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Molecular genetic diversity in the *Origanum* genus: EST-SSR and SRAP marker analyses of the 22 species in eight sections that naturally occur in Turkey

Tülin Taşcıoğlu[a], Narin Sadıkoğlu[b], Sami Doğanlar[a], Anne Frary[a,*]

[a] Izmir Institute of Technology, Department of Molecular Biology & Genetics, Urla, Izmir 35430, Turkey
[b] Department of Pharmacognosy, Faculty of Pharmacy, Inönü University, Battalgazi, Malatya 44280, Turkey

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ABSTRACT

Origanum (L.) is a genus of herbaceous perennials with culinary and medicinal uses with many species found in the Mediterranean region. The taxonomic classification of species belonging to this genus has been studied using morphological, biochemical and genetic diversity analyses. In this study, the genetic diversity of 22 Turkish *Origanum* species (including 24 taxa from eight sections) was examined with 46 herbarium specimens from the Mediterranean, Eastern Anatolian, Central Anatolian, and Black Sea regions of Turkey. Molecular marker data were generated from 25 SRAP primer pairs and six EST-SSR primers which produced 325 alleles. Dendrogram, principal coordinate and population structure analyses revealed the relationships among *Origanum* sections, species and individuals. Gene flow (*PhiPT* and *Nm*) was also studied for a deeper understanding of the relationships and hybridization patterns between sections and species. Molecular separation of the *Origanums* roughly corresponded to the taxonomy that Ietswaart proposed in 1980 but also suggested that hybridization among sections and species may result in convergence and/or divergence of different sections and species.

1. Introduction

The genus *Origanum* L. belongs to the Lamiaceae (Labiatae) family and is known by the common names oregano and marjoram. These plants have been used for tens of thousands of years (Tepe et al., 2016) as traditional remedies for diseases such as leukemia, diabetes or flu. Their efficacy lies in their essential oil and phytochemical contents which have been shown to stimulate downstream processes enhancing the immune system. As a result, *Origanum* species have worldwide value and promising commercial uses. Turkey has high oregano diversity and is one of the most *Origanum*-rich countries. Indeed, *O. onites*,"Turkish oregano", is one of the most commonly used species and has a diversified phytochemical content including important terpenoids such as thymol which is commonly used as a fungicide (Tepe et al., 2016). Turkey plays a key role in oregano trade with about 15,000 tonnes of product worth $60 million exported in 2014 (Sari and Altunkaya, 2015). In Turkey, many *Origanum* species are given the same common name, "kekik," thus leading to confusion among diverse types. However, only two species, *O. onites* and *O. vulgare*, are cultivated by farmers. In addition, most of the plants collected from the wild and sold in markets are *O. minutiflorum*, *O. onites* and *O. vulgare* subsp. *vulgare* (Gurbuz et al., 2011). Turkish oregano populations fall into eight of the ten *Origanum* sections found worldwide: *Amaracus*, *Anatolicon*, *Brevifilamentum*, *Longitubus*, *Chilocalyx*, *Majorana*, *Origanum*, and *Prolaticorolla*. The world plant checklist records 25 taxa (23 species) and 5 hybrids as the current extent of Turkish *Origanum* L. genetic resources (World Checklist of Selected Plant Families, 2017; Sadikoglu and Ozhatay, 2015).

Molecular genetic analysis has been used to discriminate different *Origanum* species and to study diversity within and among these species. For example, (Novak et al., 2008) developed 13 expressed sequence tag single sequence repeat (EST-SSR) markers that were polymorphic for two oregano taxa, *O. vulgare* and *O. majorana*. In other work, 52 transferable EST-SSR markers were developed for 12 genera of the Lamiaceae family collected from Antalya, Turkey (Karaca et al., 2013). In more recent work, 30 new simple sequence repeat (SSR) and cleaved amplified polymorphic sequence (CAPS) markers were developed for eight *Origanum* L. species collected from Antalya, Turkey (Ince et al., 2014). In addition to SSRs, random amplified polymorphic DNA (RAPD) markers were used to study phylogenetic relationships within *O. vulgare* subsp. *hirtum* populations and between seven *Origanum* L. species (Katsiotis et al., 2009). Amplified fragment length

* Corresponding author.
E-mail address: annefrary@iyte.edu.tr (A. Frary).

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polymorphism (AFLP) markers were also used to examine genetic diversity in 21 populations to evaluate the correlation between genetic structure and river flooding in Northwest Europe (Van Looy et al., 2009). In addition, Marieschi et al. (2010) developed sequence characterized amplified region (SCAR) markers for the estimation of genetic diversity among *Origanum* L. species and applied them to ten species from Italy, the USA and Germany. Recently, Aboukhalid and colleagues studied 670 Moroccan individuals from a single taxon (*Origanum compactum*) (Aboukhalid et al., 2017). The individuals were collected from 59 locations and analyzed with 15 SSR markers to evaluate their genetic diversity. Genetic diversity can also be studied with sequence-related amplified polymorphism (SRAP) markers. SRAP markers allow amplification of open reading frames as the primers have CCGG and AATT in the forward and reverse directions, respectively (Li and Quiros, 2001). An advantage of these markers is their ability to amplify fragments across genera. They are usually dominant markers and are suitable for genome wide genetic diversity studies as was shown in lavender (also in the Lamiaceae family) by Stanev et al. (2016).

In this study, we used 25 SRAP primer combinations and six cross genera EST-SSR markers developed by Novak et al. (2008) for molecular characterization of 24 *Origanum* L. taxa containing 46 individuals that grow naturally in the Mediterranean, Eastern Anatolian, Central Anatolian, and Black Sea regions of Turkey. This is the first time that genetic diversity has been examined in 24 of the 25 *Origanum* taxa found in Turkey. It is also the first SRAP and EST-SSR combination-based molecular analysis of these species. This work provides a cost-efficient approach for determining taxonomic differences between oregano species which may allow reliable selection of parental genotypes in breeding programs. The following questions were addressed by this study: (i) What is the structure of Turkish *Origanum* L. populations and the level of genetic diversity in the sampled herbarium material? (ii) Is there any significant gene flow between different Turkish oregano taxa? and (iii) How does molecular genetic differentiation compare with what is known about the origin and taxonomy of oregano species as described by Ietswaart (1980)?

2. Materials and methods

2.1. Plant material

A herbarium collection (assembled between 2005 and 2014) composed of 46 individuals belonging to 24 taxa (22 species) that grow naturally in Turkey was used as a source of plant material from eight sections (sects.) in the genus *Origanum* (Fig. 1; Table A1). The 46 accessions included all but one taxon (*Origanum brevidens*) from these sections. The number of individuals per taxa collected from each province ranged from one to 12. The most specimens were collected from Antalya involving four sections: *Chilocalyx*, *Brevifilamentum*, *Majorana*, and *Amaracus* (Fig. 1). The Osmaniye province contributed six specimens from sects. *Longitubus*, *Brevifilamentum*, *Prolaticorolla*, and *Origanum*. Five accessions (accs.) belonging to sects. *Amaracus*, *Majorana*, and *Origanum* were collected from Mersin province. Three specimens from sects. *Brevifilamentum* and *Origanum* were collected from Tunceli. Two individuals each were collected from Isparta, and Artvin while Karaman, Hatay, Adana, and Erzincan provinces provided one specimen each. Thus, specimens were sampled to include the wide distribution and diversification of the *Origanum* L. taxon in Turkey. All plant materials were obtained from the Herbarium Collection Center, Inonu University, Faculty of Pharmacy, Malatya, Turkey.

2.2. DNA extraction

Genomic DNA was isolated from dry tissue using a CTAB protocol (Doyle and Doyle, 1987) in combination with a DNA purification procedure for herbarium samples (Costa and Roberts, 2014). Amount and quality of genomic DNA were determined by SkanIt software for Multiscan Go 3.2 spectrophotometer (Thermo Scientific) and samples were run on 0.8% agarose gel, stained with ethidium bromide and visualized under UV light. DNA samples were diluted to 50 ng/µl for polymerase chain reaction (PCR) amplification.

2.3. SRAP analyses

A total of 25 SRAP primer combinations (Li and Quiros, 2001) was used to obtain random amplicons (Table A2). PCR reactions were conducted in 25 µl final volume containing 1× Tango buffer (with BSA), 3 mM $MgCl_2$, 0.125 mM deoxyribonucleotide triphosphates (dNTPs), 1 U *Taq* DNA polymerase, 2 pmol forward and reverse primers and 50 ng template DNA. The PCR reaction profile was as follows: initial denaturation step at 94 °C for 5 min, followed by two stages: first (94 °C for 1 min, 35 °C for 1 min, 72 °C for 1 min) for 5 cycles and then (94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min) for 35 cycles with final elongation at 72 °C for 10 min. PCR products were separated on 2% agarose gel electrophoresis (run at a constant 110 V for 3 h) and visualized with the BioRad (Universal Hood II) system after ethidium bromide staining.

2.4. EST-SSR analyses

Six EST-SSR markers (OR09, OR12, OR13, OR27, OR32, OR40) were used (Novak et al., 2008) (Table A3). PCR reactions were conducted in 25 µl final volume containing 1× reaction buffer, 3 mM $MgCl_2$, 0.125 mM dNTPs, 1 U *Taq* DNA polymerase, 2 pmol forward and reverse primers and 50 ng template DNA. The PCR reaction profile was as follows: initial denaturation step at 94 °C for 3 min, followed by 40 cycles (94 °C for 1 min, Tm (61–66 °C) for 1 min, 72 °C for 1 min), the final elongation was at 72 °C for 10 min. PCR products were separated and visualized as described for SRAP markers.

