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Microsatellites for genetic and taxonomic research on thyme (*Thymus* L.)

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Abstract: Microsatellites are considered the marker of choice in modern research. However, there is no application of microsatellites in the research on the genus *Thymus* due to the scarcity of specific primer pairs. Using in silico data of several genera in the family Lamiaceae, 23 microsatellite primer pairs (LT) were developed and evaluated in 48 samples representing 9 species and subspecies of the genus *Thymus*. Codominant and multiallelic LT microsatellite markers were not only useful in the determination of genetic structure and variation within and between the species but also were powerful taxonomic aids in the molecular phylogeny of *Thymus* L. Moreover, some LT primer pairs successfully cross-amplified microsatellite markers in *Salvia* and *Origanum*, indicating that they could be used in other genera in the family Lamiaceae. To the best of our knowledge, this is the first report of genic microsatellite markers for the genus *Thymus*.

Key words: Cross-species transferable markers, genetic variations, simple sequence repeats, threatened species

1. Introduction

Researchers in the food industry, academia, and medicine are increasingly interested in medicinal and aromatic plant species in the families Myrtaceae, Lauraceae, and Lamiaceae. Thyme (*Thymus* L.) is one of the most important genera in the family Lamiaceae. The number of species within this genus is still debatable but it includes about 400 species, many of which are native to the Mediterranean region (Morales, 2002). Based on morphological features, species in the genus *Thymus* were divided into 2 main subgenera, *Thymus* and *Coridothymus*, and 8 sections, which consisted of *Micantes* Velen., *Mastichina* (Mill.) Benth., *Piperella* Willk., *Teucrioides* Jalas, *Pseudothymbra* Benth., *Thymus*, *Hypodromi* (A.Kern.) Halacsy, and *Serpyllum* (Mill.) Benth (Jalas, 1971). Many species of the sections *Hypodromi* and *Serpyllum* are endemic to Anatolia and the Aegean islands (Tumen et al., 1998; Sagdic et al., 2009). Among the sections, *Hypodromi* and *Serpyllum* are taxonomically more complex. Thus, morphological and molecular approaches have limitations to identify and define species in *Hypodromi* and *Serpyllum*. For instance, the DNA barcoding approach using the *trnH-psbA*, *rbcL*, and *matK* genes failed to produce species-specific barcodes in these sections (Federici et al., 2013). New approaches to identify and define species boundaries of taxonomically complex sections are required for the genus *Thymus*.

Many species in the genus *Thymus* have high levels of natural hybridization within and between the species, probably due to the absence of incompatibility and the presence of a dimorphic breeding system, gynodioecy, in which populations comprise female and hermaphroditic individuals (Morales, 2002; Thompson, 2002; Sostaric et al., 2012; Federici et al., 2013). Polyploidy and disploidy/aneuploidy within the genus *Thymus* further complicate the determination of species boundaries. Due to the difficulties in determination of species boundaries in this genus, a "phylogenetic species concept" was suggested for the genus *Thymus*, which defines a species as the smallest aggregation of individuals diagnosable by a unique combination of character states (Sostaric et al., 2012).

The genus *Thymus* has attracted humans for centuries because several species produce high levels of some secondary metabolites such as terpenoids, phenylpropanoids, and fatty acid derivates. These secondary metabolites are increasingly used in the medical and cosmetic industries (Thompson, 2002; Boros et al., 2010; Sostaric et al., 2012; Federici et al., 2013). Among these volatile substances found in the secondary metabolites is carvacrol, which is important as a disinfectant and anti-infective, and thymol, which has antibacterial, antifungal, and antiseptic activities. Others include limonene and α-terpineol, which are used in soaps and cosmetics, 1-borneol, which has natural insect repellent properties,

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and 1,8-cineole, which is used in medicine (Giron et al., 2012). Some species of *Thymus* are also rich in polyphenols (flavonoids) and antioxidants, which regulate plant growth and provide resistance to plant diseases (Ali et al., 2012). Because of increasing concern regarding the potentially adverse effects of synthetic chemicals on humans and nature, the production of naturally produced secondary metabolites has been encouraged in food, agronomy, perfumery, cosmetics, and medicine (Brauchler et al., 2010).

Today in modern research, herbalists, agronomists, plant breeders, pharmacologists, and food scientists are increasingly taking advantage of developments in molecular biology and related fields in order to select plants with desired compounds and to identify plant materials in nature and in processed food. However, the application of DNA methods for the genus *Thymus* has lagged behind the other economically important plant species (Zhang et al., 2002; Edwards et al., 2008; Ince and Karaca, 2011a; Ali et al., 2012; Karaca et al., 2013). Over the past 3 decades, different types of DNA marker techniques have been developed and used for many plant species, but few of these molecular techniques have been used for this genus. The techniques of inter-simple sequence repeat (ISSR) and random amplified polymorphic DNA markers (RAPD) have been used in genetic characterization (Trindade et al., 2009), while amplified fragment length polymorphism (AFLP) and isozymes have been utilized in determining the genetic diversity, relationship, and population structure of the genus *Thymus* (Ali et al., 2012; Sostaric et al., 2012). However, the ISSR, AFLP, and RAPD techniques produce mainly dominant markers, which do not differentiate homozygotes from heterozygotes. Although the development of ISSR, AFLP, and RAPD markers is not required knowledge for genomic sequence information, the reliability of ISSR and RAPD is still debatable (Karaca et al., 2008; Trindade et al., 2009).

