-54: *O. onites* genotypes 55-63: *O. vulgare* L. subsp. *hirtum* genotypes.

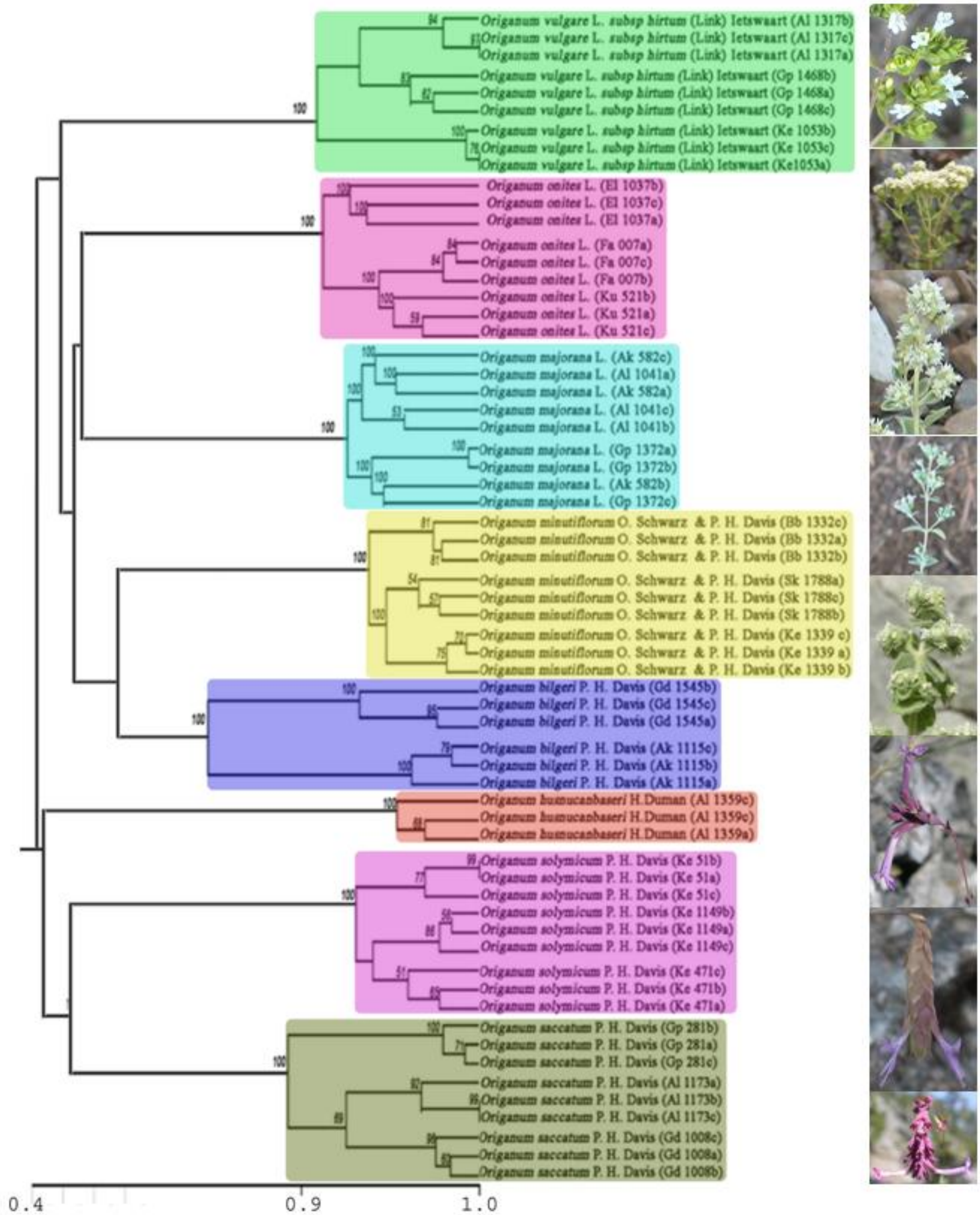

Figure 6. UPGMA analysis of eight *Origanum* species naturally grown in the flora of Antalya based on character differences of Nei and Li's genetic similarity indices (scale). Please note that numbers on nodes show bootstrap frequency values with 2000 repetitions.