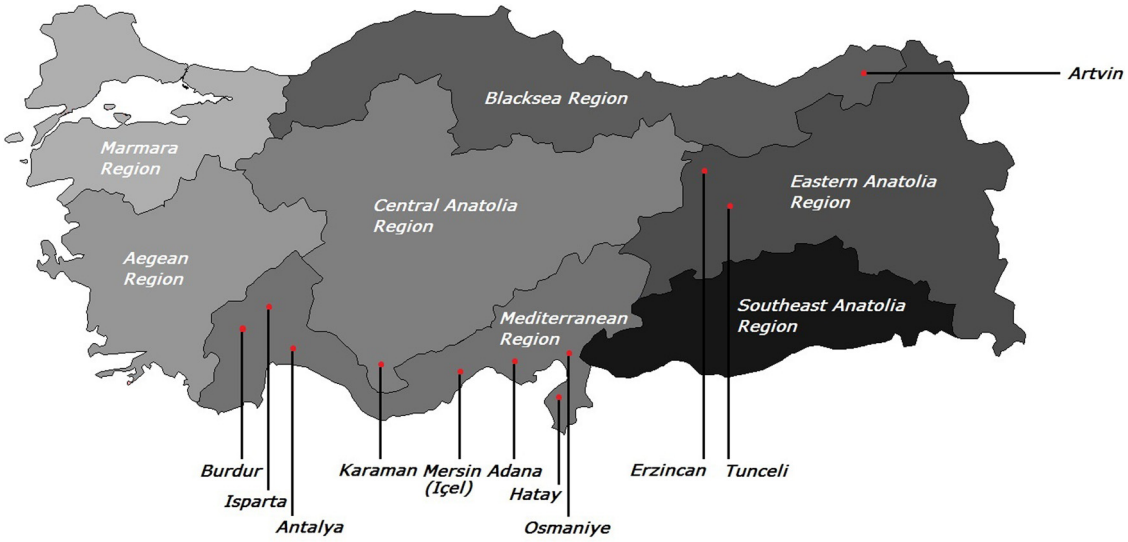
2.5. Statistical analyses

The fragments were scored according to presence "1" or absence "0" for both SRAP and EST-SSR markers. Missing data were coded as "9″ for each analysis. Forty-five individuals belonging to seven sections out of eight (except section *Longitubus* with only one individual; *O. amanum*, OAM1) were used to determine genetic diversity at the section level with GenAlEx 6.5 plugin (Peakall and Smouse, 2006, 2012). Genetic divergence within and between the 11 taxa containing more than one individual (*O. saccatum*, OSA; *O. husnucan-baseri*, OHU; *O. leptocladum*, OLE; *O. rotundifolium*, ORO; *O. majorana*, OMA; *O.onites*, OON; *O. syriacum*, OSY; *O. vulgare* subsp. *vulgare*, OVVU; *O. vulgare* subsp. *hirtum*, OVH; *O. vulgare* subsp. *viridulum*, OVVI; and *O. laevigatum*, OLA) was tested with *PhiPT* analysis (analogous to F_{ST} analysis) on binary data with the GenAlEx 6.5 plugin. *PhiPT* values less than 0.15 were assumed to indicate significant gene flow between taxa (Frankham et al., 2002). The analysis was performed with "9999″ pairwise permutations with *P* values accepted below 0.001. In addition, the number of effective alleles (*Ne*), Shannon's Index (*I*), and the mean diversity (*h*) were calculated on random binary data in the same plugin. Pairwise *Nei's* genetic distances (*NGD*) and identities (*NGI*) were also calculated. Number of migrants per generation (*Nm*) was calculated among populations in Excel using the formula (Wood and Gardner, 2007):

$$Nm = 0.25 * \left[\left(\frac{1}{PHIPT}\right) - 1\right]$$

2.6. Population structure and dendrogram analyses

Genetic diversity (GD) values for the 31 markers were calculated with GDdom software (Abuzayed et al., 2016). An unweighted Neighbor Joining (NJ) dendrogram was constructed with DARwin 6.0.8



Regions	Provinces	Sections	Sample Codes
Mediterranean	Adana Antalya Burdur Hatay Isparta Mersin Osmaniye	Brevifilamentum Amaracus Anatolicon Majorana Origanum Chilocalyx Prolaticorolla Longitubus	OON1, OHY1, OSA4, OBI1, OSI1, OVVI2, OHU1, OHU2, OMA2, OMI1, OSA1, OSA2, OSA3, OSA5, OSA6, OSA7, OSO1, OBO1, OSY1, OVH1, OVVI1, OVVU1, OVO1, OLA1, OAM1, OBA1, OLA2, OLA3, OLA4, OVVU2
Central Anatolia	Karaman	Brevifilamentum	OLE2
Eastern Anatolia	Erzincan Tunceli	Brevifilamentum Origanum	OHA1, OAC1, OMU1, OVG1
Blacksea	Artvin	Brevifilamentum	ORO1, ORO2
Unknown Region	Unknown Provinces	Amaracus Majorana Origanum Prolaticorolla	OLA5, OLA6, OLE1, OMA1, OON2, OSA8, OSY2, OSY3, OVH2

Fig. 1. Collection locations of *Origanum* L. samples used in this study.

software (Perrier and Jacquemoud-Collet, 2006) using the Jaccard dissimilarity index. Principal Coordinate Analysis (PCoA) was used to produce a graphical display of distances between units on Euclidean planes using DARwin 6.0.8 software and GenAlEx 6.5 plugin (Perrier and Jacquemoud-Collet, 2006; Peakall and Smouse, 2006).

A model-based clustering method was utilized and the structure of the population was determined with STRUCTURE 2.3.4 software (Pritchard et al., 2000) with a Bayesian approach. For an optimal value of subpopulations (*K*), the *K* was tested for 1–10 groups and 10 iterations were done for each *K*. The 100,000-initial burn-in replications were followed by 100,000 Markov Chain Monte Carlo (MCMC) replications. The optimal number of *K* subpopulations was determined using the Structure Harvester website program (Earl and vonHoldt, 2012) after the STRUCTURE run was completed. An identity threshold of 0.70 was used for membership to a given subpopulation since Scutari and Denis assumed that any value between 0.5 and 0.85 yields the same pattern (Scutari and Denis, 2014). Individuals with identity values less than this threshold were assumed to be admixed.

3. Results

3.1. Genetic diversity analyses

Genetic diversity at the section and species/taxon levels of the accessions was determined using 25 SRAP primer combinations and six EST-SSR markers. 325 polymorphic alleles were obtained. The number of polymorphic alleles per SRAP marker ranged from 3 to 20 with a mean of 12.04 (Table 1). The highest GD value for the SRAP markers was 0.35 which was obtained for primer combination em4-me3. The number of polymorphic alleles per EST-SSR marker ranged from 2 to 6 with a mean of 4 (Table 1). The most polymorphic EST-SSR marker was OR09 with a GD value of 0.49. Despite yielding fewer fragments, EST-SSR markers had higher mean GD (0.36) than SRAP markers (0.27) (Fig. A1). Mean allele frequencies (af) among all polymorphic markers ranged from 0.11 to 0.38 while individual af ranged between 0.02 and 0.96 (Fig. A2).

Within section diversity was high (83%) with the remaining 17% of diversity observed among the seven sections as determined by AMOVA analysis. Genetic diversity of the sections ranged from 0.40 to 0.42 (*h*). In addition, the *NGI* indicated that sects. *Majorana* and *Origanum* were most similar at the molecular level (0.88) while *Majorana* and *Brevifilamentum* were least similar (0.83) (Table 2). Gene flow and *Nm* in the seven oregano sections and their pairwise combinations indicated the most gene flow between sects. *Anatolicon* and *Brevifilamentum* (Table A4). The least gene flow was observed between sects. *Anatolicon* and *Prolaticorolla*.

For the 11 species with more than one individual, the mean *Ne* was 1.6, the mean value of *I* was 0.48, and *h* was 0.33. The highest *I* was

Table 1
Average number of polymorphic alleles (*Na*), allele frequency and genetic diversity (GD) for each marker listed from the lowest to highest value. A dagger (†) indicates type of marker used in this study. SE: Standard error.

Type†	Marker	*Na*	Allele Frequency	GD ± SE	Average GD
SRAP	em11-me3	11	0.14	0.14 ± 0.04	0.27
	em6-me2	3	0.38	0.18 ± 0.09	
	em1-me2	19	0.18	0.20 ± 0.03	
	em13-me3	8	0.13	0.21 ± 0.05	
	em5-me2	19	0.16	0.22 ± 0.03	
	em2-me2	11	0.11	0.24 ± 0.04	
	em5-me4	19	0.15	0.25 ± 0.03	
	em2-me4	8	0.26	0.26 ± 0.05	
	em7-me2	13	0.24	0.26 ± 0.05	
	em15-me3	7	0.23	0.26 ± 0.06	
	em3-me4	14	0.21	0.27 ± 0.04	
	em7-me4	16	0.24	0.27 ± 0.04	
	em6-me3	20	0.21	0.29 ± 0.03	
	em1-me3	16	0.18	0.29 ± 0.04	
	em7-me3	5	0.2	0.29 ± 0.07	
	em1-me4	11	0.23	0.30 ± 0.05	
	em8-me4	9	0.24	0.30 ± 0.06	
	em3-me3	13	0.25	0.31 ± 0.03	
	em5-me3	10	0.27	0.31 ± 0.05	
	em2-me3	14	0.2	0.32 ± 0.04	
	em3-me2	7	0.19	0.32 ± 0.05	
	em11-me2	10	0.28	0.33 ± 0.05	
	em15-me2	7	0.21	0.33 ± 0.05	
	em8-me2	15	0.28	0.34 ± 0.04	
	em4-me3	16	0.3	0.35 ± 0.03	
EST-SSR	Or12	6	0.16	0.25 ± 0.06	0.36
	Or32	4	0.27	0.33 ± 0.04	
	Or40	4	0.28	0.33 ± 0.07	
	Or27	4	0.25	0.37 ± 0.05	
	Or13	4	0.31	0.40 ± 0.04	
	Or09	2	0.32	0.49 ± 0.00	

$\Sigma Na = 325$.
Definitions are as follows: ΣNa = total number of polymorphic alleles.

0.50 for species OHU and OSY. Also, *h* was highest for the same two species, 0.35. Taxa OLE, OMA, OVVU and OVVI had slightly lower levels of diversity. While, the lowest diversity was seen in the OVH samples ($I = 0.44$, $h = 0.31$). The mean percentage of polymorphic loci among the 11 *Origanum* taxa was 75%. The highest percentage of polymorphic loci was 79% for the OHU population. The number of private bands was 0 for all populations (data not shown).

At the species level, AMOVA analysis indicated that the within population variation was 80%, while among population variation was 20% (Table 3). The highest *NGIs* were between OLE and ORO (0.80), and OSA and OMA (0.79) (Table 4). The least gene flow was found between taxa OHU and ORO (0.31) (*PhiPT*, Table A5). The highest gene flow was 0.04 between the OON and OMA populations (*NGD* of 0.24). As expected from these results, the highest *Nm* value, 6.04, was observed between the OON and OMA populations. The *Nm* values were also high between the following taxon pairs: OVVU-OSY (3.27), OVH-OVVU (3.07), and OVVI-OVH (3.06).

Table 3
AMOVA results for Turkish *Origanum* L. species at taxon level.

Source	df	SS	MS	Est. Var.	%
Among Pops	10	75.852	7.585	1.098	20%
Within Pops	22	97.451	4.430	4.430	80%
Total	32	173.303		5.528	100%

Definitions are as follows: df = degrees of freedom; SS = sum of squares; MS = mean of squares; Est. Var = estimated variance; % = percentage of variation.

3.2. Dendrogram analysis

Jaccard pairwise dissimilarity indices were calculated among individuals and resulted in a mean dissimilarity of 0.76 with values ranging from 0.30 to 0.90 with a cophenetic r of 0.95. NJ dendrogram analysis indicated three main clusters: A, B, and C (Fig. 2). Sections *Majorana* (φ), *Prolaticorolla* (λ), and *Longitubus* (δ) were distributed throughout cluster A. Individuals belonging to section *Origanum* (γ) were placed mainly in cluster A with one accession in cluster B. All but one of the 10 *Amaracus* (α) accessions were distributed to cluster B with one accession in cluster A. The two *Anatolicon* (β) accs. (*O. hypericifolium*, OHY1 and *O. sipyleum*, OSI1) were separately placed in clusters B and C. Section *Brevifilamentum* (χ) was dispersed throughout all clusters of the dendrogram. All three of the *Chilocalyx* (ε) accessions were found in subcluster C1.