Microsatellites, also known as simple sequence repeats (SSRs) or short tandem repeats (STRs) are stretches of DNA consisting of tandem-repeated short motifs generally 1 to 6 base pairs in length. Slippage of the DNA polymerase enzyme during genome replication and/or unequal crossing over are thought to be consequences of differences in the tandem repeat number of the core nucleotide sequences (Ince et al., 2010). Microsatellites are considered the marker of choice in many applications because (i) they are easy to develop since they are based on polymerase chain reaction (PCR), (ii) development of microsatellite markers does not require a high amount and high quality of genomic DNA, (iii) they can differentiate homozygote samples from heterozygote ones since they are codominant markers, and (iv) they are highly polymorphic since microsatellites are often multiallelic and hypervariable. However, there is no report on the use of microsatellite markers for the genus *Thymus* due to a scarcity of primer pairs.

This study was undertaken to develop a new set of microsatellite markers using in silico data. Microsatellite markers were used in 48 individuals representing 9 species and subspecies of *Thymus* previously collected from the Mediterranean region of Turkey. A total of 23 microsatellite primer pairs called LT (Lamiaceae *Thymus*) were characterized. Microsatellite markers generated by the 23 LT primer pairs were used in genetic diversity, relationship, population structure, and phylogenetic studies of *Thymus*. To the best of our knowledge, this is the first report on the development of microsatellite markers in thyme.

2. Materials and methods

2.1. In silico data

Expressed sequence tag (EST) data are essentially nonexistent for *Thymus* spp. A total of 378 sets of genomic survey sequence (GSS) and mRNA data for *Thymus* spp., 739 sets of EST, GSS, and mRNA data for *Origanum* spp., and 10,288 sets of EST data for *Salvia* spp. were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/) of the National Center for Biotechnology Information (NCBI) databases and were utilized in this study.

2.2. Microsatellite primer pairs

Each EST, GSS, and mRNA dataset was separately assembled into contiguous sequences using the software Sequencher (Gene Codes, Ann Arbor, MI, USA) to identify and remove redundant sequence data. In this study, sequences that had a minimum overlap of 100 bp and 96% identity matches were considered redundant and removed from further study. After the redundancy tests were completed, one sequence from each individual contig and all singletons that were not assembled into any contigs were used in the development of microsatellite primer pairs.

Individual sequences of contigs and singletons were analyzed using the Exact Tandem Repeats Analyzer (e-TRA 1.0) program (Karaca et al., 2005a) to identify microsatellites. Microsatellites were considered to contain repeat motifs that were between 2 and 6 nucleotides in length. Microsatellite primer pairs flanking the repeat-containing sequences were identified using PRIMER3 software (Rozen and Skaletsky, 2000) based on the criteria previously described in Ince et al. (2008).

2.3. Plant materials

Leaf samples of 48 individual plants representing 9 species and subspecies of *Thymus* naturally grown in the Mediterranean region of Antalya and collected from different latitudes, longitudes, and altitudes were used in

this study. Altitudes varied from 1346 m to 2023 m above sea level. Latitudes varied from N36°24′866″ to N37°20′737″ while longitudes varied from E29°43′531″ to E32°32′519″. During the collection of plant materials, care was taken to collect different individuals from spatially separated plants and avoid resampling individuals by collecting individuals at least 10-m apart. Three individuals were collected from each location with the exception of *T. sipyleus* Boiss. subsp. *sipyleus* var. *sipyleus*, of which 2 individual samples were collected. A total 17 locations were visited and the global position of a location was determined for the second individual collected from that location (Table 1).

2.4. DNA extraction

Young healthy leaves of 48 individual plants were used for the genomic DNA extraction studies. DNA samples were extracted according the protocol described in Karaca et al. (2005b). Some of the DNA samples were further purified according to the protocol described in Ince and Karaca (2009) and Ince et al. (2011) to increase the quality. Values for amount, purity, integrity, and enzyme accessibility of the extracted DNA samples were determined using a spectrophotometer, agarose gel electrophoresis, restriction enzyme digestion studies, and polymerase chain reaction techniques (Ince and Karaca, 2011b). All the reagents used were molecular biology grade chemicals purchased from Amresco Inc. (Solon, OH, USA) and Invitrogen Corp. (Carlsbad, CA, USA).

2.5. Touch-down polymerase chain reactions (Td-PCRs)

The Td-PCRs were carried out in 25-μL reaction volumes containing 60 ± 10 ng genomic DNA sample as a template, 0.5 μM of each LT microsatellite 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 was as follows: initial denaturation at 94 °C for 3 min, 10 cycles with denaturation at 94 °C for 30 s, annealing at 66 °C or 61 °C for 30 s in the first cycle, diminishing by 0.5 °C each cycle, and extension reactions at 72 °C for 1 min 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 or 55 °C. Denaturation and extension conditions of the reactions were the same as indicated above. The amplification reactions finished with final extension reactions at 72 °C for 10 min.

2.6. Agarose gel electrophoresis

After the Td-PCRs were completed, 5 μL of DNA loading buffer (0.25 g L^{-1} bromophenol blue, 0.25 L^{-1} xylene cyanol

Table 1. List of *Thymus* species and geographic locations.

