



Genetic relations among and within wild and cultivated *Thymus* species based on morphological and molecular markers

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Abstract

In the present study, the diversity of 11 *Thymus* species was assessed using molecular and morphological markers. Essential oil content and morphological traits were also investigated during two agronomic years. The result of the analysis of variance showed considerable differences among morphological traits. In the first and second years, the essential oil content of the species varied from 0.63 to 1.94% and 0.86 to 2.34%, respectively. *T. vulgaris* and *T. migricus* showed the highest essential oil content in two agronomic years. In this research, nine ISSR primers were also used to amplify 151 polymorphic bands in 77 accessions belonging to 11 *Thymus* species. Cluster and principal component (PCA) analyses classified the species in three major groups. Among the species, *T. vulgaris* and *T. fedtschenkoi* presented relatively higher genetic distance in comparison with other species. Analysis of molecular variance (AMOVA) revealed that 72.34% of the total variation was belonged to within-species variation, while 27.66% was associated among the species. High gene flow ($N_m = 1.11$) and genetic differentiation ($G_{st} = 0.31$) were also observed among the species. *T. transcaspicus* exhibited the highest genetic variation (0.19), polymorphism % (57.69%), and Shannon index (0.29). The STRUCTURE analysis also showed a high admixture of *Thymus* species that might be originated from a high rate of natural hybridization. Finally, based on molecular and morphological information, *T. vulgaris* and *T. carmanicus* can be suggested as good candidate species for further breeding programs in *Thymus* species.

Keywords Thyme · Essential oil · Morphology · ISSR marker · Genetic diversity

Introduction

Thyme (*Thymus* spp.) is one of the most important medicinal plants of the Lamiaceae family with more than 200 species (Tohidi et al. 2019). Mediterranean regions have been assumed as the center of origin of this genus (Ghasemi Pirbalouti et al. 2011). In Iranian flora, 14 *Thymus* species have been found in which four of them including, *T. daenensis*, *T. carmanicus*, and *T. trautvetteri*, *T. persicus* are endemic (Rahimmalek et al. 2009; Talebi et al. 2015). Thyme, a perennial herb, and aromatic plant has always been widely used in food and pharmaceutical industries due to its valuable

components (Trindade et al. 2019). Essential oils are important aromatic constituents which are derived from different part of plants such as flowers, buds, seeds, leaves, fruits, and roots (Eirini et al. 2017). From an industrial point of view, thyme has been considered as an antibacterial and antifungal plant with different applications in food industries (Rasooli et al. 2006; Ghasemi Pirbalouti et al. 2011; Mohammadi et al. 2014). In folk medicine, *Thymus* species are widely used due to their carminative, anti-inflammatory, antispasmodic, and digestive properties. So, the species of the genus *Thymus* are characterized as a biological source by having an excellent richness in essential oil (Rustaeie et al. 2013).

The current geographical distribution of plant taxa can be attributed to the present, as well as historical factors leading in the genetic structure and phylogenetic patterns (Tohidi et al. 2020). Genetic variation is a prerequisite for further breeding programs. The amount and separation of genetic variation among and within populations are also related to life history characteristics and environmental factors (Rahimmalek et al. 2009; Fadaei Heidari et al. 2016).

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Knowledge of genetic diversity among and within species can provide new information regarding the evolutionary process as well as new insights for conservation management of the plant species. Interspecies genetic variations lead to some phenotypical changes. In this respect, knowledge of interspecies genetic variations may also be beneficial to manage the extinction risks brought about by different intrinsic or extrinsic factors such as inbreeding and environmental factors (Lu et al. 2018). So, evaluating of inter- and intraspecies genetic variation can also be useful for selecting further breeding programs.

Nowadays, molecular markers can provide reliable information in comparison to morphological characteristics. Among the molecular markers, ISSR (inter simple sequence repeat) markers have widely been applied because of high reproducibility, high polymorphism, and simple use. ISSR marker amplifies the sequences between simple sequence repeats (Fadaei Heidari et al. 2016; Verma et al. 2017). ISSR is a PCR-based marker with high polymorphism and affordability that made it a strong tool for genetic diversity studies in plant species (Gupta et al. 1996). ISSR have been efficiently applied for genetic diversity analysis of *T. carmanicus* (Hadian et al. 2014) and *T. daenensis* (Rahimmalek et al. 2009) natural populations.

Thymus is one of the most important medicinal plant genera worldwide, and most of the research has been focused on its secondary metabolites (Tohidi et al. 2017). However, there are some reports regarding the genetic diversity of some *Thymus* species in different countries including *T. deanaensis* (Rahimmalek et al. 2009; Talebi et al. 2015), *T. caespititius* (Trindade et al. 2008, 2009), *T. kotschianus* (Yousefi et al. 2015), *T. carmanicus* (Hadian et al. 2014) and *T. sibthorpii* (Abraham et al. 2018). Most of the previous researches used one molecular marker, one or limited studied species. However, comprehensive research has not yet been performed to assess the inter- and intraspecific variation of *Thymus* species. In Iran, 14 *Thymus* species have

been reported in which in the present research for the first time, inter and intraspecies variation of 11 species viz. *Thymus migricus* Klokov & Des.-Shost., *T. fallax* Fisch. & C. A. Mey., *T. serpyllum* L., *T. trautvetteri* Klokov & Desj.-Shost., *T. transcaspicus* Klokov, *T. carmanicus* Jalas, *T. fedtschenkoi* Ronneger, *T. daenensis* Celak subsp. *daenensis*, *T. pubescens* Boiss. & Kotschy ex Celak, *T. kotschyanus* Boiss. & Hohen., and *T. vulgaris* L. in two years was evaluated using molecular, morphological, and essential oil features.