Cluster A was composed of 22 individuals from six sections. All but one (*O. vulgare* subsp. *gracile*; OVG1) of the accessions belonging to section *Origanum* were found in subcluster A1. Among these individuals, two OVH individuals subclustered within A1. OVVU and OVVI were most closely related to these OVH individuals. OVVU2 was placed with individuals from section *Majorana* in cluster A1. OON and OSY, species in section *Majorana,* were also found in cluster A1 with OON1 and OON2 most closely related to the *Origanum* individuals. OSY1, OSY2, and OSY3 formed their own subcluster within A1. All six *Prolaticorolla* individuals (OLA) were found in cluster A2. Cluster A3 had an interesting distribution of single individuals belong to *Majorana* (OMA1 and OMA2), *Brevifilamentum* (OLE1), *Longitubus* (*O. amanum*; OAM1), and *Amaracus* (*O. boissieri*; OBO1).

Cluster B was composed of 17 individuals from four sections. Subcluster B1 contained all but one individual (*O. boissieri*, OBO1) of section *Amaracus* which included the species OSA, OBO and *O. solymicum* (OSO). All but one of the OSA (OSA3) individuals were found in this subcluster. OSA3 was most closely related to OSO1 and OHY1 (section *Anatolicon*). OHU individuals clustered together in subcluster B2 which also included OLE2 from the same section, *Brevifilamentum*. Three more *Brevifilamentum* taxa were found in cluster B3: *O. munzurense* (OMU), *O. acutidens* (OAC), and *O. haussknechtii* (OHA). This cluster also contained OVG1, the only *Origanum* accession in cluster B.

Cluster C was composed of seven individuals in three sections. The three individuals [*O. vogelii* (OVO), *O. bilgeri* (OBI), and *O. minutiflorum* (OMI)] from section *Chilocalyx* were distributed together in subcluster

Table 2
Nei's genetic distances (below diagonal) and *Nei's* genetic identity values (above diagonal) are given below for eight oregano sections. Bold character indicates highest *Nei's* genetic distance between sections *Majorana* and *Brevifilamentum,* while *italic* character displays the lowest genetic distance between sections *Origanum* and *Majorana*.

Nei's Genetic Distance vs. *Nei's* Genetic Identity	*Amaracus*	*Anatolicon*	*Brevifilamentum*	*Chilocalyx*	*Majorana*	*Origanum*	*Prolaticorolla*
Amaracus	–	0.84	0.86	0.86	0.85	0.87	0.85
Anatolicon	0.17	–	0.86	0.84	0.84	0.86	0.84
Brevifilamentum	0.15	0.15	–	0.84	0.83	0.85	0.84
Chilocalyx	0.15	0.18	0.17	–	0.85	0.87	0.86
Majorana	0.16	0.17	**0.19**	0.16	–	0.88	0.85
Origanum	0.14	0.15	0.16	0.14	*0.13*	–	0.87
Prolaticorolla	0.16	0.18	0.17	0.15	0.17	0.14	–

Table 4
Pairwise population matrix with *Nei's* genetic distance below the diagonal and *Nei's* genetic identity above the diagonal at taxon level.

	OSA	OHU	OLE	ORO	OMA	OON	OSY	OVVU	OVH	OVVI	OLA
OSA	–	0.77	0.74	0.7	0.79	0.76	0.76	0.76	0.72	0.75	0.76
OHU	0.27	–	0.76	0.7	0.76	0.74	0.75	0.78	0.75	0.77	0.77
OLE	0.30	0.28	–	0.8	0.75	0.77	0.74	0.76	0.73	0.75	0.75
ORO	0.32	0.29	0.27	–	0.76	0.76	0.73	0.73	0.73	0.75	0.73
OMA	0.24	0.27	0.29	0.3	–	0.78	0.77	0.74	0.73	0.76	0.76
OON	0.28	0.30	0.27	0.3	0.24	–	0.74	0.75	0.70	0.73	0.74
OSY	0.27	0.29	0.30	0.3	0.26	0.30	–	0.78	0.76	0.75	0.76
OVVU	0.28	0.24	0.27	0.3	0.30	0.28	0.25	–	0.75	0.75	0.77
OVH	0.33	0.29	0.32	0.3	0.31	0.36	0.28	0.29	–	0.74	0.76
OVVI	0.28	0.26	0.28	0.3	0.28	0.32	0.29	0.29	0.30	–	0.75
OLA	0.28	0.26	0.29	0.3	0.27	0.30	0.27	0.27	0.28	0.28	–

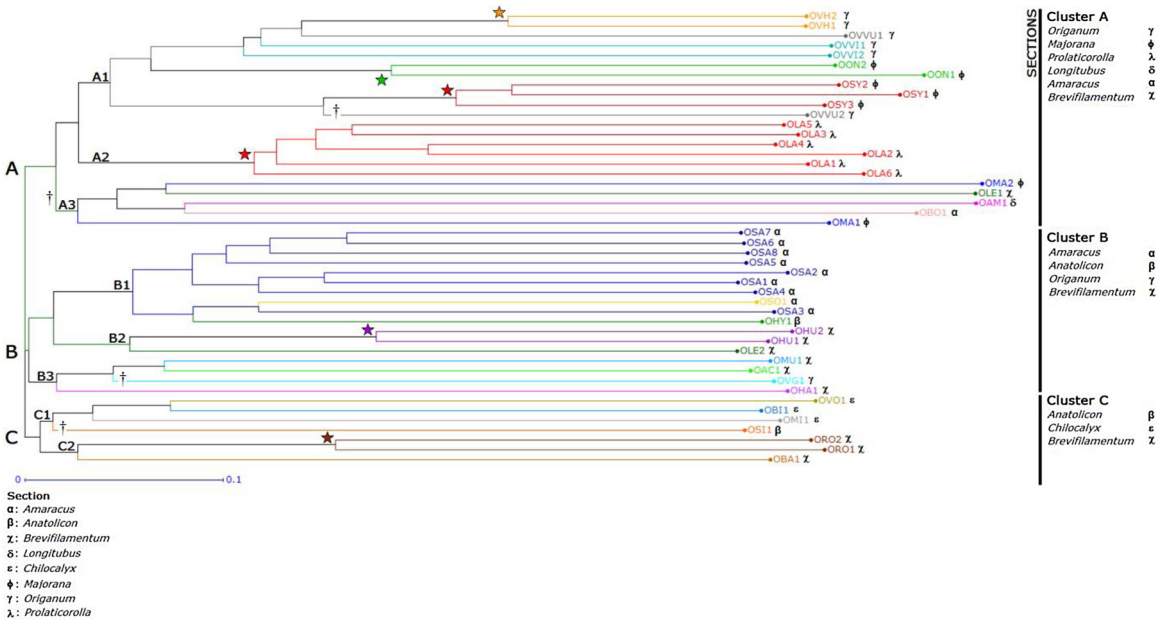


Fig. 2. Neighbor joining dendrogram for the oregano accessions (colored stars indicate the taxa for which all individuals grouped together in a single sub cluster. Accessions/clusters which may require re-examination are represented by symbol: †. Sections in each cluster are listed to the right of the dendrogram.

C1. This subcluster also included the OSI individual from section *Anatolicon*. Subcluster C2 included ORO and the *O. bargyli* (OBA) individual from the same section, *Brevifilamentum*.

3.3. Principle coordinate analysis (PCoA)

The Principle coordinate analysis (PCoA) graph was plotted and the eigen values explained 11.93, 8.49, and 6.52% of the variance, respectively. The PCoA graph resulted in three major clusters for the 24 taxa (Fig. 3). The distribution of sections was as follows: *Prolaticorolla* (cluster I); *Origanum*, *Majorana*, and *Longitubus* (cluster II); and *Amaracus, Anatolicon*, *Brevifilamentum*, and *Chilocalyx* (cluster III). Interestingly the individuals OLE1 (*Brevifilamentum*) and OAM1 (*Longitubus*) were placed at the intersection of clusters II and III. Cluster I consisted of only OLA individuals which belong to section *Prolaticorolla*. As indicated, cluster II contained four different sects.: *Majorana* with all seven individuals from three different taxa (OMA, OON, and OSY); *Origanum* with its seven individuals from different taxa (OVVU, OVG, OVH, and OVVI); *Longitubus* with its single OAM individual and *Brevifilamentum* with one of the ten individuals from the section. The third cluster was composed of four different sects.: *Amaracus*, *Anatolicon*, *Brevifilamentum*, *Chilocalyx*. Overall, most of the taxa that had more than one individual per population, namely, OLA, OSY, OVVU, OVVI, OVH, OON, OHU, OSA and ORO were clustered as expected in two dimensions. In contrast, OMA (OMA1 and OMA2) and OLE (OLE1 and OLE2) individuals were not closely clustered in the plot.

3.4. Population structure analysis

The population structure analysis resulted in two possible optimal numbers of subpopulations (*Ks*) because the most significant delta *K* [Δ(*K*)] values were observed for $K = 3$ and $K = 8$. An identity threshold value greater than 0.70 was used for classifying individuals into subpopulations or as admixed (Table A6). Because the oregano material encompassed eight sections and $K = 8$ gave the highest likelihood value (Fig. A3a), the hypothesis of eight subpopulations was examined first (Fig. 4). The graph for $K = 8$ showed that *Prolaticorolla* formed its own subpopulation (SPVIII). In addition, eight of the 10 *Amaracus* individuals, specifically those belonging to OSA, fell into a single subpopulation (SPV). Section *Brevifilamentum* individuals fell into four subpopulations with the ORO and OHU individuals forming exclusive subpopulations (SPIV and VI, respectively). Section *Majorana* accessions fell almost equally into two subpopulations: SPII and III. Five of the seven accessions from section *Origanum* were admixed while the remainder (OVH individuals) formed their own subpopulation, SPVII. The individuals from sects. *Longitubus* and *Chilocalyx* fell into subpopulations that they shared with other sections (SPI and III) while both *Anatolicon* accessions were admixed.

Overall, six of the eight (75%) subpopulations consisted of a single section (Table A6). Of these exclusive subpopulations, SPIV with two