No	Species collected	Location	GPS		
			Latitude	Longitude	Altitude (m)
1	*T. cilicicus* Boiss. & Bal.	Alanya	N36°34′993″	E32°21′033″	1401
		Gazipaşa	N36°30′067″	E32°27′363″	1386
		Gündoğmuş	N36°52′019″	E32°04′706″	1463
2	*T. revolutus* Celak.	Saklıkent	N36°53′847″	E30°27′644″	1733
		Kemer	N36°30′286″	E30°23′125″	1439
3	*T. cherlerioides* Vis. var. *isauricus* Jalas	Gündoğmuş	N36°52′141″	E32°09′017″	1523
4	*T. leucotrichus* Hal. var. *austroanatolicus* Jalas	Gazipaşa	N36°24′866″	E32°32′519″	2131
		Akseki	N37°07′135″	E31°47′153″	1279
5	*T. zygioides* Griseb. var. *lycaonicus* (Celak) Ronniger	Elmalı	N36°49′016″	E29°42′074″	1493
		Saklıkent	N36°43′837″	E30°21′771″	1613
		Korkuteli	N36°56′736″	E30°07′347″	1346
6	*T. sipyleus* Boiss. subsp. *sipyleus* var. *sipyleus* L.	Elmalı	N36°43′581″	E29°43′531″	1599
		Beydağları	N36°51′685″	E30°15′535″	2000
7	*T. sipyleus* Boiss. subsp. *sipyleus* var. *davisianus* Ronniger	Saklıkent	N36°49′921″	E30°19′600″	2023
8	*T. sipyleus* Boiss. subsp. *rosulans* (Borbas) Jalas	Gazipaşa	N36°25′167″	E32°33′113″	2005
9	*T. longicaulis* C. Presl subsp. *chaubardii* var. *chaubardii* (Boiss. & Heldr. ex Reichb. fil.) Jalas	Bozburun	N37°20′737″	E31°01′730″	1476
		Elmalı	N36°35′856″	E30°02′627″	1720

GPS = global positioning system, GPS data were obtained using eTrex (Garmin, Taiwan).

Table 2. Microsatellite primer pairs and related information.

GI*	Primer ID	Sequence (5'–›3')	Tm	Motif	Size (bp)	PIC
51952291	**LT01**	F: AGTATTTGTGCCGAGGGTTG R: ACAGGAAAGGGAGAGGGAGA	59.9 60.2	$[TC]_{15}$	200–220	0.728
51953394	**LT02**	F: GAGGAGGCAGGCAGAAGG R: TGTTAGGTGTCATCGGCTCAC	61.1 61.1	$[TGA]_{7}$	300–320	0.850
51955305	**LT03**	F: CAAATCCAGCCCCAAATCA R: TTCCTCTTTCAGGTTCCATCAG	61.8 60.6	$[CT]_{13}$	170–180	0.081
51956167	**LT04**	F: CACGAGGCACACAAGCAC R: TTGAACAGAACCCATCTCCTTC	60.0 60.5	$[GA]_{12}$	190	0.00
51958121	**LT05**	F: GGAGCTGGAGAAAGAGAACA R: TGCAAGAAAAGCAAGCTACA	57.2 57.5	$[TA]_{14}$	160–190	0.732
51959777	**LT06**	F: CGCAATCCTCCCTCATAAAT R: GACCTTCTTCACGCTGGTG	59.0 59.4	$[TC]_{19}$	240–260	0.615
99077744	**LT07**	F: GGGGCTGTGGTGTTTCAT R: TTTCTCATCTGGGCTATCAAGA	63.0 59.3	$[TC]_{19}[TC]_{6}$	150–280	0.894
99077745	**LT08**	F: AAGCGTGAGAAGAGCAGCAC R: CCACCACAACAGGAGAGACC	58.9 60.6	$[TG]_{14}$	190–250	0.791
99077749	**LT09**	F: GAGCATCTCGAAGCGAAAGT R: CGGCATAAGCAACCTCTTTT	59.7 59.4	$[TC]_{13}$	186–210	0.809
99077751	**LT10**	F: AAGTTTGGGACGGAGTTAGT R: CTGAAGCACCTTTTGATTTG	55.4 56.0	$[AG]_{15}$	220–350	0.922
99077753	**LT11**	F: GATCCACCTCAATTTCAAGA R: TGTGCCTCCTTCTATTCATC	55.1 55.3	$[AC]_{7}[GA]_{11}$	172–192	0.901
99077754	**LT12**	F: GTAGGGATTGTCGCCGTTG R: CCTCCGCCATTTTCATTTCT	61.4 61.3	$[AG]_{10}$	210–220	0.761
99077749	**LT13**	F: GTGAAGTAACGCTTCCATGAGAG R: GAGTACAAAAGAGCTACAGATG	60.3 51.2	$[AG]_{12}$	190–210	0.765
99077744	**LT14**	F: TTTGCGCAGATCTCAAGTGC R: AAGCGGTGACTGACGGAGAC	62.6 62.8	$[TG]_{13}$	160–190	0.453
99077745	**LT15**	F: GGATGATGCTGAGTTGGTGATAAG R: CCTGACACGCCACAAAAGTG	62.1 62.7	$[AG]_{15}$	250–310	0.872
99077747	**LT16**	F: GGGCATTAAAGCTAAGGAGCG R: CAGCCGATCACCTGTCCTTC	62.3 63.1	$[CT]_{12}$	220–280	0.432
99077752	**LT17**	F: CACACGCACTGGTGAGGTG R: TTCCCGCAGATCTCCAGAAC	62.5 62.6	$[AG]_{11}$	210–240	0.143
99077754	**LT18**	F: CCAAGAATGCCGATGTCAAAG R: CTCCACCTCCTAGTTTCTTGGC	62.8 61.5	$[TC]_{14}$	190–240	0.876
99077749	**LT19**	F: GAAAAGCGAAGCCGTTGAAG R: TGCTGAGCCTTTGCCCTTAG	62.3 62.8	$[GA]_{6}$	300–900	0.121
557836588	**LT20**	F: AGCCAAACTCGCTGCTTCTG R: GGTAAAAAGGGTAATAGACGTGG	63.1 58.1	$[TAAAA]_{5}$	230–250	0.231
99077754	**LT21**	F: AAGATCGAAGGCATCGATCG R: GGTGAAAAATGAATACAGTGGGC	62.5 61.8	$[AG]_{12}$	260–310	0.876
528746517	**LT22**	F: CATCAAGTTCAATAATGCTGTG R: CAGATAGTTGCATCGAGGTTAG	56.0 56.7	$[AAAC]_{5}$	220–240	0.589
190361713	**LT23**	F: TCCCATCATTTTCCTCCGTC R: CCCCACTACAGCAGAAACCG	62.1 62.9	$[ACG]_{5}$	190–260	0.312

PIC: Polymorphic information content, GI: GenBank identification number, *: Please note that some GIs in the table may not show the motif since their homologous sequences were used for the design of primer pairs. Tm is calculated using Primer3 program.