The aims of the present research were (1) to assess the genetic variation among and within 11 *Thymus* species using ISSR markers; (2) to evaluate the morphological characteristics and essential oil content of studied species in two agronomic years.

Materials and methods

Plant materials and field experiment

The seeds of 11 *Thymus* species comprising *T. daenensis*, *T. migricus*, *T. vulgaris*, *T. fallax*, *T. trautvetteri*, *T. serpyllum*, *T. transcaspicus*, *T. pubescens*, *T. fedtschenkoi*, *T. kotschyanus*, *T. carmanicus*, were collected from gene bank of Research Institute of Forests and Rangeland, Tehran. The species were identified by Dr. Mozaffarian (Research Institute of Forests and Rangeland, Tehran), according to Flora Iranica (Rechinger 1963). The species codes and the detailed information of the species are exhibited in Table 1. A randomized block design (RCBD) with three replicates was used as a statistical design.

The study was performed at Isfahan University of Technology experimental farm. The area had a clay loam soil (pH 7.5) with an average bulk density of 1.48 g/cm³ in the top 60-cm layer of the soil surface. The mean annual precipitation and temperature were 140 mm and 15 °C, respectively. Seeds were planted with 35 cm distance on the rows and

Table 1 Gene bank codes, accession codes and origins of 11 *Thymus* species

Accession no	Gene bank codes	Accession codes	Species	Origin
1	33918	Tm	<i>T. migricus</i>	Shahedieh, Yazd, Iran
2	33916	Tf	<i>T. fallax</i>	Shahedieh, Yazd, Iran
3	33920	Ts	<i>T. serpyllum</i>	Shahedieh, Yazd, Iran
4	39341	Tp	<i>T. pubescens</i>	Shahedieh, Yazd, Iran
5	33921	Ttv	<i>T. trautvetteri</i>	Shahedieh, Yazd, Iran
6	3507	Tts	<i>T. transcaspicus</i>	Khorasan Razavi, Hezar Masjed, Iran
7	18316	Tc	<i>T. carmanicus</i>	Zarand, Kerman, Iran
8	10126	Td	<i>T. daenensis</i>	Fereydonshahr, Isfahan, Iran
9	18063	Tfed	<i>T. fedtschenkoi</i>	Naqadeh, Azarbaijan Gharbi, Iran
10	14287	Tv	<i>T. vulgaris</i>	London, England, United Kingdom
11	17090	Tk	<i>T. kotschyanus</i>	Qazvin, Qazvin, Iran

50 cm space between the rows at 0.5 cm depth in a 2×1 m² plots. The aerial parts of studied *Thymus* species were collected at the flowering period in the two agronomic years of 2018 and 2019.

DNA extraction

Young leaves in full flowering stage were used to extract for DNA using the CTAB method with minor modifications (Murry and Thompson 1980). NanoDrop evaluated the quantity and quality of DNA at the ratio of OD₂₆₀/OD₂₈₀. Suitable DNA concentration was observed between the ratios of 1.6–1.8. The quality of DNA was also investigated by agarose gel in which the sharpened bands without any smearing was useful to select DNAs with higher quality.

ISSR analysis

Among 13 ISSR stock primers, nine produced high numbers of repeatable bands. The obtained information of the ISSR primers is illustrated in Table 2. Amplification reactions were conducted using a thermocycler (Bio-Rad) in a total volume of 15 µL, as described previously (Fadaei Heidari et al. 2016). The optimum annealing temperature was determined according to the melting temperature for each primer. The PCR was performed under the following operation program: 2 min of initial denaturation (94 °C) followed by 40 cycles of 1 min at 94 °C; 1 min at the specific annealing temperature; extension at 72 °C for 2 min; and final extension step of 10 min at 72 °C. DNA amplified fragments were separated in a 1.2% agarose gel in 1 × TBE buffer and visualized on a gel image system.

Essential oil isolation

Aerial parts were collected at the full flowering stage. The plants were dried in shade conditions at 25 °C. Clevenger-apparatus was applied for essential oil extraction from thyme plants. For this purpose, 500 ml of distilled water was added to 40 g powder of each leaf sample and boiled for 5 h. The obtained essential oil was transferred to glass containers and stored at 4 °C. Essential oil content was measured from 77 accessions belonging to 11 *Thymus* species, and the average of essential oil content in each species was calculated. The essential oil content was evaluated according to dry matter, and measurements were performed in triplicates (Tohidi et al. 2017).

Morphological trait analysis

The morphological traits were measured by randomly selecting individuals of each replicate. The means of each studied species were considered for further analysis. The studied morphological traits were plant height (cm), days to flowering, days to 50% flowering, days to 100% flowering, dry weight (g/plant), fresh weight (g/plant), canopy extension (cm), leaf length (mm), leaf width (mm), stem diameter (mm), inflorescence diameter (mm), inflorescence length (mm), number of floret in inflorescence, and essential oil content (% v/w) (Table 3).