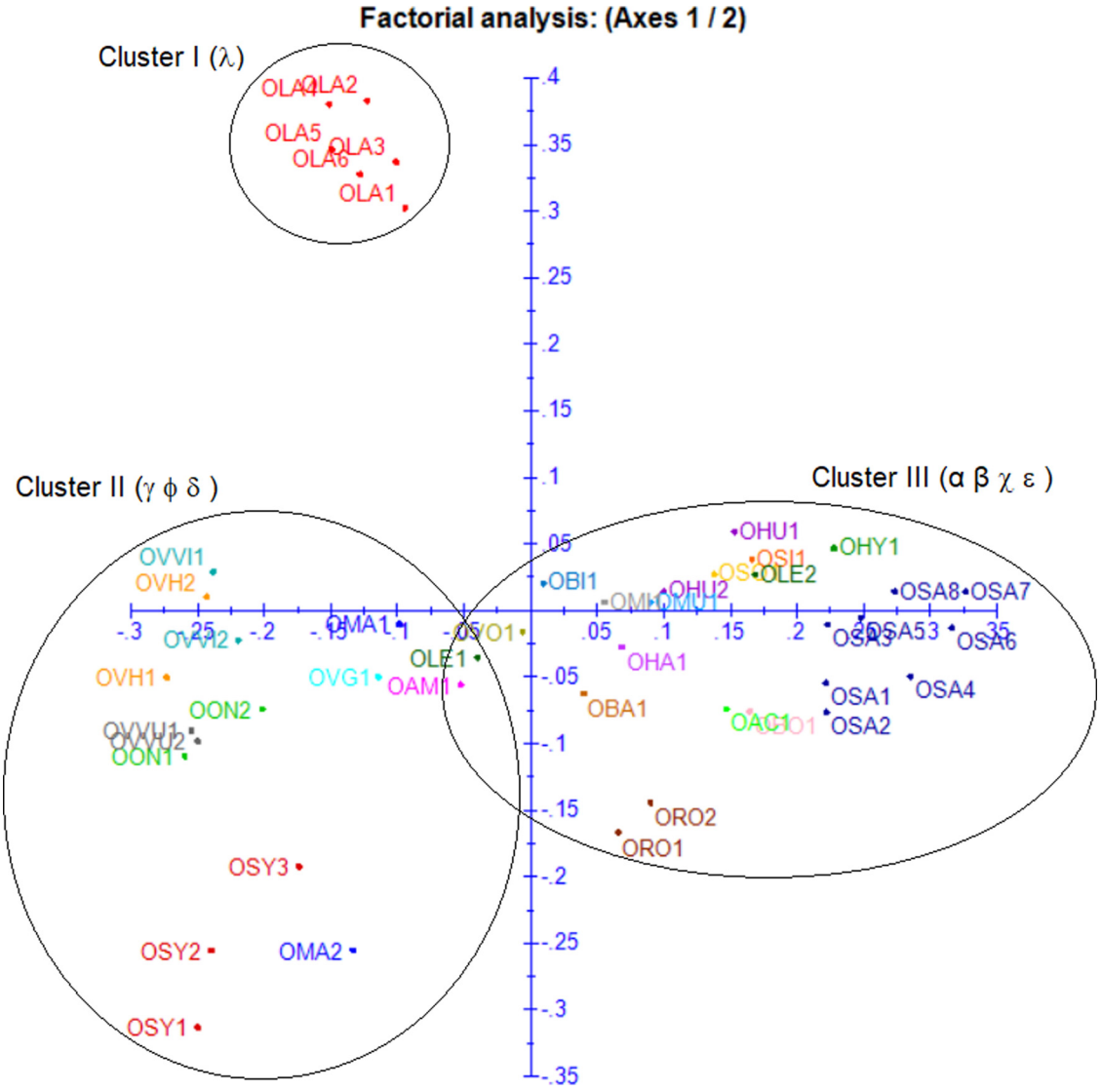


Fig. 3. Principle Coordinate Analysis (PCoA) plot of the oregano material showing three main clusters. Sections are shown in bold with following symbols: **α:** *Amaracus*, **β:** *Anatolicon*, **χ:** *Brevifilamentum*, **δ:** *Longitubus*, **ε:** *Chilocalyx*, **φ:** *Majorana*, **γ:** *Origanum*, and **λ:** *Prolaticorolla*.

ORO accessions had the highest expected heterozygosity (*He*) value, 0.24 with moderate gene flow (F_{ST} = 0.49) (Table A7). The lowest heterozygosity and gene flow were observed in SPVII which contained only OVH. Overall, SPI with two sects. (*Brevifilamentum* and *Chilocalyx*)

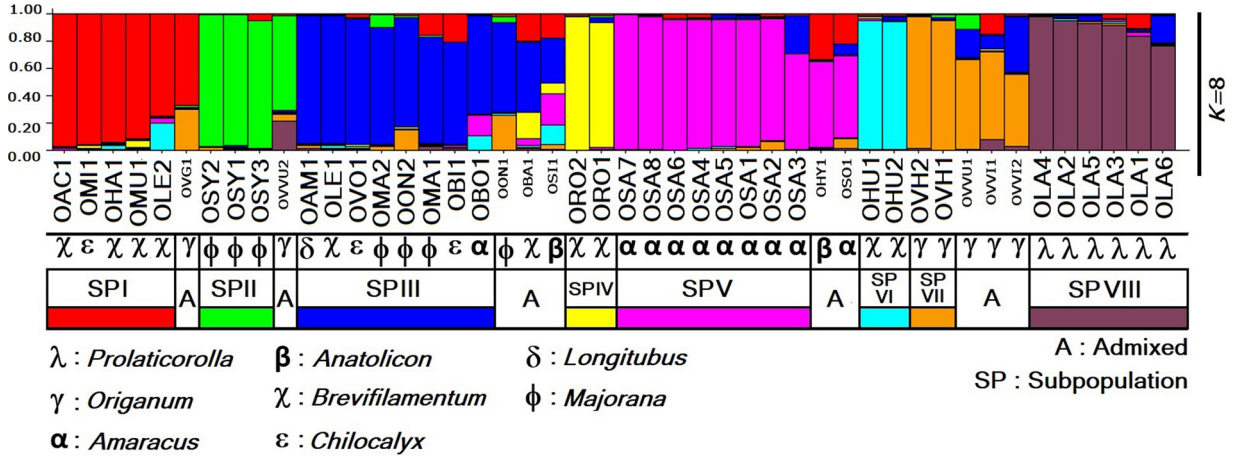


Fig. 4. STRUCTURE graph for eight subpopulations ($K = 8$). The distribution of sections *Brevifilamentum*, *Majorana*, *Amaracus*, *Origanum*, and *Prolaticorolla* in separate sub populations is displayed.

and five different taxa (OAC, OMI, OHA, OMU, OLE) had the highest *He* and gene flow, values of 0.34 and 0.02, respectively. All but one of these species (OLE) was represented by only one accession.

An alternative result from the population structure analysis was that the oregano accessions fell into three subpopulations. This hypothesis was supported by the highest Δ(*K*) for all tested values of *K* (Fig. A3b). Based on these results (Fig. A3c), section *Amaracus* fell into an exclusive subpopulation, SPII, containing all OSA individuals but excluding OSO1 and OBO1 individuals which were admixed. Accessions from sects. *Prolaticorolla*, *Origanum*, *Majorana*, *Longitubus* and *Brevifilamentum* fell into SPIII. The remaining *Brevifilamentum* individuals were found in SPI (six individuals) and in the admixed group (three accs.). One accession from section *Chilocalyx* was in SPI and the other two were admixed. The two *Anatolicon* accessions were admixed. As seen with $K = 8$, the SP containing *Brevifilamentum* and *Chilocalyx* accessions had the highest gene flow and heterozygosity (Table A7).

4. Discussion

4.1. Origanum L. in Turkey

Origanum L. is an important medicinal plant with a wealthy essential oil content and special uses in the herbal medicine and food industries. In recent years, there has been a remarkable increase in the number of metabolic and genetic studies of herbs which necessitate that species are correctly identified. The use of morphological characters for taxonomical classification is difficult because these features do not always explain differences between species and their genetic relationships (Lukas et al., 2013). Misclassification confuses both the public's and scientists' knowledge about these plants. For example, Polat and Satıl (2012) identified two *Origanum* species (*O. onites* and *O. vulgare* subsp. *hirtum*) as commonly sold medicinal plants in Balıkesir province in Turkey. Although they correctly indicated that these species belong to the Lamiaceae family, they misclassified them as "thyme;" thus, potentially confusing its genus and common name identifications. Furthermore, there is great difficulty in the identification of various closely related *Origanum* taxa (Efe, 2013). The complex and indefinite taxonomical classification of *Origanum* species hinders efforts toward maintaining its sustainability as environmental and human forces change its natural habitat and threaten plant populations. In Turkey, as elsewhere, *Origanum* specimens that are sold in the market are commonly collected from nature. As a result, harvest and trade, including exportation, are usually uncontrolled. Recently, the Turkish ministry of agriculture has provided incentives to farmers for cultivation of oregano (Gurbuz et al., 2011) which has resulted in increased demand for properly labelled seeds. Thus, many factors contribute to the need for conservation of the fifteen *Origanum* taxa which are endemic to Turkey.

In this study, the gene pool consisted of 22 *Origanum* species including 24 taxa growing naturally in Turkey representing eight sections: *Amaracus*, *Anatolicon*, *Brevifilamentum*, *Longitubus*, *Chilocalyx*, *Majorana*, *Origanum,* and *Prolaticorolla*. Fifteen of these are endemic species (Table A1). The *Origanum vulgare* L. accessions included four subspecies: *vulgare*, *gracile*, *hirtum*, and *viridulum*. These subspecies were included in our study as different taxa. The only endemic taxon that was not included was *O. brevidens* (Bornmüller) Dinsmore (in section *Brevifilamentum*). This species was only recorded once in 1933 as growing in the Amanos Mountains (Hatay, Turkey) with a limited specimen taken for the herbarium collection which was not sufficient for inclusion in this study (Ietswaart, 1980; Duman et al., 1995; Guzelmansur and Lise, 2013).

4.2. SRAP and EST-SSR markers in Origanum

The genetic diversity among *Origanum* species was investigated in 46 individuals from 24 taxa with SRAP and EST-SSR markers. The SRAP markers yielded 12.04 fragments per primer combination and were highly informative in oregano as is often expected from genetic markers that are based on random or non-sequence specific amplification. Similar results (an average of 13.30 markers) were obtained with RAPD markers in 27 Greek *O. vulgare* subsp. *hirtum* accessions (Katsiotis et al., 2009).

The EST-SSR markers were also highly polymorphic with an average of four fragments per primer pair. The markers were developed by Novak et al. (2008) who obtained an average of 0.87 fragments in 39 *O. vulgare* and *O. majorana* accessions. This discrepancy in polymorphism is probably due to the fact that the current study examined more accessions from a wider variety of taxa. Some of the same EST-SSR markers were also used to examine *O. vulgare* populations from Tunisia and found to be highly polymorphic with an average of five fragments per marker (Mechergui et al., 2017). Additional EST-SSR markers were developed by Ince et al. (2014). Thirty of these markers were tested on 65 samples from eight *Origanum* species (Ince et al., 2014) while 15 were surveyed on 670 *O. compactum* individuals (Aboukhalid et al., 2017). In both studies, the markers yielded a similar number of fragments (one to six) as obtained in our work.

4.3. Genetic diversity of Turkish Origanum

Overall genetic diversity of the Turkish *Origanum* accessions was high with a mean Jaccard dissimilarity index of 0.76. Ince et al. (2014) also used EST-SSR primers to examine genetic diversity of Turkish *Origanum*. They analyzed 65 individuals from eight species and obtained an average genetic dissimilarity of 0.46. Higher diversity was expected in our study given the broad range of material examined: 46 individuals representing 24 of the 25 *Origanum* taxa. Nearly all *Origanum* species have identical chromosome numbers ($2n = 30$) (Ietswaart, 1980) and the genus can be either self or cross-pollinated with a reported lack of hybridization barriers between species and taxa (Kitiki et al., 1997). Cross-pollination and the resulting high diversity are also aided by the geographical proximity of different *Origanum* populations (Loveless and Hamrick, 1984). Because the samples were collected from contiguous regions in Turkey, the transfer of pollen or seed from one population into another can result in enhanced gene flow (Loveless and Hamrick, 1984). Furthermore, rivers or streams can also spread seed or pollen of *Origanum* species as described by Van Looy et al. (2009).

4.3.1. Section Amaracus

Ten accessions from three species were sampled from section *Amaracus*. The average diversity of the section was 0.41 (*h*) with highest *NGI* to section *Origanum* (0.87). In the dendrogram and PCoA analyses, the section was most closely related to sections *Anatolicon* and *Brevifilamentum*. This agreed with the gene flow analysis which indicated that *Amaracus* had the highest flow and number of migrants with these two sections. In addition, *Amaracus* and *Brevifilamentum* species have similar habit and inflorescence structure (Ietswaart, 1980).