As shown in Figure 6, eight species of *Origanum* naturally occurring in the flora of Antalya were divided into 5 main groups corresponding to five sections. At the base of tree, two species, *O. saccatum* and *O. solymicum,* were the oldest group. Within this group, *O. saccatum,* which is distributed in east of Antalya, was sampled from Gazipaşa, Alanya, and Gündoğmuş. The Gazipaşa location (281 m) is separated from Alanya (1173 m) and Gündoğmuş (1008 m) by the difference in elevation. The other species of this group is *O. solymicum*, a species endemic locally to the Kemer region. Individual plants were collected from elevations of 51 m, 471 m, and 1149 m in this region. Individuals sampled from the 51 m elevation location, which were collected from calcareous rocks, were separated from those sampled at the 471 m and 1149 m elevations, which were collected from forest and roadside slopes. The bootstrap values and the UPGMA tree obtained from the analysis also confirmed at 100% for each species. Distinctions between these two species and their relations were also confirmed using PCoA analysis as shown in Figure 7.

O. saccatum and *O. solymicum* species have common morphological characters in section Amaracus. It is known that these species are evolutionarily the most primitive section of this genus, which is widespread in Türkiye, and include large, drooping spikelet inflorescences; circular floral whorls with two flowers; a calyx with one or two lips that are morphologically quite different from each other; a corolla with saccules and stamens extending from the corolla; and filaments of approximately equal length to the corolla. The most obvious morphological character that distinguishes these species from other species of this genus distributed in Antalya is their generally drooping, large spike-shaped inflorescences (Elmasulu, 2010).

The second group consisted of *O. husnucanbaseri*, which belongs to Brevifilamentum section, a locally endemic species found between 1200 and 1500 m along the Alanya-Hadim road (Elmasulu, 2010) It is morphologically similar to *O. saccatum* and *O. solymicum,* as indicated with UPGMA and PCoA results (Figure 6 and Figure 7). Section Brevifilamentum differs morphologically from section Amaracus by having several flowers (rather than just two) in circular inflorescences, well-developed teeth on both the upper and lower lips of the calyx (rather than being toothless), a bilabiate corolla that lacks saccules (in contrast to those that have saccules), stamens of significantly varying lengths (more or less unequal), and filaments that are considerably shorter than the corolla (instead of being nearly equal in length). *O. husnucanbaseri* is the sole member of Brevifilamentum section in Antalya. As seen in UPGMA and PCoA results, *O. saccatum*, *O. solymicum*, and *O. husnucanbaseri* are related (Figure 1). Based on the morphological characteristics mentioned above, *O. husnucanbaseri* is evolutionarily more advanced than the species in section Amaracus.

The third group consisted of *O. bilgeri* and *O. minutiflorum,* both belonging to section Chilocalyx. *O. bilgeri* and *O. minutiflorum* species are endemic to Türkiye, exclusively located in Antalya. They are characterized by erect inflorescences that take the form of small spikelet, with circular inflorescences that are two-flowered. The calyx can be one- or two-lipped and often features small teeth. The corolla is two-lipped and is approximately twice the length of the calyx. The stamens are nearly equal in length and remain enclosed within the corolla, while the filaments are about half the length of the corolla. Samples of *O. bilgeri* were collected from Gündoğmuş (1545 m) and Akseki (1115 m). Samples of *O. minutiflorum* were collected from Saklıkent (1788 m), and Kemer (1339 m), and the Bozburun Mountain forest road (1332 m). An examination of the bootstrap values and UPGMA tree for *O. minutiflorum* shown in Figure 6 reveals that this grouping is consistent, with samples from the eastern Bozburun location distinct from the other two locations.