Data analysis

The polymorphic ISSR bands in each gel were recorded as absent (0) or present (1). The principal coordinate (PCoA) and cluster analyses were carried out through NTSYSpc,

Table 2 Polymorphism number, annealing temperature and PIC of studied *Thymus* species

No	Primer	Sequence (5' → 3')	Anneling temp ^b	No.total bands	No.PB ^c	PPB ^d (%)	PIC
1	P ₇	5'-AGAGAGAGAGAGAGAGYT-3'	48.8	9	8	88.88	0.26
2	P ₈	5'-ACACACACACACACYG-3'	52.4	12	11	91.66	0.37
3	P ₉	5'-AGAGAGAGAGAGAGAGT-3'	52.4	35	34	97.14	0.36
4	P ₁₂	5'-GAGAGAGAGAGAGAGAT-3'	48	32	32	100	0.32
5	P ₁₃	5'-CTCTCTCTCTCTCTRG-3'	48	9	9	100	0.24
6	P ₁₅	5'-BDBTCCTCCTCCTCCTCCTCC-3'	60	8	7	87.5	0.24
7	P ₁₆	5'-HVHCCTCCTCCTCCTCCTCC-3'	60	7	7	100	0.25
8	P ₁₇	5'-AGAGAGAGAGAGAGAGC-3'	48	36	35	97.22	0.39
9	P ₂₂	5'-TCCTCCTCCTCCTCCWS ^a -3'	52.4	8	7	87.5	0.26
Total				156	151	-	-
Average				17.33	16.77	94.43	0.29

^aWS: wobbles

^bDegrees celsius (°C)

^cNumber of polymorphic band

^dPercentage of polymorphic band

Table 3 Means of morphological characters and essential oil yield of *Thymus* species in two years of 2018 and 2019