NGI and gene flow between the *Amaracus* species could not be measured because two of the taxa (OBO and OSO) had only one sample. The OSA accessions formed their own cluster with OSO indicating their close genetic relationship which was expected given their membership in the same section as well as the geographical proximity of their collection locations in Burdur and Antalya. Unexpectedly, the OBO1 accession was clustered with a mixed group of individuals from various sections in the dendrogram (cluster A3) and population structure

analyses. This discrepancy suggests that this particular sample should be re-examined for its morphology and that additional samples of this species should be collected and molecularly characterized. Based on these results, it should be clear if the position of OBO1 is due to its genetic distinctness from the other *Amaracus* species or if it has been misidentified.

4.3.2. Section Anatolicon

Two accessions from two species were sampled from section *Anatolicon* with an average heterozygosity of 0.41. *Anatolicon* had highest genetic identity (0.86) with *Origanum* and *Brevifilamentum*. Both *Anatolicon* accessions were admixed according to the population structure analysis and were not closely related in the dendrogram. Instead, the accessions clustered with individuals from sects. *Brevifilamentum*, *Chilocalyx* and *Amaracus*. In agreement with this result, *Anatolicon* had high gene flow with these three sections. Indeed, the most migrants observed between sections in the entire study was seen between *Anatolicon* and *Brevifilamentum*. Moreover, natural hybrids between *Anatolicon* species OSI and *Brevifilamentum* (OBA) have been documented (Ietswaart, 1980). Thus, high rates of cross-pollination could result in the admixed population structure seen in the OHY and OSI samples. Both OHY and OSI are widely distribution in Turkey (Sadikoglu, 2012) which may also contribute to their admixed structure. *Anatolicon* has also been reported to hybridize with individuals from section *Majorana* and may hybridize with section *Origanum* (Ietswaart, 1980). However, especially high gene flow was not observed between these sections in our study. More samples from these species should be collected and analyzed before any conclusions about their genetic structure and relationships with other taxa can be made.

4.3.3. Section Brevifilamentum

Ten accessions from seven species were analyzed from section *Brevifilamentum*. The section had an average diversity of 0.40 (*h*) and highest genetic identity with section *Origanum* (0.85). Individual samples were widely spread in the dendrogram and population structure analysis. The section had the highest gene flow and *Nm* with *Anatolicon*. High gene flow were also observed with *Amaracus, Chilocalyx, Origanum* and *Majorana*. The dendrogram and gene flow results indicate the diverse genetic background of *Brevifilamentum*. These findings are in an agreement with Ietswaart's hypothesis (1980) that many of the species in this section arose through ancient hybridizations when *Origanum* populations were restricted to mountainous regions due to climate change and a more arid environment. Natural hybrids between *Brevifilamentum* and *Majorana* were identified by Ietswaart (1980). The tested *Brevifilamentum* samples were collected from all regions included in our study which spanned from the Mediterranean to the Black Sea and Eastern Anatolia. Thus, the section is widely dispersed in Turkey and has the opportunity for cross-pollination with many Origanum species.

Of the three *Brevifilamentum* species that had more than one sample, OHU had the highest *h* (0.35) followed by OLE (0.34) and ORO (0.32). *NGI* between these species ranged from 0.75 to 0.76. The two OHU samples clustered together in the dendrogram and formed their own subpopulation. These samples were collected in eastern Antalya and were most closely related to OLE2 which was from the adjacent province, Karaman. Interestingly, in the dendrogram, the other OLE accession was placed in the mixed group from various sections (cluster A3) as described previously. The PCoA agreed with this placement as OLE1 was found at the junction between Clusters II and III. Therefore, it would be useful to test other OLE individuals to determine where they are placed in the analyses. The two ORO accessions formed their own subcluster in the dendrogram and PCoA and their own subpopulation in structure analysis. These samples were most closely related to the OBA individual which is also from section *Brevifilamentum*. The single OAC, OMU and OHA samples clustered in the dendrogram and were in a subpopulation which contained mainly other *Brevifilamentum* accessions. These three samples (OAC, OMU, OHA) were collected from Tunceli and Erzincan which share a border in Eastern Anatolia. These results suggest cross-pollination between these individuals. However, it is necessary to collect additional samples from these species to get an estimate of their gene flow.

4.3.4. Section Longitubus

Only one sample was used from section *Longitubus* which is comprised of only one species, OAM. The accession clustered with the mixed group of accessions from different sections (Cluster A3) that contained OBO1 (see Section *Amaracus*) and was found at the junction between Clusters II and III in the PCoA. Natural hybrids between species of *Longitubus* and *Prolaticorolla* have been described (Ietswaart, 1980); however, due to a lack of samples, we could not measure gene flow between these sections. Thus, it is evident that additional samples from this species must be collected and analyzed to understand more about the genetic diversity and relationships of this species and section.

4.3.5. Section Chilocalyx

Three samples from the three species (OBI, OVO, OMI) in section *Chilocalyx* were examined and had an *h* of 0.42. *Chilocalyx* had the highest *NGI* with *Origanum* (0.87). Indeed, putative hybrids between these two sections have been recorded (Ietswaart, 1980). The *Chilocalyx* samples formed a distinct cluster in dendrogram analysis and their own subcluster in PCoA. They were most closely associated with the *Brevifilamentum* accessions, ORO and OBA; and overall, had high gene flow with this section. Significant gene flow was also observed between *Chilocalyx*, *Anatolicon* and *Majorana*. The samples from these sections were collected in the Mediterranean region, thus, facilitating cross-pollination.

4.3.6. Section Majorana

Seven accessions from the three species in section *Majorana* were analyzed and had an average genetic diversity of 0.41 (*h*). *Majorana* had the highest *NGI* with *Origanum* (0.88). All of the samples were found in the same cluster of the dendrogram. Moreover, OSY and OON accessions formed their own distinct subclusters. The *Majorana* samples were most closely related to individuals from section *Origanum* as seen in the dendrogram and PCoA. These results were confirmed by a high gene flow and *Nm* between these two sections. Relatively high gene flow was also observed between *Majorana*, *Brevifilamentum* and *Chilocalyx*. Natural hybridization was previously observed between *Majorana* and sects. *Anatolicon*, *Brevifilamentum*, *Origanum* and *Prolaticorolla* (Ietswaart, 1980) which indicates that *Majorana* alleles may migrate even more than observed in our study.

Within *Majorana*, OSY (0.35) had slightly higher heterozygosity than OMA (0.34) and OON (0.33). High genetic diversity was also identified in the ITS (internal transcribed spacer) region of OSY (Lukas et al., 2013). Genetic identity among the *Majorana* species ranged from 0.74 to 0.78. As in the dendrogram analysis, population structure analysis indicated that OSY accessions formed their own subpopulation. Interestingly, these samples had high *NGI* (0.78) with and were most closely related to one of the two OVVU samples. This relationship was also reflected in a high gene flow between OSY and OVVU. As previously mentioned, the OON samples formed their own dendrogram subcluster. These results are consistent with those of Lukas et al. (2013) who examined the ITS sequences of species in *Majorana* and found that OON individuals formed a distinct group that did not include OSY or OMA. OON is also unusual in the genus Origanum because its morphology is relatively homogenous and can be easily distinguished from

other species (Lukas et al., 2013). Highest gene flow for OON was with OMA and OVVI.

The two OMA samples did not cluster most closely with each other but were both found in the mixed cluster A3 and in a subpopulation with multiple sections according to population structure analysis. This situation is mirrored at the morphological level in that OMA has characters which vary in natural populations and are not distinct from OSY (Ietswaart, 1980). OMA is an interesting species because, depending on the expert/genebank, it may or may not include individuals from *O. dubium,* a morphologically similar taxon (Lukas et al., 2013). Ietswaart (1980) classified *O. dubium* as a synonym of OMA. However, others who have examined samples at the molecular level argue that *O. dubium* arose by hybridization between OSY and OON (Lukas et al., 2013). Therefore, it is possible that one of our OMA samples could be *O. dubium*, thereby explaining why they did not cluster closely in the dendrogram. OMA had relatively high gene flow with the OLE in *Brevifilamentum* and OVVI in *Origanum*. The close relationships and high gene flow between OSY, OON and the *O. vulgare* subspecies may be a result of their geographical proximity as many of the samples were collected in Mersin, Antalya and their surrounding regions.

4.3.7. Section Origanum

Seven accessions from four *Origanum* subspecies were analyzed and found to have an average genetic diversity of 0.42 (*h*). In the dendrogram, all but one of the accessions, OVG1 grouped in cluster A1. However, OVG1 did cluster with all the *Origanum* samples in the PCoA. This disparity could be due to exceptional situation of OVG1 as being the only *Origanum* accession not collected from the Mediterranean. Instead OVG1 was sampled from Tunceli, a remote region in Eastern Anatolia. This region does not contain natural populations of any of the other *O. vulgare* subspecies. Thus, OVG was isolated from the rest. The Origanum accessions were most closely related to each other and to *Majorana* accessions. This close relationship with *Majorana* was also reflected by a high level of gene flow and migrants between the two sections and a high value for *NGI* (0.88). In addition, both natural and artificial hybrids have been reported for sections *Origanum* and *Majorana* (Ietswaart, 1980). *Origanum* also had high gene flow with *Brevifilamentum*.

The three subspecies with more than one individual (OVVU, OVH, and OVVI), had similar levels of genetic diversity. The OVH individuals formed their own subpopulation and their own subcluster in dendrogram analysis. OVH also clustered separately from OVVU in the work of Mechergui et al. (2017), which, in common with our work, used the EST-SSRs developed by Novak et al. (2008). Moreover, separate clustering of OVH and OVVU was reported by Katsiotis et al. (2009) using sequence from the ITS1-5.8S-ITS2 region. The remaining *Origanum* individuals were considered to be admixed in terms of population structure when the number of subpopulations was assumed to be eight, in agreement with Ietswaart's classification (1980). Gene flow was high between OVH, OVVU and OVVI as expected given that they are subspecies and not distinct species. Genetic identity between the subspecies was moderate (~0.74) with some "non-*Origanum*" taxa more closely related to *O. vulgare* subspecies. For example, OVVU had more genetic identity with OHU and OSY (~0.78) than with OVH and OVVI. These results are in an agreement with the gene flow analysis which indicated high interchange with both *Majorana* and *Brevifilamentum* species.

4.3.8. Section Prolaticorolla

Section *Prolaticorolla* contains only one species, *O. laevigatum* (OLA), and six samples from this species were analyzed in the study. The section had *h* of 0.41 and highest *NGI* with *Origanum*. The section did not have high gene flow with any other section. Average genetic diversity of OLA was 0.33 and this species had relatively high gene flow with only OVVU. All the OLA samples formed a distinct subcluster in the dendrogram and PCoA. This species was most closely related to both *Origanum* and *Majorana* accessions. Natural hybrids between *Prolaticorolla* and *Majorana* have been described as well as between *Prolaticorolla* and *Longitubus* (Ietswaart, 1980). The reason for the seeming genetic distinctness of OLA is unknown given the fact that interspecific hybrids occur and the species is not geographically isolated.