FF, and 400 g L^{-1} sucrose in water) was added to each 25 µL of amplified product and 8–10 µL of these mixtures were loaded on 3% high-resolution Serva agarose gels (Serva Electrophoresis GmbH, Heidelberg, Germany) containing 0.05 µg of ethidium bromide per mL. Samples were electrophoresed at 5 V cm^{-1} at a 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 (UV) transilluminator for analysis.

2.7. Confirmation of microsatellites

Five single amplicons (alleles) randomly selected among the amplified products of the 23 LT primer pairs were recovered from the agarose gels, purified using a DNA purification kit (PureLink Quick Gel Extraction & PCR Purification Combo Kit, Invitrogen Corporation, Carlsbad, CA, USA), and cloned using an InsTAclone Cloning Kit/TransformAid Vectors (Fermentas Life Sciences, Hanover, MD, USA). Plasmids were extracted from bacterial strains (JM107) using a GeneJET Plasmid Miniprep Kit (Fermentas Life Sciences, Hanover, MD, USA) and the target regions were commercially sequenced (Macrogen Inc., Amsterdam, Netherlands).

2.8. Data analysis

Amplified products (alleles) of primer pairs were scored as present (1) or absent (0). The scores were used in the determination of polymorphic information content (PIC) values of each primer pair (Table 2). The scores were also used in calculation of Nei and Li's genetic similarity indices and phylogenetic studies. The PIC value of each microsatellite locus was calculated according to Smith et al. (1997), as follows: $PIC = 1 - \sum f_i^2$, where f_i is the frequency of the *i*th allele in the species examined.

2.9. Genetic similarity indices within and between species and subspecies

A 48 × 48 genetic similarity matrix was obtained using Nei and Li's genetic similarity index values (GSIs), which were calculated using the formula $GSI_{XY} = 2a/(a + b) + (a + c)$, where X and Y are the numbers of microsatellite 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. The analysis was done using Multi Variety Statistical Package software (MVSP 3.13O, Kovach Computing Services, Pentraeth, UK).

Mean genetic similarity indices within and between the species and subspecies were calculated using the similarity indices values of the matrix. The genetic relationships among individuals from the 9 species and subspecies were determined using a principal coordinate analyses (PCoA) generated from allelic frequencies for the polymorphic loci using the MVSP 3.13O program based on mean character differences. PCoA is an ordination method similar to principal component analysis, except that PCoA uses a distance matrix rather than the values to plot the axes (Karaca et al., 2008).

2.10. Molecular phylogeny

The microsatellite data were analyzed in the Bayesian method using the MrBayes v3.2.1 x64 software program. One cold and 3 heated chains were run starting from a random tree for 10 million Markov chain Monte Carlo (MCMC) generations, with chains sampled every 100th cycle (Ronquist and Huelsenbeck, 2003). The nucleotide model used was 4 by 4 and the number of substitution type was selected as "1" (F81 model). The binary data were executed using MCMC methods that included random walk Monte Carlo method algorithms and a 50% majority-rule consensus phylogenetic tree constructed with the posterior probabilities indicated at the nodes (Ronquist and Huelsenbeck, 2003; Karaca et al., 2013).

3. Results

3.1. Plant species

A total of 81 individual plants representing 9 species and subspecies of thyme were intended to be used in this study. Seventeen locations whose global positioning values are given in Table 1 were selected as described in *Flora of Turkey* (Jalas, 1982). It was expected that among the 17 locations visited at least 3 would have wild populations of plant materials suitable to study. However, some of the species or subspecies could not be collected since they were not found or the collected materials were not identified as the expected species by the senior taxonomists who kindly helped during the study. *T. cilicicus*, *T. zygioides* var. *lycaonicus*, and *T. sipyleus* subsp. *sipyleus* var. *sipyleus* could be collected from 3 different locations. On the other hand, *T. cherlerioides* var. *isauricus*, *T. leucotrichus* var. *austroanatolicus*, *T. sipyleus* subsp. *sipyleus* var. *davisianus*, and *T. sipyleus* subsp. *rosulans* were found in 1 of the 17 locations while the other species and subspecies were collected from 2 different locations (Table 1).

Although Turkey is still considered the richest of the European countries in terms of flora diversity and endemic species, there has been limited study about the sustainability of its natural resources. The last 2 decades have witnessed dramatic changes in the Mediterranean region of Turkey in terms of loss of wildlife habitat and reduction in plant diversity due to human activities such as clearing for wide road and shopping center construction, stone mining, and play areas. These activities threatened some thyme species reported in Davis (1982) so much that they are no longer surviving in their natural growing areas. Several locations from which individual plant samples were collected in 2008 were revisited in 2012. Unfortunately, it was

witnessed that some of the thyme species naturally found in limited locations of the Mediterranean region of Turkey were threatened, mainly by overharvesting and clearing for road construction and stone mining. Furthermore, overharvesting due to the growing global market demand for some aromatic and medicinal plant species, including thyme, resulted in increasing destruction of natural habitats (Giron et al., 2012).