Species	Days to flowering		Days to 50% flowering		Days to 100% flowering		Leaf length (mm)		Leaf width (mm)		Inflorescence diameter (mm)		Canopy extension (cm)	
	2018	2019	2018	2019	2018	2019	2018	2019	2018	2019	2018	2019	2018	2019
<i>T. migricus</i>	170.5±1.0	169.2±0.1	191.8±0.1	191.7±0.1	201.0±1.7	200.2±0.1	7.8±1.0	7.5±0.0	3.83±0.6	4.84±0.0	12.7±0.5	12.8±0.7	36.7±2.2	35.6±0.1
<i>T. fallax</i>	183.1±3.6	181.3±0.0	192.0±1.7	192.6±0.0	201.6±1.2	202.2±0.1	10.1±0.5	9.5±0.0	4.57±0.4	5.43±0.0	18.2±3.8	19.5±0.5	23.8±1.6	23.4±0.5
<i>T. serpyllum</i>	179.3±0.8	178.4±0.3	187.0±0.6	185.4±0.1	201.3±1.0	201.7±0.1	14.8±1.1	12.4±0.0	4.01±0.5	3.65±0.0	21.7±2.4	22.9±0.4	40.1±0.9	39.6±0.3
<i>T. pubescens</i>	165.0±1.1	166.4±0.1	181.7±0.1	180.3±0.0	195.6±1.1	196.7±0.1	14.1±1.2	11.0±0.0	3.99±0.4	3.69±0.0	19.1±1.8	19.4±0.3	51.7±0.2	51.2±0.1
<i>T. trautvetteri</i>	174.8±1.0	176.2±0.1	185.6±1.8	184.6±0.0	200.7±1.1	200.7±0.1	16.2±0.3	12.2±0.1	3.65±0.1	3.07±0.0	22.2±1.1	23.6±0.0	46.9±3.3	46.7±0.5
<i>T. transcaspicus</i>	174.7±0.1	174.7±0.5	180.2±0.1	180.2±0.1	197.8±0.1	197.8±0.1	9.0±1.3	8.3±0.1	4.86±0.8	3.93±0.0	17.7±6.5	20.0±1.0	33.6±0.2	33.7±0.2
<i>T. carmanicus</i>	180.2±0.6	180.3±0.3	196.0±0.6	196.5±0.5	201.8±0.3	204.1±0.1	11.9±0.5	11.1±0.0	5.15±0.2	6.64±0.0	17.5±1.3	21.4±0.1	27.1±0.7	25.9±0.1
<i>T. daenensis</i>	176.0±0.0	176.0±0.0	182.7±0.1	182.7±0.1	198.8±0.1	198.8±0.1	11.9±1.8	11.3±0.1	3.04±0.3	4.45±0.0	19.9±2.4	20.8±0.6	41.9±0.1	41.9±0.2
<i>T. fedtschenkoi</i>	167.8±2.7	169.2±0.1	178.0±0.5	178.6±0.0	199.4±0.8	198.7±0.1	13.3±1.4	9.2±0.0	4.52±0.5	4.06±0.0	22.0±2.2	21.2±0.0	31.8±1.9	31.8±0.2
<i>T. vulgaris</i>	176.4±0.3	177.6±0.0	185.6±0.8	186.3±0.0	201.8±0.5	203.2±0.1	5.3±0.6	5.8±0.0	2.93±0.3	2.99±0.0	13.4±2.5	12.5±0.4	31.7±1.2	30.7±0.3
<i>T. kotschyanus</i>	168.5±0.7	169.3±0.0	182.1±1.6	180.3±0.0	194.1±3.7	195.7±0.1	12.3±1.7	9.4±0.0	3.99±0.6	5.45±0.0	19.4±1.6	21.4±0.8	41.5±0.6	40.8±0.0
LSD	2.71	0.43	1.76	0.34	2.51	0.33	2.11	0.15	0.91	0.05	3.88	1.04	2.34	0.49
Species	Inflorescence length (mm)		Stem diameter (mm)		Plant height (cm)		Number of floret in inflorescence		Fresh weight (g/plant)		Dry weight (g/plant)		Essential oil content (% v/w)	
	2018	2019	2018	2019	2018	2019	2018	2019	2018	2019	2018	2019	2018	2019
<i>T. migricus</i>	16.0±1.2	16.6±0.3	0.84±0.0	0.87±0.0	15.9±2.6	14.7±0.3	33.3±2.3	34.2±0.1	49.3±1.4	76.7±0.5	16.2±0.7	50.2±0.9	1.93	2.20±0.2
<i>T. fallax</i>	23.5±1.5	23.2±0.2	0.82±0.1	0.65±0.0	13.7±1.2	15.6±0.0	68.6±6.5	75.8±1.3	27.2±4.3	53.0±1.5	9.6±0.6	24.5±1.3	0.63	0.92±0.0
<i>T. serpyllum</i>	25.2±0.6	30.8±1.0	1.01±0.0	1.17±0.0	12.8±0.5	13.4±1.4	91.3±1.3	91.5±1.0	26.9±4.1	60.6±1.3	10.9±0.5	26.0±1.8	0.96	1.74±0.0
<i>T. pubescens</i>	23.9±2.1	22.9±0.3	0.86±0.1	0.95±0.0	18.5±4.8	19.7±0.0	86.8±0.3	86.1±0.5	27.1±1.0	53.1±2.8	10.6±0.4	22.8±2.8	0.82	0.93±0.0
<i>T. trautvetteri</i>	25.7±2.3	28.4±0.6	1.02±0.0	0.93±0.0	20.1±2.6	19.9±0.1	84.8±2.1	85.2±0.3	54.8±3.1	84.9±3.9	20.8±0.8	92.0±3.4	1.22	1.38±0.0
<i>T. transcaspicus</i>	21.0±2.5	23.9±0.1	0.96±0.0	1.32±0.0	11.9±1.8	13.7±0.1	84.3±1.1	84.5±0.5	53.2±2.5	98.4±3.9	17.9±0.6	55.0±1.7	1.54	1.70±0.0
<i>T. carmanicus</i>	26.2±0.6	31.1±0.8	1.03±0.0	0.74±0.0	14.9±1.7	16.1±0.2	63.8±0.9	63.3±0.3	24.0±0.5	43.4±1.4	9.2±0.2	18.0±1.3	0.67	0.85±0.0
<i>T. daenensis</i>	13.8±1.0	22.4±0.6	0.85±0.0	0.74±0.0	18.8±1.9	15.6±1.4	65.2±3.0	75.8±2.5	34.1±4.1	65.3±1.7	11.3±1.1	26.8±0.7	0.82	0.95±0.0
<i>T. fedtschenkoi</i>	24.6±0.4	28.4±0.1	0.89±0.0	0.74±0.0	13.7±3.0	18.9±0.6	63.2±4.4	66.7±0.9	47.1±1.6	77.3±2.5	18.1±0.1	44.1±0.2	1.82	1.87±0.0
<i>T. vulgaris</i>	21.3±2.8	17.8±0.3	0.95±0.3	0.67±0.0	21.3±6.6	21.1±0.0	34.5±1.2	36.2±0.6	86.4±3.2	123.8±0.6	28.9±0.6	48.7±2.6	1.87	2.33±0.0
<i>T. kotschyanus</i>	28.8±3.6	29.0±0.3	0.93±0.0	1.31±0.0	14.0±0.8	16.9±0.2	76.2±2.2	77.0±0.8	48.2±2.5	83.1±3.8	16.7±0.5	35.9±2.2	1.53	1.78±0.0
LSD	3.30	0.70	0.20	0.009	5.26	1.00	4.62	1.55	5.06	4.84	1.15	4.04	0.05	0.14

Means having difference less than the LSD provided are not significantly different ($p < 0.05$)

ver. 2.02 (Rohlf 1998). The polymorphic information content (PIC) was computed based on previous research (Anderson et al. 1993). Genetic similarity among samples was assessed based on the Jaccard's similarity index (Jaccard 1908). The genetic structure parameters were assessed using Pop Gene software, ver. 32 (McDermott and McDonald 1993). The Arlequin ver. 3 software was also used to estimate the variation among and within the *Thymus* species. Also, STRUCTURE software was used to assess the admixture of genotypes based on Evanno's Method (Evanno et al. 2005). The *K* value (2–10) was evaluated based on those explained by Pritchard et al. (2000). Morphological and essential oil data were analyzed using SAS ver. 9.4 and Statgraphics ver. 18.1.08.