4.4. Origanum taxonomy

The origin of the different Origanum sections was proposed by Ietswaart in 1980. According to this hypothesis which assumes 10 sections, sections *Amaracus, Majorana,* and *Origanum* were directly descended from species in the ancestral genus Saturejeae (Ietswaart, 1980). Thus, taxa in these sections are the oldest Origanum species which may explain why OON (sect. *Majorana*) and OVVU (sect. *Origanum*) are the most numerous and widely distributed oregano species in Turkey, respectively (Sadikoglu, 2012). The prevalence of natural populations of OON may also be explained by its high genetic diversity which has conferred its adaptability to different environments (Ayanoglu et al., 2006). Over time, cross-hybridization among individuals led to gene flow among populations and species. This is evident in the large numbers of migrants that were observed between sections *Majorana* and *Origanum* in our study as well as the natural hybrids identified previously (Ietswaart, 1980). Interestingly, despite its ancient origins *Amaracus* did not exhibit high gene flow with the other two original sections in our work. This may be attributed to the very limited distribution of *Amaracus* species in Turkey (Sadikoglu, N; personal communication).

Cross-hybridization is common in the genus Origanum and has played a significant role in speciation (Ietswaart, 1980). According to Ietswaart (1980), section *Anatolicon* arose from many years of hybridization between species in sects. *Origanum* and *Amaracus*, and genus Thymus. The admixed population structure of the *Anatolicon* accessions studied in this work may reflect the diverse origin of this section. This idea is also supported by the high gene flow observed between *Anatolicon* and *Amaracus* which could be a remnant of the section's origin or could arise from more recent hybridizations.

According to Ietswaart (1980), section *Brevifilamentum* arose from hybridizations involving *Amaracus* and the genus Saturejeae. This hybrid origin may still be evident in the fact that the *Brevifilamentum* species were the most widely distributed in the dendrogram and population structure analyses. In addition, the results can be attributed to the high gene flow that is ongoing between *Brevifilamentum* and most of the other sections as well as between some of the *Brevifilamentum* species. Another key factor is the known endemism of most of these species. For example, OHU is endemic to only two locations (Sadikoglu, N; personal communication) and formed its own subpopulation in the structure analysis ($K = 8$). Similarly, ORO which has unknown endemism but is considered to be local (Sadikoglu, N; personal communication), also formed its own subpopulation. Both OHU and ORO had very low gene flow with the other species as expected for populations with restricted geographical locations.

Longitubus is hypothesized to be originated from genus Saturejeae and section *Amaracus* from genus Origanum (Ietswaart, 1980). According to the same source, *Longitubus* has unique corolla and stamen morphology which distinguish it from the other Origanum sections. In our molecular genetic study, only one individual was included from the section and it was found to cluster with a variety of species from other sections. Thus, it is apparent that morphological differences are not

necessarily reflected at the molecular level. Of course, additional *Longitubus* individuals must be examined to learn more about the genetic relationships of this section with the other oregano species.

Chilocalyx arose by hybridizations involving sects. *Majorana*, *Origanum* and an unknown genus (Ietswaart, 1980). In our work, *Chilocalyx* had the highest genetic identity with section *Origanum* reflecting its origins. In addition, significant gene flow was observed between *Chilocalyx* and its other progenitor, *Majorana*. All the *Chilocalyx* species are endemic and our samples were collected from adjoining Mediterranean locations. Individuals belonging to this section grouped together in our analyses indicating a shared gene pool, however, not enough accessions were studied to examine gene flow and genetic distance.

Prolaticorolla originated from hybridizations involving section *Origanum* and the genus Saturejeae (Ietswaart, 1980). Indeed, the section had the highest *NGI* with section *Origanum* as may be expected from its origin. The section contains only one species which is endemic to the eastern Mediterranean region of Turkey (Sadikoglu, 2012). Perhaps as a result of this endemism, gene flow between this section and the others was the lowest overall and OLA formed its own subpopulation and very distinct clusters in the other analyses.

The population structure analysis of the eight Origanum sections based on molecular genetic data did not have complete concordance with Ietswaart's classification based on morphology (1980). When the results for eight subpopulations are examined, most of the *Amaracus* (80%, all of the OSA acc.), *Prolaticorolla* (100%) and *Origanum* (71%) individuals fell into distinct subpopulations as expected based on their section assignments. *Anatolicon* individuals (2 acc.) were admixed and did not fall into any subpopulation which was also true of some *Amaracus* (1 acc.), *Origanum* (5 accs.), *Brevifilamentum* (1 acc.) and *Majorana* (1 acc.) accessions. The remaining individuals in *Brevifilamentum* fell into four subpopulations. Two of the subpopulations were exclusive containing only the individuals from one *Brevifilamentum* species, ORO (2 accs.) and OHU (2 accs.). The other two subpopulations also included species from other sections. These mixed subpopulations contained individuals from *Longitubus* and *Chilocalyx*. The members of section *Majorana* were equally split between two subpopulations, one of which was exclusively OSY (3 acc.) from *Majorana*.

Thus, the main difference between Ietswaart's classification and our subpopulations assignments lies in the occurrence of mixed subpopulations. These were most notable for sects. *Brevifilamentum* and *Majorana*. This difference may be simply the result of examining the material at two different levels—morphological versus DNA. Although morphological differences require changes at the DNA level, many more mutations occur in the genome than are apparent from morphology. In addition, the results suggest the possibility that cross-hybridization in these two sections might lead to divergence and speciation. This hypothesis is in agreement with Ietswaart's work (1980) which indicated that hybridization is the main driver of speciation in the genus. A much weaker factor in speciation is geographical isolation (Ietswaart, 1980). This hypothesis is also supported by our finding that endemic species such OHU formed their own subpopulations.

More recently, some researchers have suggested a departure from Ietswaart's classification and have proposed that section *Majorana* be categorized as its own genus (Kaufmann and Wink, 1994). According to this research which examined *rbcL* sequence, the genetic distance between *O. majorana* (syn. *Majorana hortensis*) and *O. vulgare* (subsp. not given) was 1.4%, a value which is typical for inter-genera comparisons. However, our results did not support this hypothesis as the *Majorana* accessions did not form an outgroup. Ince et al. (2014) obtained similar results with a different set of SSR markers. Moreover, high gene flow (this study) and natural hybridization (Ietswaart, 1980) were observed between section *Majorana* and other *Origanum* sections. Thus, our work suggests that *Majorana* should remain within the genus.

Hybridization is the key factor in *Origanum* speciation (Ietswaart, 1980) and is ongoing in natural populations. Consequently, the species are continuing to evolve. In some cases, this has led to divergence at the molecular and morphological levels while; in other cases, species have become admixed or more similar. This behavior can have different effects on adaptation of the genus to its local environment and result in highly endemic species such as OMU which is only found in one location in Tunceli. It can also result in broadly distributed species like OON. From this perspective, it is clear that the number of sections and species in the genus can change over evolutionary time. In our study, population structure analysis at $K = 8$ suggested that some of the species from sections such as *Brevifilamentum* and *Majorana* may eventually form their own sections. $K = 8$ was selected because it matches Ietswaart's taxonomy and gave the highest likelihood value. However, $K = 3$ gave the highest delta K value indicating that the data also fit the hypothesis of three subpopulations. According to this hypothesis, 11 of the accessions were admixed, *Amaracus* formed its own subpopulation, most of the *Brevifilamentum* samples formed another subpopulation and the remaining sections were mixed in the third subpopulation. Thus, our results stress that the hybridization behavior of *Origanum* has complicated its taxonomy and that both morphological and molecular data should be considered when proposing revisions to the genus. In addition, it is evident that more samples from each species must be examined to understand diversity and genetic relationships at the species and section levels in more depth. At the same time such work will aid in conservation efforts by providing methods for species identification. Thus, seeds of known specimens can be collected from nature for tissue culture, greenhouse, field and breeding applications. Such conservation will allow researchers to examine more samples and to devise a robust classification which prevents misidentification and mislabeling of seeds, plants, and culinary and medicinal products.

Data accessibility

EST-SSR and SRAP marker sequences are available in this article (Tables A2 and A3). All herbarium specimens can be obtained from the Herbarium Collection Center, Inonu University, Faculty of Pharmacy, Malatya, Turkey.

Author contributions

S.D., A.F., and N.S. designed the study. T.T. conducted both experiments and analyses. N.S. provided herbarium specimens from Inonu University herbarium collection. T.T. and A.F. wrote the manuscript with input from all co-authors.

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Acknowledgement

We are grateful to Inonu University (Faculty of Pharmacy) for providing herbarium specimens.

Appendix A

Table A1
Origanum L. materials used in this study, their collection coordinates and other details.