The first specimens of *O. minutiflorum* were collected from Tahtalı Mountain in 1947 by P. H. Davis, the editor of the Flora of Türkiye, and were introduced to the scientific community in 1949 alongside O. Schwarz. In Antalya, *O. minutiflorum* is found in the easternmost regions of Tahtalı Mountain, the Bey Mountains, Saklıkent, and their immediate surroundings, as well as in the westernmost regions of Bozburun Mountain and its vicinity. As Ietswaart et al. (1982), who revised the genus *Origanum*, noted in his Flora of Türkiye, some specimens displaying morphological characteristics of both species are found in locations such as Bozburun Mountain, where the current distribution of these species, endemic to Antalya, at transitions.

The strongest morphological evidence demonstrating that these specimens exhibit transitional characteristics is seen in the general structure and pubescence characteristics of their calyxes and their proportions to their bracts.

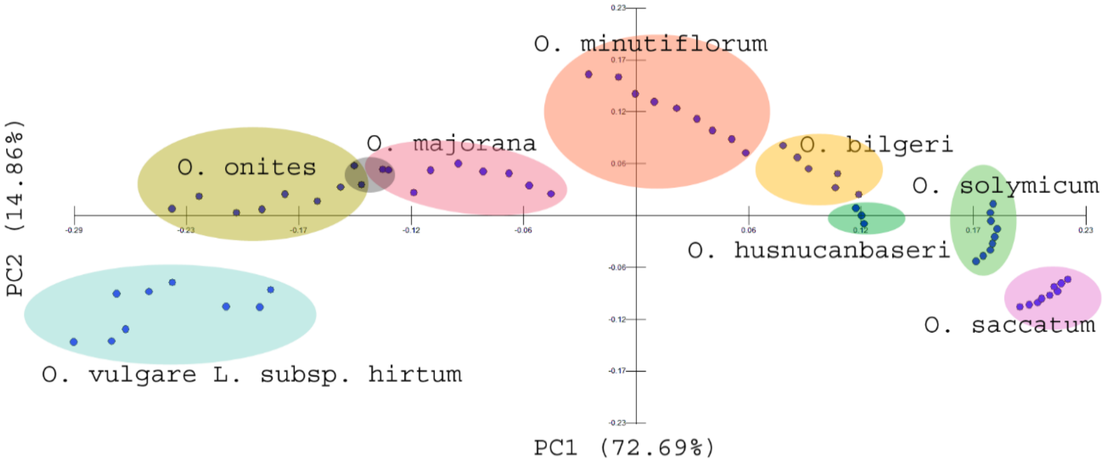

Figure 7. Principle coordinates analysis of 63 samples from eight *Origanum* species naturally grown in the flora of Antalya based on character differences of Nei and Li's genetic similarity indices.

The most significant morphological differences between the two species are seen in the stem and leaf hair structure, bract and corolla size, and the general structure of the calyx. It was noted that in this study *O. bilgeri* and *O. minutiflorum* were similar species and clustered at 100% bootstrap values. The stem and leaf pubescence of *O. minutiflorum* is pilose (sparsely hairy). However, the same feature is sparsely tomentose or pubescent in *O. bilgeri*. Furthermore, the bracts and corollas of *O. bilgeri* are wider and longer than those of *O. minutiflorum*. During the fieldwork for this study, it was noted that specimens from the Akseki Serebil well and its surrounding area exhibit a mixture of these features. Specimens collected from the Akseki Serebil well region share a general stem and leaf pubescence with *O. minutiflorum*, but their bracts bear a closer resemblance to *O. bilgeri* (Elmasulu, 2010). These specimens, which are transitional between the calyx and calyx length, are also distributed on Bozburun Mountain, as noted by Ietswart in Flora of Türkiye. Davis (1982) stated that the specimens he collected from this region in 1945 were transitional between these two species and emphasized the need for further taxonomic study to fully define their taxonomic position.