Results and discussion

Molecular results

In a pre-screening of primers, 13 ISSR primers were tested in which nine showed high polymorphism and sharpness in the tested genotypes. The products showed the range of 150–2000 bp in size. The average polymorphism (%) was 94.43%. The number of polymorphic bands in primer varied from 7 to 35, with an average of 16.77 (Table 2). The average value of the PIC was 0.29 (Table 2). P13 and P15 showed the lowest PIC (0.24), while P17 revealed the highest value (0.39). *Thymus* species have shown different polymorphism content, including *T. daenensis* (89%) (Rahimmalek et al. 2009), *T. daenensis* (81.5%) (Heydari et al. 2019), *T. sibthorpii* (71%) (Abraham et al. 2018) and higher polymorphism observed in this study might be attributed to inter and intrageneric variation of 11 *Thymus* species. Similar inter- and intra-variation was also obtained in similar studies on *T. carmanicus* (Hadian et al. 2014) and *T. serpyllum* (Sostaric et al. 2012).

ISSR genetic structure

Based on AMOVA (Analysis of the molecular variance), 72.34% of the variation was attributed to “within species”, while the “among species” variation assigned for 27.66% of the variation (Table 4). These results were in line with the results of Kumar et al. (2019). The results also showed a relatively moderate differentiation in allele frequencies ($F_{st}=0.27$) and relatively high gene flow ($N_m=0.92$) among the studied species (Table 4). Besides, most of the genetic variation was obtained within species (Table 4). The genetic structure criteria are shown in Table 5. The highest values for genetic variation (0.19), the number of effective alleles (1.29), and the Shannon index (*I*) (0.29) were obtained for *T. transcaspicus* from Khorasan Razavi. In contrast the lowest values were observed in *T. serpyllum* and *T. trautvetteri* from Shahedieh (Table 5).

Cluster analysis was performed to investigate the genetic relationships among the species. According to the Mantel test, the highest correlation was obtained between the Jaccard similarity coefficient and cophenetic matrices ($r=0.81$). Cluster analysis was done using the UPGMA algorithm. The clustering classified *Thymus* species into four main clusters. The first main cluster (Group I) included five species, including *T. carmanicus*, *T. transcaspicus*, *T. pubescens*, *T. fedtschenkoi*, and *T. trautvetteri* (Fig. 1). The second main cluster consisted of *T. daenensis*, *T. fallax*, *T. migricus*, and *T. kotschyanus* (Group II). The third cluster (Group III) included *T. vulgaris* (Fig. 1), and the rest of the species were classified in group IV (Fig. 1). However, the classification did not distinctly separate the species. For instance, some of the accessions of one species were classified in different groups, while *T. vulgaris* was classified in one group. This kind of discrepancy in the cluster might be interpreted by ploidy level, cross-pollination, high gene flow, and natural hybridization (Fadaei Heidari et al. 2016; Ghafouri and Rahimmalek 2018).

Principal coordinate analysis (PCoA) also confirmed the dendrogram data (Fig. 2). The first three principal coordinates explained 37.2% of the total variation implying high distribution of ISSR markers throughout the genome.

Table 4 Analysis of molecular variance (AMOVA) among and within from *Thymus* species ISSR markers

Source of variation	<i>df</i> ^a	Mean of squares	Percentage of variation	<i>P</i> value	<i>F</i> _{st}	<i>N</i> _m ^b	<i>G</i> _{st} ^c
Among species	10	47.85	27.66	<0.001			
Within species	66	13.01	72.34				
Total	76	17.60			0.27	0.92	0.35

^aDegrees of freedom

^bGene flow. N_m =estimate of gene flow from *G*_{st} or *G*_{cs}. E.g., $N_m=0.5(1 - G_{st})/G_{st}$; McDermott and McDonald, Ann. Rev. Phytopathol. 31:353–373 (1993)

^cDiversity among species

Table 5 Summary of genetic variation statistics for ISSR markers in 77 used in *Thymus* species

Species	Sample size	Na ^a	Ne ^b	H ^c	I ^d	NPL ^e	PPL ^f (%)	He ^g
<i>T. migricus</i>	7	1.51	1.24	0.16	0.25	81	51.92	0.18
<i>T. fallax</i>	7	1.53	1.26	0.17	0.26	84	53.85	0.19
<i>T. serpyllum</i>	7	1.50	1.27	0.17	0.26	78	50.00	0.20
<i>T. pubescens</i>	7	1.56	1.29	0.18	0.28	88	56.41	0.21
<i>T. trautvetteri</i>	7	1.50	1.23	0.15	0.24	78	50.00	0.18
<i>T. transcaspicus</i>	7	1.57	1.29	0.19	0.29	90	57.69	0.22
<i>T. carmanicus</i>	7	1.52	1.25	0.16	0.25	82	52.56	0.19
<i>T. daenensis</i>	7	1.57	1.27	0.17	0.27	89	57.05	0.20
<i>T. fedtschenkoi</i>	7	1.55	1.26	0.17	0.27	86	55.13	0.20
<i>T. vulgaris</i>	7	1.53	1.25	0.16	0.25	83	53.21	0.19
<i>T. kotschyanus</i>	7	1.55	1.27	0.17	0.27	86	55.13	0.20
Multi population	77	1.99	1.43	0.26	0.41	–	–	0.27

^aObserved number of alleles^bEffective number of alleles^cGene diversity^dShannon's information index^eNumber of polymorphic loci^fPercentage of polymorphic loci^gExpected heterozygosity