Sample Code		Species Name	Subspecies Name (Herbarium Identifier)	Section	Year	Endemism (E)	Location	Coordinates
OBO	OBO1	*O. boissieri* Ietsw.	*O. boissieri*/Narin/kekik/141	*Amaracus* (Gleditsch) Bentham	DD	E	Mersin	N37° 14′ 282″ E34° 37′ 740″
OSA	OSA1	*O. saccatum* P.H. Davis	*O. saccatum*/2012/6		2012	E	Burdur & Antalya	N36° 49′ 652″ E31° 56′ 797″
	OSA2		*O. saccatum*/2012/5		2012			N36° 46′ 426″ E31° 46′ 905″
	OSA3		*O. saccatum*/2009/17		2009			N36° 35′ 494″ E30° 27′ 624″
	OSA4		*O.saccatum*/2009/15		2009			N37° 37′ 965″ E30° 41′ 926″
	OSA5		*O. saccatum*/2009/33		2009			N36° 32′ 145″ E32° 14′ 892″
	OSA6		*O. saccatum*/2009/35		2009			N36° 33′ 237″ E32° 19′ 467″
	OSA7		*O. saccatum*/2009/20		2009			N36° 39′ 137″ E32° 07′ 694″
	OSA8		*O. saccatum*/2009/6a		2009			DD
OSO	OSO1	*O. solymicum* P.H.Davis	*O. solymicum*/2009/18		2009	E	Antalya	N36° 35′ 494″ E30° 27′ 624″
OHY	OHY1	*O. hypericifolium* O. Schwarz et P.H. Davis	*O. hypericifolium*/2009/16	*Anatolicon* Bentham	2009	E	Burdur	N37° 42′ 057″ E30° 18′ 812″
OSI	OSI1	*O. sipyleum* L.	*O. sipyleum*/2009/14		2009	E	Isparta	DD
OAC	OAC1	*O. acutidens* (Hand.-Mazz.) Ietsw.	*O. acutidens*/2014/1	*Brevifilamentum* Ietswaart	2014	E	Tunceli	N39° 21′ 864″ E39° 12′ 538″
OBA	OBA1	*O. bargyli* Mouterde	*O. bargyli*/2009/41		2009	DD	Osmaniye	N37° 01′ 373 ″E36° 26′ 982″
OHA	OHA1	*O. haussknechtii* Boiss.	*O. haussknechtii*/2009/2		2009	E	Erzincan	N39° 09′ 750″ E38° 37′ 167″
OHU	OHU1	*O. husnucan-baseri* H. Duman, Aytac & A. Duran	*O. husnucan-baseri*/2009/34		2009	E	Antalya	N36° 33′ 237″ E32° 19′ 467″
	OHU2		*O. husnucan-baseri*/2009/21		2009			N36° 33′ 100″ E32° 19′ 023″
OLE	OLE1	*O. leptocladum* Boiss	*O. leptocladum*/2005		2005	E	Karaman	DD
	OLE2		*O. leptocladum*/2009/22		2009			N36° 38′ 084″ E32° 41′ 564″
OMU	OMU1	*O. munzurense* Kit Tan & Sorger	*O. munzurense*/2014/2		2014	E	Tunceli	N39° 21′ 864″ E39° 12′ 538″
ORO	ORO1	*O. rotundifolium* Boiss	*O. rotundifolium*/2012/4		2012	DD	Artvin	N41° 18′ 972″ E41° 44′ 138″
	ORO2		*O. rotundifolium*/2012/3		2012			N40° 50′ 901″ E41° 32′ 411″
OAM	OAM1	*O. amanum* Post	*O. amanum*/Narin/kekik/143	*Longitubus* Ietswaart	DD	E	Osmaniye	DD
OBI	OBI1	*O. bilgeri* P.H. Davis	*O. bilgeri*/2012/7	*Chilocalyx* (Briq.) Ietswaart	2012	E	Antalya	N36° 45′ 948″ E32° 08′ 606″
OVO	OVO1	*O. vogelii* Greuter & Burdet	*O. vogelii*/Narin/kekik/80		DD	E	Adana	DD
OMI	OMI1	*O. minutiflorum* O. Schwarz et P.H. Davis	*O. minutiflorum*/2009/13		2009	E	Antalya	N37° 22′ 798″ E30° 56′ 427″
OMA	OMA1	*O. majarona* L.	*O. majarona*/2009/32	*Majorana* (Miler) Bentham	2009	DD	Antalya	DD
	OMA2		*O. majarona*/2009/19		2009			N36° 34′ 326″ E32° 00′ 942″
OON	OON1	*O. onites* L.	*O. onites*/2009/12		2009	DD	Isparta	N37° 37′ 866″ E30° 52′ 287″
	OON2		*O. onites*/2009/11		2009			DD
OSY	OSY1	*O. syriacum* L. subsp. *bevanii* (Holmes) Ietsw.	*O. syriacum*/2009/37		2009	DD	Mersin & Osmaniye & Hatay	N37° 12′ 595″ E34° 38′ 081″
	OSY2		*O. syriacum*/2009/6		2009			DD
	OSY3		*O. syriacum*/2009/24		2009			DD
OVVU	OVVU1	*O. vulgare* L. subsp. *vulgare* Linnaeus	*O. vulgare* subsp. *vulgare*/2009/8	*Origanum*	2009	DD	Osmaniye & Mersin	N37° 15′ 081″ E34° 45′ 830″
	OVVU2		*O. vulgare* subsp. *vulgare*/2009/7		2009			N37° 21′ 100″ E36° 27′ 680″
OVG	OVG1	*O. vulgare* L. subsp. *gracile* (K. Koch) Ietsw.	*O. vulgare* L. subsp. *gracile*/2014/3		2014	DD	Tunceli	N39° 21′ 864″ E39° 12′ 538″
OVH	OVH1	*O. vulgare* L. subsp. *hirtum* (Link) Ietsw.	*O. vulgare* L. subsp. *hirtum*/2009/36		2009	DD	Mersin	N37° 08′ 345″ E34° 41′ 560″
	OVH2		*O. vulgare* L. subsp. *hirtum*/2009/9		2009			DD
OVVI	OVVI1	*O. vulgare* L. subsp. *viridulum* (Martrin-Donos) Nyman	*O. vulgare* L. subsp. *viridulum*/2009/38		2009	DD	Isparta & Mersin	N37° 14′ 282″ E34° 37′ 740″
	OVVI2		*O. vulgare* L. subsp. *viridulum*/2009/10		2009			N37° 34′ 848″ E31° 10′ 560″
OLA	OLA1	*O. laevigatum* Boiss.	*O. laevigatum*/2009/23	*Prolaticorolla* Ietswaart	2009	E	Osmaniye & Hatay	N36° 52′ 685 E36° 16′ 770″
	OLA2		*O. laevigatum*/2009/39		2009			N37° 05′ 290″ E36° 20′ 116″
	OLA3		*O. laevigatum*/2009/42		2009			N37° 03′ 795″ E36° 23′ 131″
	OLA4		*O. laevigatum*/2009/5		2009			N37° 20′ 003″ E36° 27′ 320″
	OLA5		*O. laevigatum*/2009/40		2009			DD
	OLA6		*O. laevigatum*/St.Simean/27.11.2009		2009			DD

Notations are as follows: E = endemic; DD = data deficient.

Table A2
SRAP primer combinations and their sequence information.

Primer Type	Pair Code	Forward	Sequence of Forward (5' to 3')	Reverse	Sequence of Reverse (5' to 3')
SRAP	**1**	**me2**	TGAGTCCAAACCGGAGC	**em1**	GACTGCGTACGAATTAAT
	2			**em2**	GACTGCGTACGAATTTGC
	3			**em3**	GACTGCGTACGAATTGAC
	4			**em5**	GACTGCGTACGAATTAAC
	5			**em6**	GACTGCGTACGAATTGCA
	6			**em7**	GACTGCGTACGAATTATG
	7			**em8**	GACTGCGTACGAATTAGC
	8			**em11**	GACTGCGTACGAATTTCG
	9			**em15**	GACTGCGTACGAATTCGT
	10	**me3**	TGAGTCCAAACCGGAAT	**em1**	GACTGCGTACGAATTAAT
	11			**em2**	GACTGCGTACGAATTTGC
	12			**em3**	GACTGCGTACGAATTGAC
	13			**em4**	GACTGCGTACGAATTTGA
	14			**em5**	GACTGCGTACGAATTAAC
	15			**em6**	GACTGCGTACGAATTGCA
	16			**em7**	GACTGCGTACGAATTATG
	17			**em11**	GACTGCGTACGAATTTCG
	18			**em13**	GACTGCGTACGAATTGGT
	19			**em15**	GACTGCGTACGAATTCGT
	20	**me4**	TGAGTCCAAACCGGACC	**em1**	GACTGCGTACGAATTAAT
	21			**em2**	GACTGCGTACGAATTTGC
	22			**em3**	GACTGCGTACGAATTGAC
	23			**em5**	GACTGCGTACGAATTAAC
	24			**em7**	GACTGCGTACGAATTATG
	25			**em8**	GACTGCGTACGAATTAGC

Table A3
EST-SSR primers and their sequence information.

Primer Type	Name	Primer	Sequence (5′ to 3′)	Repeat Pattern
EST - SSR	OR09	Forward	TTGAAGCATTGTTGGAGGTAGATG	$(TTTTTC)_4(T)_5(TTTTTC)_1$
		Reverse	TCCCAACTAGGGAGAAATGTGC	
	OR12	Forward	GCCCCTGCAGTGACTCCTAC	$(AG)_7G(AG)_3$
		Reverse	AAAAAGGCTTCGGACTCGATC	
	OR13	Forward	GAGAGAATCCAAGCCTCCGC	$(AAC)_7AGC(AAC)_1$
		Reverse	TGAAGGAGTCCGATGTTGACG	
	OR27	Forward	TCAGAAACAATGAAGGCCGC	$(CCT)_6$
		Reverse	CCGTACAGGTCAAACACCGG	
	OR32	Forward	TCTTGCCAATTTATGCGTGTTC	$(AG)_6GG(AG)_2GA(AG)_5GG(AG)_1$
		Reverse	GAAACAAGCATCTTTTCCTGAATTC	
	OR40	Forward	GCCCAAGGACATCCAACTTG	$(GGT)_4GTT(GGT)_1$
		Reverse	CAACTGAACACCTCCCACAATG	

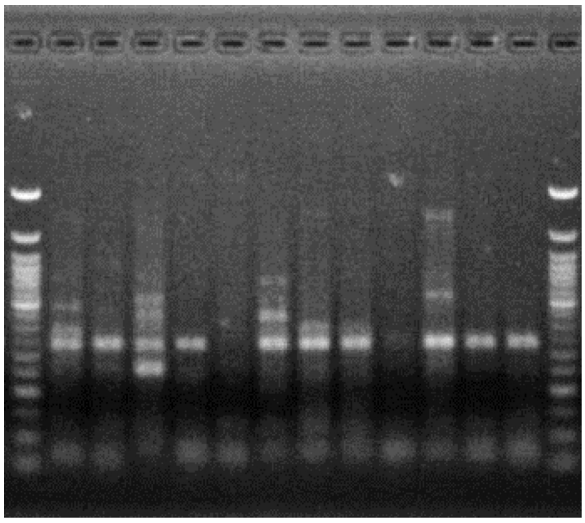

Fig. A1. Allelic pattern of SRAP marker combination, em4-me3, on an agarose gel image.

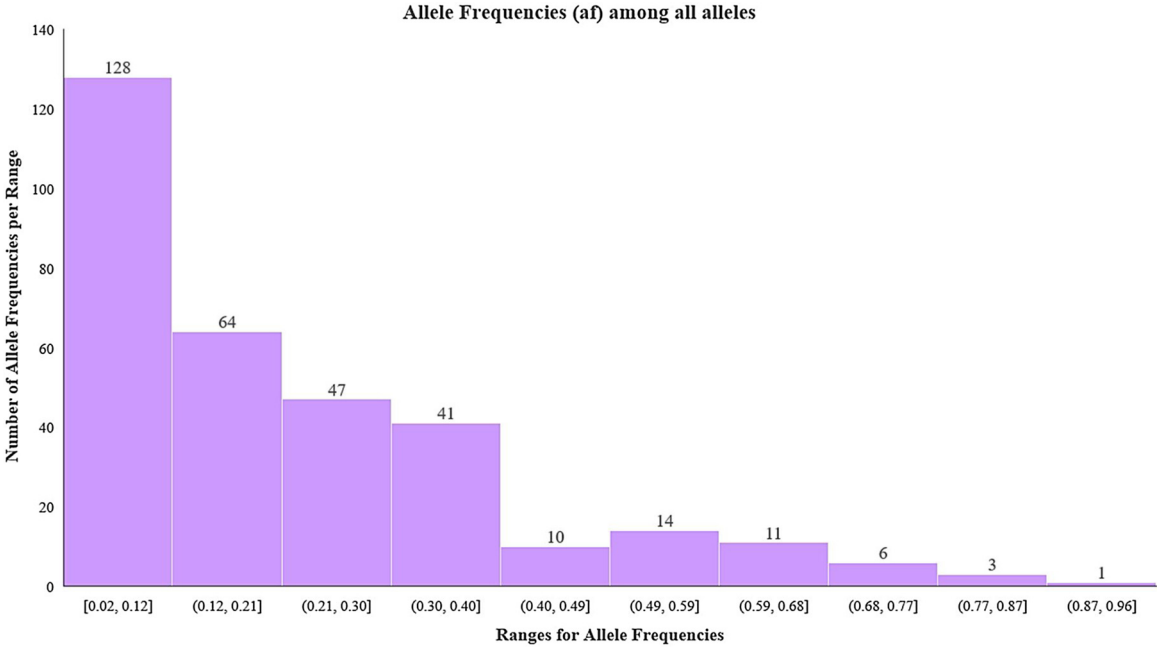

Fig. A2. Distribution of individual allele frequencies (af) among all alleles for both SRAP and EST-SSR markers.

Table A4
Gene flow in the eight sections of *Origanum* L. *PhiPT* values are shown below the diagonal. Values accepted as significant (< 0.15) are in bold characters indicating relatively low allelic differentiation between taxa. Number of migrants per generation (*Nm*) for sections of *Origanum* L. genera are shown above the diagonal and bold characters indicate relatively high gene flow between taxa (*PhiPT* < 0.15).