3.2. Primer pairs

A total of 50 primer pairs were designed, 27 of which were not considered microsatellite primer pairs since some produced amplified products larger than 1 kb, some produced multiple products or no amplifications, and some produced very weak amplifications. However, 23 primer pairs called LT microsatellite primer pairs (Table 2) amplified genomic DNA samples of 48 individuals representing 9 different species and subspecies of thyme. Sequence analyses of the amplified products confirmed the presence of microsatellite motifs as expected from sequence information (Figure 1). A total of 23 LT primer pairs numbered from LT01 to LT23 amplified microsatellite patterns. LT microsatellite primer pairs LT01 to LT06 were designed from *Salvia* ESTs and LT07 to LT23 were designed from EST, GSS, and mRNA sequences of *Thymus* and *Origanum*.

The calculated PIC values of the 23 microsatellite primer pairs ranged from 0 to 0.92 with a mean value of 0.307 (±0.307). Among the 23 LT microsatellite primer pairs, 15 had a PIC value greater than 0.5 (Table 2). PIC values greater than 0.5 indicated that the microsatellites developed in this study have a high level of polymorphism. The number of amplified products (alleles) varied from 1 to 8 depending on the primer pairs and species. The majority of the LT primer pairs produced codominant and multiallelic amplification patterns in the thyme samples used in this study (Figure 2). The use of codominant and multiallelic amplification patterns of LT primer pairs was not only helpful to identify homozygote and heterozygote individuals at a particular locus but also useful in the determination of hybridizations and population structure.

3.3. Genetic variations

Microsatellite loci amplified with LT primer pairs were used to study interspecific and intraspecific genetic variations of thyme naturally growing in the Mediterranean region of Turkey. Genetic variation within a species means that polymorphism at the intraspecific level was observed within individuals of the same species. Genetic variation between species means polymorphism at the interspecific level was observed among individuals of different species. Interspecific and intraspecific genetic variations were calculated based on Nei and Li's GSIs. Genetic variations were usually low (higher genetic similarity values) among the individuals collected from the same location while variations reached a maximal value among the species naturally found at different locations.

The mean GSI at the intraspecific level was 0.79 (±0.089) and ranged from 0.76 (±0.14) in *T. revolutus* to 0.87 (±0.06) in *T. cherlerioides* var. *isauricus* (Table 3). A mean GSI value of 0.76 with a relatively high standard deviation (±0.14) indicated higher intraspecific variations among the individuals of *T. revolutus*. Further observation among the amplified products of LT primer pairs showed that 3 individual *T. revolutus* specimens collected from the Saklıkent location contained alleles of other species growing in the same location. The same allelic patterning of different species growing in the same location was an indication of natural hybridization at the interspecies level. The mean GSI at the interspecific level was 0.373 (±0.05) and ranged from 0.199 for *T. revolutus* and *T. longicaulis* subsp. *chaubardii* var. *chaubardii* to 0.657 for *T. sipyleus*. subsp. *rosulans* and *T. sipyleus* subsp. *sipyleus* var. *davisianus* (Table 3). This indicated that the most unrelated species used in this study were *T. revolutus* and *T. longicaulis* subsp. *chaubardii* var. *chaubardii*, while the most related species were *T. sipyleus*. subsp. *rosulans* and *T. sipyleus* subsp. *sipyleus* var. *davisianus*. Certain morphologic features of these 2 species were also very similar in comparison to other species.

A PCoA based on the mean character differences (Karaca et al., 2008) of the microsatellite markers was performed on all 48 individuals representing 9 species and subspecies (Figure 3). The first 2 dimensions of the PCoA accounted for 63.4% of the total variation, to which axis 1 and axis 2 contributed 48.2% and 15.2%, respectively. The analysis divided the 48 individual samples into 7 distinct groups. One of the 7 groups consisted of individuals representing 3 subspecies of *T. sipyleus* (*T. sipyleus* subsp. *sipyleus* var. *sipyleus*, *T. sipyleus*. subsp. *sipyleus* var. *davisianus*, and *T. sipyleus* subsp. *rosulans*). There were also clear differences among the subspecies of *T. sipyleus*. Of the 7 groups, 6 consisted of a single species (Figure 3). The overall findings of the PCoA clearly indicated that LT microsatellite markers could be used as aids in identification of species of unknown individual and genetic studies for *Thymus* species.

3.4. Molecular phylogeny

The 2 most commonly used distance-based methods for building trees, the unweighted group method with arithmetic mean (UPGMA) and neighbor joining (NJ) were also used in this study. These 2 methods produced very similar trees (data not shown). Since the Bayesian method shows posterior probabilities at nodes that indicate the reliability of phylogeny, this analysis was selected and performed on 48 thyme individuals using microsatellite alleles. Among the 9 species, 6 individuals of *T. longicaulis* subsp. *chaubardii* var. *chaubardii* were in the



Figure 1. Confirmation of microsatallite domains in amplified products. Panels a, b, c, d, e, and f represent flanking region of primer pairs LT06, LT07, LT08, LT09, LT10, and LT11. Underlined sequences are primer binding sites; boxes show microsatallite domains with tandem repeats.

Table 3. Genetic similarity indices within and between the species and subspecies of *Thymus* used in this study.