The fourth group of UPGMA analysis consisted of *O. majorana* and *O. onites*, located within the Majorana section (Figure 6). *O. majorana* samples were collected from Akseki (582 m), Alanya (1041 m), and Gazipaşa (1372 m). *O. onites* samples were collected from the Elmalı district (1037 m) in the northwest of Antalya and from Phaselis (-7 m) and Kumluca (521 m). UPGMA results differentiated the sample collected from the coastal locations of Phaselis and Kumluca and Elmalı, located in the interior of Antalya. When examined in terms of elevation difference, Elmalı location was distinguished from the other two locations by 1037 m. In this section differences existed between UPGMA and PCoA results (Figure 7), although each species was supported by 100% bootstrap values. The situation clearly supports both the data obtained during field studies conducted within the thesis and the findings of the morphological characters of the species, which range from primitive to advanced. *O. majorana* and *O. onites* differ from *O. bilgeri* and *O. minutiflorum* in that the bracts envelop the calyx; the calyx is flat, bract-like, and single-lipped without any division; the corolla is flat; and the stamens are of varying lengths and are external to the corolla.

It was reported that Majorana is one of the taxonomically problematic sections since morphological characters fail to explain the exact boundaries and evolutionary relationships of specie (Lukas et al., 2013). Elmasulu et al. (2011) reported that morphology-based classification failed to differentiate some samples of *O. majorana* and *O. onites*. In the present study, it was noted that UPGMA clustering differentiates *O. majorana* and *O. onites* with 100% bootstrap values (Figure 6). On the other hand, PCoA analysis based on Nei and Li genetic similarity indices revealed that two *O. majorana* and two *O. onites* were probably natural hybrid naturally occurred (Figure 7). Although interspecies hybridization is one of the sources and causes of speciation, it makes it difficult to classify *Origanum* species. Previous studies revealed that interspecies hybridization occurred in natural populations of the genus *Origanum*. It is known that occurrences of interspecies hybridization happen relatively often when the species distribution overlaps (Arabaci et al., 2021; Alekseeva et al., 2023b).

The fifth group consisted of *O. vulgare* subsp. *hirtum* collected from Kemer (1053 m) in the west of Antalya, as well as from Alanya (1317 m) and Gazipaşa (1468 m) in the east. All three locations are above 1000 m, but the Alanya and Gazipaşa locations are higher than Kemer, closer to 1500 m. Both the elevation and the direction differences are reflected in the UPGMA tree. Among the species assessed in this study, all but *O. vulgare* possesses one- or two-lipped calyxes. *O. vulgare*, belonging to the *Origanum* section, exhibits a five-toothed calyx structure. This species, which has four subspecies in Türkiye, is represented by subsp. *hirtum* in Antalya Province. It is distinguished from other species in the study by its unique calyx structure and several other traits: the inflorescences are small spikelet that stand upright, two flowers arranged in circular flower rings, the corolla is about 2.5 times longer than the calyx, the stamens vary in length and possess short outgrowths from the corolla, and the filaments are half the length of the corolla.

Analyses revealed that certain plant samples displayed differences between locations or among genotypes within the same location. A high level of variation and genetic heterozygosity were detected in most species studied. Geographic and ecological factors also appeared to significantly influence the varying levels of genetic variation observed within the same species at different locations or even within the same location. The identified genetic relationships in this study are fully consistent with the taxonomic rankings derived from morphological characteristics (Ietswaart, 1982; Ince et al., 2014) The information obtained from this study emphasizes the importance of selecting and propagating superior genotypes found in natural flora, which is crucial for determining the genetic diversity of hybridization combinations in future breeding studies, preserving natural genetic resources, and minimizing redundancy in existing germplasm resources. Diagnostic DNA markers capable of distinguishing between *Origanum* species identified in this study could facilitate the identification of species with taxonomic difficulties.