High variation among the species might be due to processes like a recombination of genes and chromosomes, gene mutation, the genetic structure of species, and the number of effective individuals (Ghafouri and Rahimmalek 2018). Hadian et al. (2014) and Talebi et al. (2015) also observed high genetic diversity among the *Thymus* species. High genetic variation within the species can be due to the different fertility of seeds, dispersal, and reproduction method (Gupta et al. 1999). Furthermore, AMOVA revealed significant variation among the species ($P < 0.001$). In this research, the F_{st} value of 0.27 was obtained, which represented the mean difference between species. *T. serpyllum* and *T. pubescens* showed the highest similarity (0.945), while the least was gained between *T. trautvetteri* and *T. fallax* (0.830). The highest and lowest genetic distance was also observed between *T. fedtschenkoi* and *T. vulgaris* (0.191), and *T. pubescens* and *T. serpyllum* (0.056), respectively (Table 6). The low gene diversity within *T. pubescens* and *T. serpyllum* species might be interpreted as the low gene flow, as compared with other species (Fadaei Heidari et al. 2016).

In the present study, high genetic differentiation was observed among the species ($G_{st} = 0.35$). Three classification types have been reported for the coefficient of genetic differentiation including strong ($G_{st} > 0.15$), moderate ($0.15 > G_{st} > 0.05$), and low ($G_{st} < 0.05$) (Ghafouri and Rahimmalek 2018). Based on this classification, the value of G_{st} showed a high genetic differentiation in the *Thymus* species. The results were in agreement with those obtained by Ray et al. (2018) in *Hedychium coronarium*. STRUCTURE analysis was also performed for the better expounding of

admixture in some species obtained via cluster and principal component analysis (PCA). Accordingly, six autonomous runs produced consistent outcomes. For the given data collection, the K value was appraised by the posterior probability of the data for a given K , $Pr(X|K)$ (Pritchard et al. 2000). The highest value was afforded at $K = 3$. Thus, the analysis was accomplished according to three colors viz. red (A), blue (B), and green (C) for the studied *Thymus* species, as displayed in Fig. 3. The admixture model with correlated allele frequency was applied to inter genetic structure commentary. One of these clusters (Cluster A in Fig. 3) included the species containing *T. carmanicus*, *T. fedtschenkoi*, and *T. transcaspicus*. Cluster B contained species such as *T. daenensis*, *T. fallax*, *T. migricus*, and *T. kotschyanus*. The remaining species including, *T. pubescens*, *T. vulgaris*, *T. serpyllum*, and *T. trautvetteri* were classified in cluster C. The high admixture was perceived in this analysis. For instance, *T. fallax*, *T. pubescens*, and *T. serpyllum* showed high admixture in this study. One eventual reason for this admixture could be due to natural hybridization in some *Thymus* species out of lack of self-pollination strategies such as incompatibility mechanism.

Essential oil content

In the first year (2018), essential oil content ranged from 0.63 to 1.94%, with a mean value of 1.26% (Fig. 4). The highest essential oil content and the lowest was obtained in *T. migricus* (1.94%), and *T. fallax*, respectively. In 2019, essential oil content varied from 0.86 to 2.34% to exhibit