PhiPT values vs. *Nm* values	*Amaracus*	*Anatolicon*	*Brevifilamentum*	*Chilocalyx*	*Majorana*	*Origanum*	*Prolaticorolla*
Amaracus	–	**2.97**	**1.91**	1.09	0.98	0.81	0.68
Anatolicon	**0.08**	–	**6.08**	**1.67**	1.34	0.82	0.62
Brevifilamentum	**0.12**	**0.04**	–	**3.48**	**1.64**	**1.51**	1.17
Chilocalyx	0.19	**0.13**	**0.07**	–	**1.51**	0.88	0.81
Majorana	0.20	0.16	**0.13**	**0.14**	–	**2.18**	0.97
Origanum	0.24	0.23	**0.14**	0.22	**0.10**	–	0.77
Prolaticorolla	0.27	0.29	0.18	0.24	0.20	0.25	–

Table A5
Gene flow in the oregano accessions. *PhiPT* values are shown below the diagonal. *PhiPT* values that were accepted as significant (< 0.15) are in bold characters indicating relatively low allelic differentiation between taxa. Number of migrants per generation (*Nm*) for *Origanum* L. populations are shown above the diagonal. Bold characters indicate relatively high gene flow between taxa (*PhiPT* < 0.15).

	OSA	OHU	OLE	ORO	OMA	OON	OSY	OVVU	OVH	OVVI	OLA
OSA	–	1.18	1.38	0.91	0.87	0.89	0.89	**1.45**	1.06	0.97	1.09
OHU	0.18	–	1.16	0.56	0.6	0.67	0.64	0.91	0.64	0.79	0.87
OLE	0.15	0.18	–	1.15	**2.66**	1.36	0.89	**1.48**	**1.81**	1.33	1.1
ORO	0.22	0.31	0.18	–	0.62	0.64	0.65	0.73	0.58	0.65	0.67
OMA	0.22	0.29	**0.09**	0.29	–	**6.04**	0.72	0.91	0.96	**1.51**	1.03
OON	0.22	0.27	0.16	0.28	**0.04**	–	0.81	1.23	1.33	**2.43**	1.09
OSY	0.22	0.28	0.22	0.28	0.26	0.24	–	**3.27**	0.89	0.92	0.84
OVVU	**0.15**	0.22	**0.14**	0.26	0.22	0.17	**0.07**	–	**3.07**	**2.17**	**1.52**
OVH	0.19	0.28	**0.12**	0.30	0.21	0.16	0.22	**0.08**	–	**3.06**	0.98
OVVI	0.20	0.24	0.16	0.28	**0.14**	**0.09**	0.21	**0.10**	**0.08**	–	1.29
OLA	0.19	0.22	0.19	0.27	0.19	0.19	0.23	**0.14**	0.20	0.16	–

Table A6
Subpopulations and admixed individuals as determined by population structure analysis at $K = 8$ and $K = 3$.

***K*=8**				***K*=3**			
Individual	**Inferred Ancestry**	**Significance Value**	**Section**	**Individual**	**Inferred Ancestry**	**Significance Value**	**Section**
OSO1	Admixed	0.60	***Amaracus, Anatolicon, Brevifilamentum, Majorana,* and *Origanum***	OBO1	Admixed	0.52	***Amaracus, Anatolicon, Brevifilamentum, Chilocalyx Majorana,* and *Origanum***
OHY1	Admixed	0.62		*OSO1*	Admixed	0.60	
OSI1	Admixed	0.33		*OHY1*	Admixed	0.62	
OBA1	Admixed	0.51		*OSI1*	Admixed	0.43	
OON1	Admixed	0.66		OBA1	Admixed	0.57	
OVVU1	Admixed	0.66		OHU1	Admixed	0.59	
OVVU2	Admixed	0.69		OHU2	Admixed	0.58	
OVG1	Admixed	0.70		OBI1	Admixed	0.60	
OVVI1	Admixed	0.65		OVO1	Admixed	0.59	
OVVI2	Admixed	0.53		OMA1	Admixed	0.62	
OAC1	SP I	0.97	***Brevifilamentum* and *Chilocalyx***	OSY3	Admixed	0.54	
OHA1	SP I	0.94		*OVG1*	Admixed	0.63	
OLE2	SP I	0.75		OAC1	SP I	0.97	***Brevifilamentum* and *Chilocalyx***
OMU1	SP I	0.91		OHA1	SP I	0.92	
OMI1	SP I	0.96		OLE2	SP I	0.89	
OSY1	SP II	0.96	***Majorana***	OMU1	SP I	0.94	
OSY2	SP II	0.96		ORO1	SP I	0.87	
OSY3	SP II	0.94		ORO2	SP I	0.97	
OBO1	SP III	0.73	***Amaracus, Brevifilamentum, Longitubus, Chilocalyx,* and *Majorana***	OMI1	SP I	0.96	
OLE1	SP III	0.93		OSA1	SP II	0.91	***Amaracus***
OAM1	SP III	0.94		OSA2	SP II	0.87	
OBI1	SP III	0.75		OSA3	SP II	0.78	
OVO1	SP III	0.92		OSA4	SP II	0.92	
OMA1	SP III	0.78		OSA5	SP II	0.94	
OMA2	SP III	0.86		OSA6	SP II	0.90	
OON2	SP III	0.80		OSA7	SP II	0.99	
ORO1	SP IV	0.92	***Brevifilamentum***	OSA8	SP II	0.96	
ORO2	SP IV	0.98		OLE1	SP III	0.79	***Brevifilamentum, Longitubus, Majorana, Origanum,* and *Prolaticorolla***
OSA1	SP V	0.93	***Amaracus***	OAM1	SP III	0.85	
OSA2	SP V	0.90		OMA2	SP III	0.86	
OSA3	SP V	0.70		OON1	SP III	0.84	
OSA4	SP V	0.95		OON2	SP III	0.80	
OSA5	SP V	0.94		OSY1	SP III	0.80	
OSA6	SP V	0.95		OSY2	SP III	0.92	
OSA7	SP V	0.99		OVVU1	SP III	0.98	
OSA8	SP V	0.97		OVVU2	SP III	0.92	
OHU1	SP VI	0.94	***Brevifilamentum***	OVH1	SP III	0.98	
OHU2	SP VI	0.94		OVH2	SP III	0.98	
OVH1	SP VII	0.95	***Origanum***	OVVI1	SP III	0.89	
OVH2	SP VII	0.96		OVVI2	SP III	0.96	
OLA1	SP VIII	0.84	***Prolaticorolla***	OLA1	SP III	0.75	
OLA2	SP VIII	0.95		OLA2	SP III	0.92	
OLA3	SP VIII	0.92		OLA3	SP III	0.78	
OLA4	SP VIII	0.98		OLA4	SP III	0.96	
OLA5	SP VIII	0.93		OLA5	SP III	0.97	
OLA6	SP VIII	0.77		OLA6	SP III	0.95	

Definitions are as follows: Individual codes with *italic* font indicate the observed admixed individuals for $K = 3$ and $K = 8$; SP = Sub population.

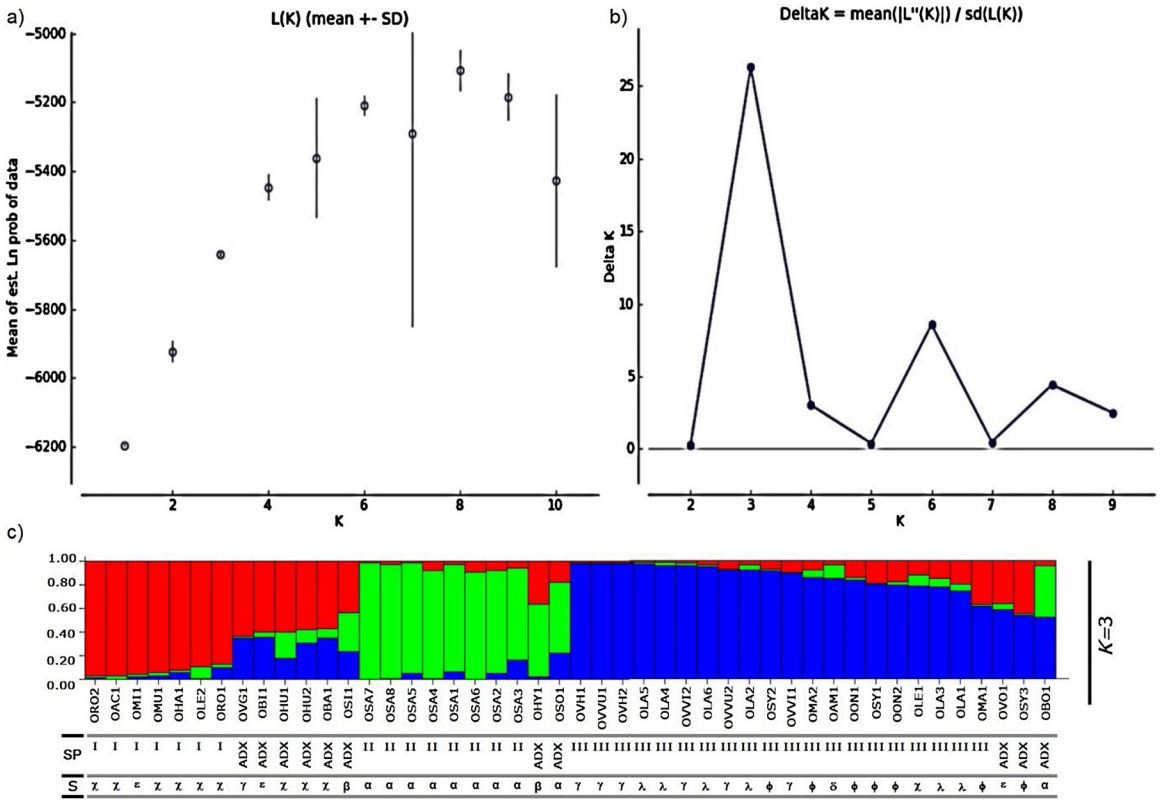

Fig. A3. (a) Likelihood values from Structure Harvester analysis are plotted for the ten different subpopulations tested in the analysis. (b) Delta *K* is plotted for each *K* with peaks indicating a high probability that the number of subpopulations was $K = 3$or $K = 8$. (c) Structure graph for $K = 3$.

Table A7
The results for Bayesian clustering for $K = 8$ and $K = 3$.

Sub population	*Ns*	*ΣNi*	*He*	F_{ST} values
$K = 8$				
SP I	2	5	0.34	0.02
SP II	1	3	0.15	0.65
SP III	4	8	0.23	0.32
SP IV	1	2	0.24	0.49
SP V	1	8	0.22	0.39
SP VI	1	2	0.16	0.66
SP VII	1	2	0.09	0.8
SP VIII	1	6	0.15	0.58
Admixed	4	10		
$K = 3$				
SP I	2	7	0.36	0.0008
SP II	1	8	0.21	0.42
SP III	5	19	0.21	0.39
Admixed	5	12		

Notations are as follows: SP = Sub population; *Ns* = Number of sections for that cluster; *ΣNi* = Total number of individuals; *He* = Expected heterozygosity; F_{ST} = Fixation index.

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