No	1	2	3	4	5	6	7	8	9
1	**0.79 (±0.06)**								
2	0.592 (±0.046)	**0.76 (±0.14)**							
3	0.435 (±0.047)	0.408 (±0.040)	**0.87 (±0.06)**						
4	0.313 (±0.053)	0.330 (±0.047)	0.589 (±0.083)	**0.77 (±0.03)**					
5	0.307 (±0.047)	0.292 (±0.046)	0.291 (±0.044)	0.387 (±0.057)	**0.79 (±0.069)**				
6	0.257 (±0.044)	0.227 (±0.055)	0.251 (±0.043)	0.342 (±0.033)	0.559 (±0.052)	**0.81 (±0.09)**			
7	0.279 (±0.041)	0.246 (±0.079)	0.284 (±0.145)	0.310 (±0.045)	0.510 (±0.050)	0.583 (±0.037)	**0.84 (±0.04)**		
8	0.233 (±0.053)	0.310 (±0.054)	0.237 (±0.024)	0.251 (±0.046)	0.468 (±0.038)	0.495 (±0.049)	0.657 (±0.052)	**0.81 (±0.05)**	
9	0.217 (±0.048)	0.199 (±0.041)	0.235 (±0.044)	0.251 (±0.031)	0.432 (±0.055)	0.538 (±0.043)	0.596 (±0.047)	0.628 (±0.046)	**0.84 (±0.048)**

Numbers: 1 = *T. cilicicus*, 2 = *T. revolutus*, 3 = *T. cherlerioides* var. *isauricus*, 4 = *T. leucotrichus* var. *austroanatolicus,* 5 = *T. zygioides* var. *lycaonicus*, 6 = *T. sipyleus* subsp. *sipyleus* var. *sipyleus*, 7 = T. *sipyleus* subsp. *sipyleus* var. *davisianus*, 8 = *T. sipyleus* subsp. r*osulans*, and 9 = *T. longicaulis* subsp. *chaubardii* var. *chaubardii.* Numbers in bold are intraspecific genetic similarity indices.

section *Serpyllum* and rooted trees clearly separated this species from the rest of the species belonging to the section *Hypodromi* (data not shown).

The phylogenetic relationship between *T. cherlerioides* var. *isauricus* and *T. leucotrichus* var. *austroanatolicus* was supported with a higher posterior probability value (Figure 4). On the other hand, natural hybridization between individuals of *T. revolutus* with other species interfered with the inference of phylogeny. Three individuals of *T. revolutus* clustered with *T. cilicicus* at a node with 76.5% posterior probability while 3 other individuals of the same species clustered with *T. cherlerioides* var. *isauricus* at a node with 96.5% posterior probability (Figure 4). Nine individuals of *T. zygioides* var. *lycaonicus* were clearly separated from the other species at a node with a 99.9% posterior probability value. A total of 12 individuals belonging to subspecies of *T. sipyleus* clustered together at a node with 97.9% posterior probability. A Bayesian analysis based on codominant and multiallelic microsatellite markers taxonomically differentiated 9 species and subspecies of *Thymus*. The results of this study indicate that a species node with posterior probability greater than 80% could be used as a valued aid in the molecular phylogeny of *Thymus.*

4. Discussion

Spain and Turkey are the main thyme trading countries (Loziene, 2009). In Turkey, wild populations of thyme are the major source of exported leaves and flowering parts. Due to the increasing use of natural extracts in the pharmaceutical, cosmetic, and perfume industry, and as flavoring and preservation in food industry, the last 2 decades have witnessed the continuous eradication of genetic variability and a reduction in the population size of *Thymus* naturally growing in the Mediterranean region of Turkey. Today, *Thymus* species are collected from scattered small populations showing different levels of destructions due to human activities. The protection of thyme populations in the Mediterranean region and in the other natural growing areas is a priority to avoid their further reduction from clearing for constructions of roads, houses, and shopping centers, stone mining, and overharvesting. The destruction of *Thymus* populations in Spain has already resulted in the classification of *Thymus albican* as an endangered species due to habitat fragmentation and degradation (Giron et al., 2012). Due to human activity, several species of *Thymus* growing in the Mediterranean region of Turkey are also threatened.