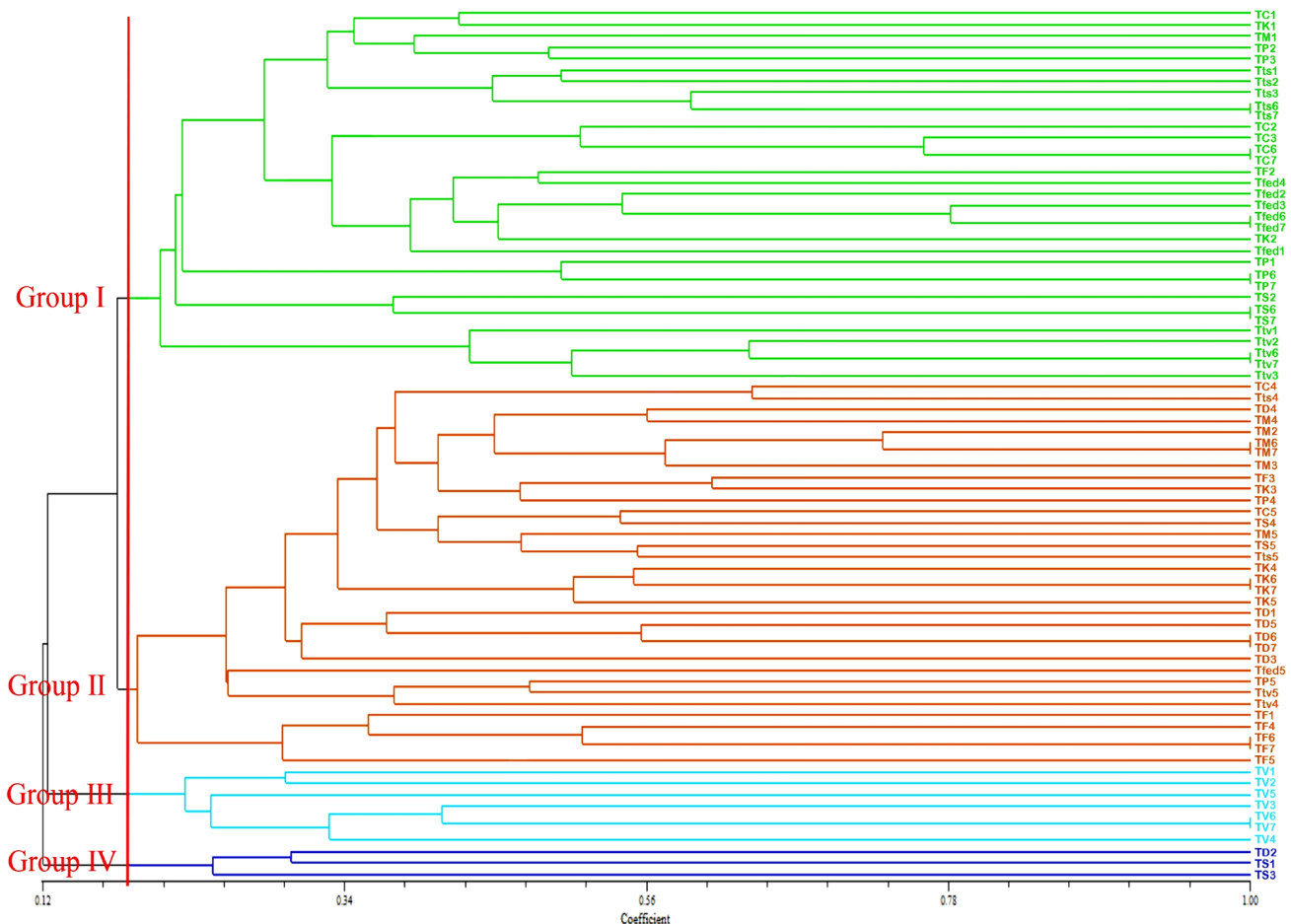


Fig. 1 Dendrogram of 77 studied *Thymus* genotypes based on ISSR markers according to the Unweighted Pair Group Mean Algorithm (UPGMA) with the Jaccard similarity index ($r=0.81$). TM: *T. mig-*

ricus; TF: *T. fallax*; TC: *T. carmanicus*; TK: *T. kotschyanus*; TP: *T. pubescens*; TD: *T. daenensis*; Ttv: *T. trautvetteri*; Tts: *T. transcaspicus*; Tfed: *T. fedtschenkoii*; TV: *T. vulgaris*; TS: *T. serpyllum*

a significant variation with an average value of 1.52% (Fig. 4). *T. vulgaris* and *T. carmanicus* revealed the highest (2.34%) and lowest (0.86%) essential oil content.

The following descending arrangement was gained for essential oil content in both years:

First year : Tm > Tv > Tfed > Tts > Tk
> Ttv > Ts > Td, Tp > Tc > Tf,

Second year : Tv > Tm > Tfed > Tk > Ts
> Tts > Ttv > Td > Tp > Tf > Tc.

Environmental factors and plant species might highly effect on essential oil content (Llorens et al. 2014; Tohidi et al. 2017). Previous researches reported different ranges for the essential oil content in various *Thymus* species, including *T. pannonicus* (1.29%) (Arsenijević et al. 2019) and *T. capitatus* (2.37%) (Tammar et al. 2018).

In the present study, high variation was observed in terms of essential oil content in two studied years.

Similarly, Arabsalehi et al. (2018) revealed a high variation in *Stachys lavandulifolia* essential oil content in two agronomic years.

Morphological trait analysis

Analysis of variance was performed to specify the effects of replication, species, year, and their interactions on morphological traits of *Thymus* species (Table 7). In both agronomic years, the coefficient of phenotypic variation for plant height was higher than other traits (CV = 13.80%). However, the lowest value of the coefficient of variation belonged to days to 50% flowering (CV = 0.39%) (Table 7).

The results of the two-year analysis of variance showed considerable differences among the studied species for all the traits. Also, the effect of species was significant for all the traits, and interactions of S × R (Species × Replication) was meaningless for all the characters (Table 7).