Amplified fragment length polymorphism (AFLP), random amplification of polymorphic DNA (RAPD), inter-simple sequence repeats (ISSR), and sequenced repeat amplified polymorphism (SRAP) have been used in genetic diversity studies in *Origanum*. However, these techniques usually produce dominant-type markers (Ince et al., 2014). Although these markers are dominant (AFLP, RAPD, SRAP, and ISSR) they are easy to apply since they do not require previous knowledge of the sequence of organisms. On the other hand, dominant type markers provide limited information regarding the allelic state of the loci. Moreover, data generated by dominant markers is often difficult to reproduce in various laboratories, as several factors can affect the PCR-based banding pattern due to the non-locus-specific character of the primers used. All these significantly hamper identification of species, breeding lines and cultivars in the genus *Origanum*. Among the DNA markers, SSRs have special properties and are widely used in many crop species (Ince et al., 2011; Karaca and Ince, 2011). However, the use of SSR markers in *Origanum* and in many members of the Lamiaceae family has lagged other plant species due to the lack of genome sequences (Ince et al., 2014). SSRs are usually codominant, almost randomly distributed in the genome, and highly polymorphic markers. These properties of SSR markers have resulted in them being commonly used to assess population genetic structure and in molecular ecological studies (Ince et al., 2014; Sun et al., 2023; Alekseeva et al., 2023b). The levels of polymorphisms in SSR are mainly due to replication slippage and unequal recombination. It is known that the level of marker polymorphism tends to decrease as the motif length increases since shorter motifs allow more possible replication slippage events per unit length of DNA (Karaca and Ince, 2011). Some of the SSR markers identified in this study are valuable for species identification since they show species-specific amplification fashions. This study reported species-specific, location-specific, and genotype-specific SSR markers that could be used for breeding and identifying genotypes belonging to the 8 *Origanum* species naturally occurring in the flora of Antalya. Species-specific amplification patterns of SSR markers could be used to identify herbal materials and are very useful for tracing adulteration in commercial herbal materials (Ince et al., 2014; Papaioannou et al., 2020; Sun et al., 2023; Alekseeva et al., 2023b).

4. Conclusion

In this study, SSR primer pairs were reported, and the user of primer pairs provided an effective means for assisting in species identification and served as an unambiguous tool for species discrimination in the genus *Origanum*. Since most species in the genus *Origanum* have complex taxonomy, complicated by a considerable amount of morphological variations and hybridization, development of new SSR markers is important in *Origanum*. It was concluded that eight species of *Origanum* naturally grown in the flora of Antalya could be easily differentiated using molecular markers reported in this study since genetic relationships among the species used in the present study agreed with the Iteswaart classifications. Each species was assigned with SSR markers such as OR-SSR201 (*O. vulgare* subsp. *hirtum*), OR-SSR202 (*O. saccatum*), OR-SSR209 and OR-SSR212 (*O. onites*), OR-SSR213 (*O. minutiflorum*), OR-SSR218 and OR-SSR219 (*O. onites*), OR-SSR227 (*O. saccatum*), OR-SSR231 (*O. majorana*), OR-SSR231 (*O. solymicum*), OR-SSR238 (*O. majorana*), OR-SSR240 (*O. husnucanbaseri*), OR-SSR244 (*O. vulgare* subsp. *hirtum*), and OR-SSR267 (*O. onites*). SSR markers reported in the present study are useful not only to clarify taxonomic uncertainties within the genus but also to provide anchoring points for the integration of genetic maps among species. In addition, these markers are useful to investigate commercial aspects related to traceability of herbal materials since these molecular markers are stable, easy to assay, and amenable to high-throughput screening of large series of samples. Furthermore, increasingly growing market demand for some *Origanum* species on a global level resulted in destruction of natural habitats due to lack of regulations and standards, heavy harvesting, and global warming that limit the growth and reproduction of some species. These are threatening some species of the genus *Origanum*. According to the National Red Data Book, *Origanum minutiflorum* is near threatened at the national level due to overharvesting. Genetic diversity within some species of *Origanum* differed significantly, which can affect the overall health of these species. Based on the data it was speculated that some species of *Origanum* in the flora of Antalya might be fragmented due to forest fires, the opening of new sand and marble quarries, the creation of new agricultural areas, as well as drought and seasonal shifts caused by climate change.

Author Contributions

The percentages of the authors' contributions are presented below. All authors reviewed and approved the final version of the manuscript.

	S.E.	M.K.
C	50	50
D	30	70
S	40	60
DCP	70	30
DAI	50	50
W	60	40
CR	40	60
SR	50	50
PM	20	80
FA	10	90

C=Concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Consideration

Ethics committee approval was not required for this study because of there was no study on animals or humans.

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