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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

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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

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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