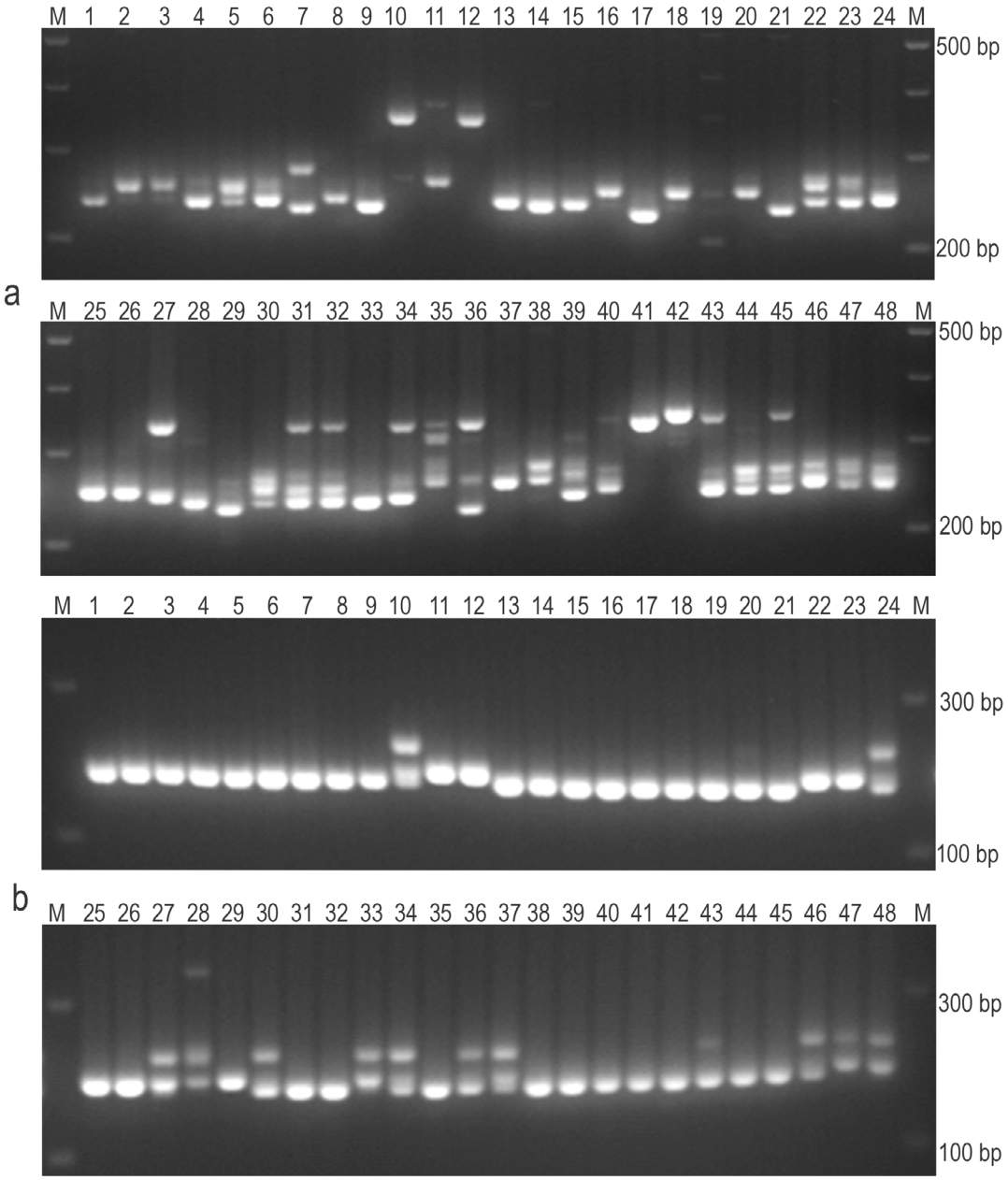


Figure 2. Representative gels showing amplification profiles of microsatellite markers resolved in 3% agarose gel along with DNA size markers. Samples in panels a and b are amplified products of primer pairs LT10 and LT06, respectively. Lanes 1–9 are *T. cilicicus*, 10–15 are *T. revolutus*, 16–18 are *T. cherlerioides* var. *isauricus*, 19–21 are *T. leucotrichus* var. *austroanatolicus,* 22–30 are *T. zygioides* var. *lycaonicus*, 31–36 are *T. sipyleus* subsp. *sipyleus* var. *sipyleus*, 37–39 are *T. sipyleus* subsp. *sipyleus* var. *davisianus*, 40–42 are *T. sipyleus* subsp. *rosulans*, and 43–48 are *T. longicaulis* subsp. *chaubardii* var. *chaubardii.*

Knowledge of population structure, genetic variations, and the levels of hybridization could be used in preservation and management studies. Since the ability of a population to respond to selection forces is directly proportional to the level of genetic variation present, a new tool useful in the analysis of the genetic diversity is important in conservation and breeding programs (Trindade et al., 2009; Ali et al., 2012; Giron et al., 2012; Sostaric et al., 2012; Federici et al., 2013). New DNA markers could be used for the genus *Thymus* to assess the population structure and genetic variations, and could be used as aids in taxonomic studies. Despite the great importance of the genus *Thymus* in world and local markets, the application of DNA markers for this genus is surprisingly low in comparison to other

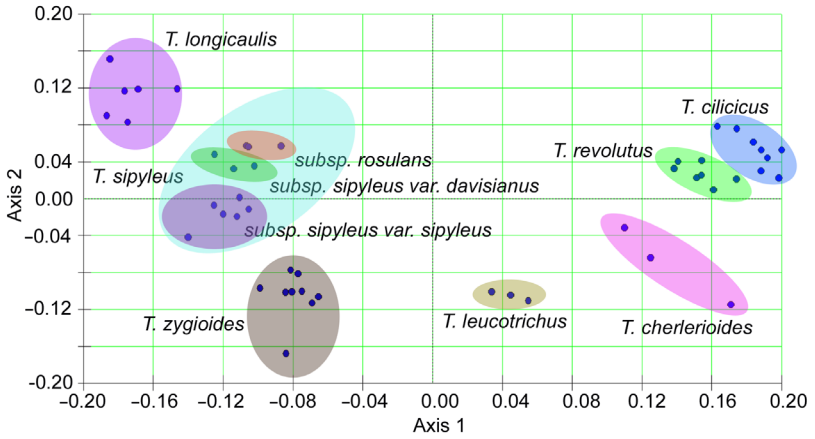


Figure 3. PCoA analysis graph of the first 2 axes for 48 *Thymus* species individuals.

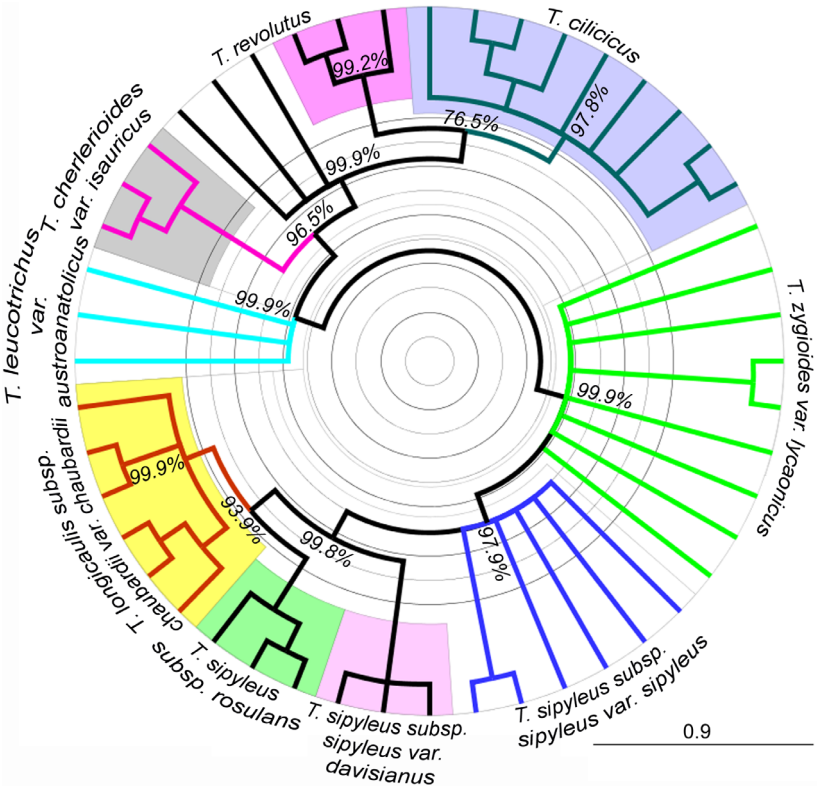


Figure 4. A 50% majority-rule consensus tree of some species of *Thymus* L. The numbers at the nodes are posterior probabilities. The scale shows the branch lengths measured in expected substitutions per site.

plant species. Among the DNA marker techniques, RAPD, AFLP, and ISSR have been used for thyme (Trindade et al., 2009; Sostaric et al., 2012). However, these markers have some disadvantages due to (i) comigration of fragments of the same size originating from different loci among different samples, (ii) comigration of paralogous markers, (iii) amplicons derived from overlapping fragments due to primer pairs flanking the same regions, (iv) heteroduplex formation, and (v) the presence of 2 or more equally sized different fragments within a single band. Furthermore, the levels of polymorphism and the scoring of these markers are problematic in comparison to microsatellite markers (Karaca et al., 2004; Edwards et al., 2008; Karaca et al., 2013).

The microsatellite markers developed in this study do not require capillary and polyacrylamide gel electrophoresis platforms, but the resolution of an agarose gel system may be very low in comparison to capillary and polyacrylamide gel electrophoresis. In order to get high resolution of alleles produced with LT microsatellite primer pairs, the use of a capillary electrophoresis system is recommended. In addition, with the use of a capillarity system, most of the primer pairs reported in this study could be used in multiplex PCRs. The use of multiplex PCRs will definitely reduce the reaction costs and labor time for population genetic studies of *Thymus* species.

The LT microsatellite primer pairs listed in Table 2 were obtained from ESTs and mRNAs and had a high level of cross-transferability between different genera. Although microsatellite markers developed from genomic DNA sequences usually have a lower degree of cross-species transferability, they show a high level of polymorphism (Karaca et al., 2013). The LT microsatellite primer pairs produced cross-genera transferable polymorphic markers among *Thymus*, *Salvia*, and *Origanum*. A high level of cross-transferability of LT markers makes them very useful in comparative mapping studies of different genera in the family Lamiaceae. Furthermore, higher degrees of cross-species and cross-genera transferability of LT markers make them useful in detection of individuals in herbal mixtures.

The majority of LT primer pairs produced codominant and multiallelic markers within the 9 species and subspecies of *Thymus*. The use of co-dominant markers in the determination of population structure is critical since they inform researchers about the gene flow within and between species. Codominant markers could be used to select and pair homozygote parental genotypes or select traits at the homozygous states that are difficult to measure using phenotypic assays.

The allelic patterns and polymorphisms of the 48 thyme individuals clearly showed that genetic variations at the interspecific level were greater than at the intraspecific level. Based on the allelic patterning, it was found that hybridizations were common in *T. revolutus, T. zygioides* var. *lycaonicus*, *T. sipyleus* subsp. *sipyleus* var. *sipyleus*, and *T. sipyleus* subsp. *rosulans*. A PhD study supervised by the first author of this study also showed that the 48 thyme individuals contained several types of plastids, confirming the hybridization events. The findings of our study, in accordance with the results of previous reports, reveal that in natural populations of the genus *Thymus* with 2 or more species living together hybridizations are very common and species boundaries are difficult to identify due to the absence of genetic incompatibility (Morales, 2002; Thompson, 2002; De Mattia et al., 2011; Ali et al., 2012).

Several studies were interested in the integration between essential oil compound data and molecular characterization but there are contradictory results in the literature. For instance, a good correlation has been identified between RAPD profiles and essential oil analysis for *T. caespititius* (Echeverrigaray et al., 2001), while a low correlation between these 2 has also been obtained (Trindade et al., 2008). The essential oil compositions of several *Thymus* species used in this study were different from one location to another (Karaca et al., 2011). Comparison between the allelic patterns of the microsatellite markers developed in this study revealed that the chemotypes of some *Thymus* species grown in different locations could be determined using the LT microsatellite markers. These findings indicate that the composition of essential oils and allelic patterns of microsatellite markers could be successfully used as taxonomic aids in the characterization of the species, in the detection of hybridizations in natural populations, and in detection in herbal mixtures. The existence of correlation between allelic patterns of the microsatellite markers developed in this study and the essential oil composition previously detected (Karaca et al., 2011) indicates that these microsatellite markers could be utilized in future breeding programs to produce desired genotypes.

In conclusion, this study reported a new set of microsatellite markers using in silico data. LT microsatellite markers offer the following advantages over previously used DNA markers in *Thymus*: (i) since the majority of the markers are parts of the expressed genome, they represent the functional part of the genome; (ii) since they are cross-genera and cross-species transferable markers, they are very valuable in comparative genomic studies; and (iii) since they are codominant and multiallelic markers, they are valuable in population structure, conservation, and taxonomic studies.

LT microsatellite markers were used to study the genetic structure of 48 individuals of *Thymus*. It was found that there was greater genetic diversity at the intraspecific and interspecific level. The number of microsatellite alleles increased as the altitude increased while the level of hybridizations increased as the distance between species and individual decreased. According to the genetic diversity parameters used in this study, the majority of species showed a high level of variation suggesting that the populations constitute a valuable germplasm for conservation and improvement work.

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