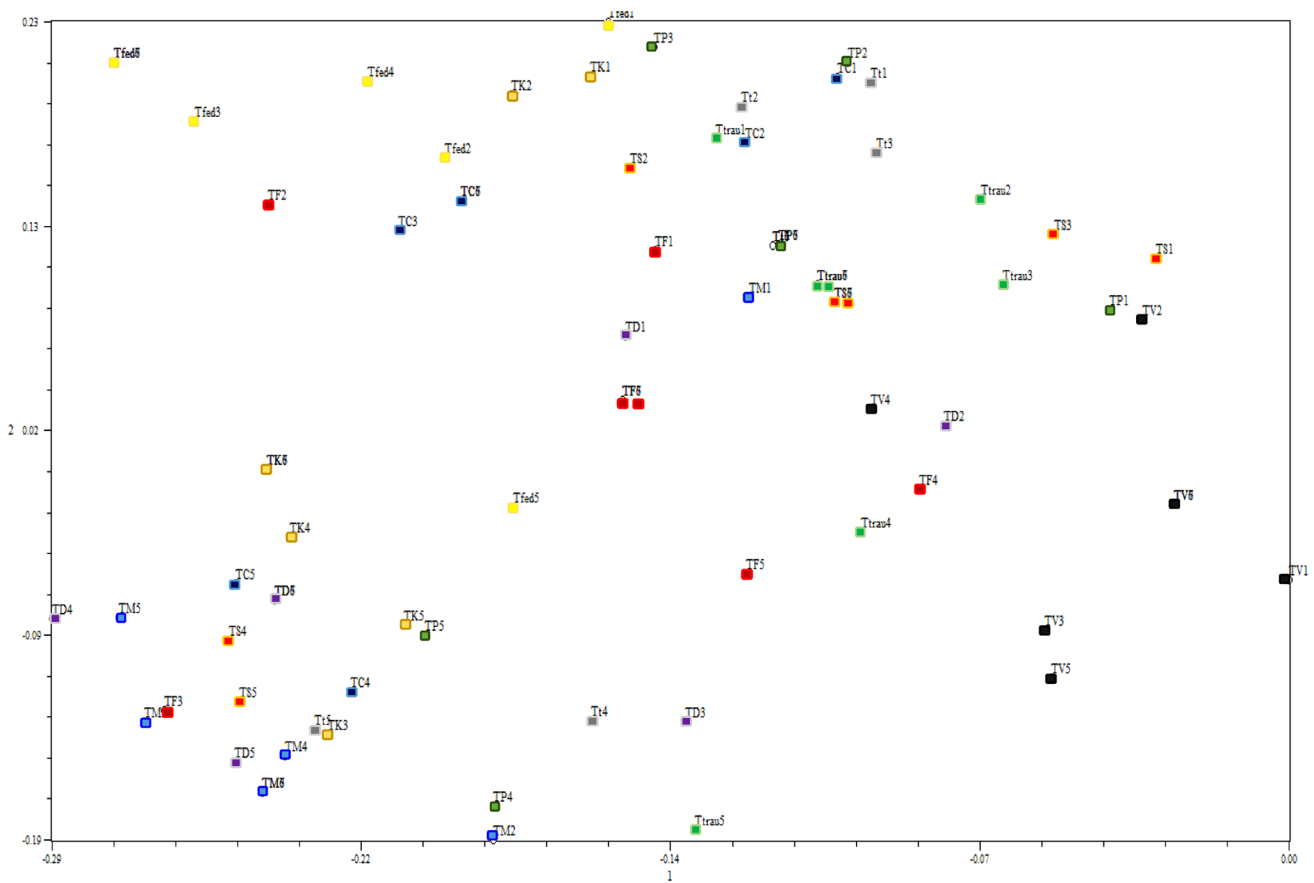


Fig. 2 Plot of *Thymus* samples by principal coordinate analysis using the Jaccard's similarity coefficients. *T. migricus*; *T. fallax*; *T. carmanicus*; *T. kotschyanus*; *T. pubescens*; *T. daenensis*, *T. trautvetteri*; *T. transcaspicus*; *T. fedtschenkoii*; *T. vulgaris*; *T. serpyllum*

Table 6 Nei's genetic identity (above diagonal) and genetic distance (below diagonal) in *Thymus* species

Species	Tc	Td	Tf	Tfed	Tk	Tm	Tp	Ts	Tts	Ttv	Tv
Tc	0.000	0.8948	0.8901	0.8916	0.8994	0.8739	0.9100	0.9111	0.9045	0.8422	0.8438
Td	0.1112	0.000	0.9041	0.8770	0.9178	0.9304	0.9177	0.9200	0.8935	0.8655	0.8590
Tf	0.1164	0.1008	0.000	0.8803	0.9203	0.8861	0.9196	0.8990	0.8807	0.8305	0.8713
Tfed	0.1148	0.1312	0.1275	0.000	0.9033	0.8370	0.9000	0.9157	0.8810	0.8560	0.8261
Tk	0.1060	0.0858	0.0830	0.1017	0.000	0.9198	0.9322	0.9197	0.9186	0.8716	0.8759
Tm	0.1347	0.0722	0.1209	0.1779	0.0836	0.000	0.9025	0.8971	0.8897	0.8397	0.8536
Tp	0.0943	0.0859	0.0838	0.1053	0.0702	0.1025	0.000	0.9451	0.9325	0.8981	0.8985
Ts	0.0931	0.0834	0.1065	0.0881	0.0837	0.1086	0.0564	0.000	0.9009	0.9055	0.9057
Tts	0.1004	0.1126	0.1271	0.1267	0.0849	0.1169	0.0699	0.1043	0.000	0.8696	0.8658
Ttv	0.1718	0.1445	0.1857	0.1555	0.1375	0.1747	0.1075	0.0992	0.1398	0.000	0.8736
Tv	0.1699	0.1520	0.1377	0.1910	0.1325	0.1583	0.1071	0.0990	0.1441	0.1351	0.000

During the first agronomic year, *T. fallax* displayed the highest value for days to flowering (183.1 days), while *T. pubescens* (165 days) showed the lowest one (Table 3). The highest value of days to 100% flowering belonged to *T. carmanicus* and *T. vulgaris*, while the lowest was related to *T. kotschyanus* (Table 3). Leaf length varied from 5.3 in *T. vulgaris* to 16.2 mm in *T. trautvetteri* (Table 3). *T. pubescens*

revealed the highest canopy extension, while *T. fallax* had the latest canopy extension (Table 3). Plant height ranged considerably from 11.9 (*T. transcaspicus*) to 21.3 cm (*T. vulgaris*) (Table 3). *T. vulgaris* and *T. carmanicus* possessed the highest and lowest fresh weight, respectively. Dry weight varied from 9.2 (*T. carmanicus*) to 28.9 (*T. vulgaris*) (g/plant) (Table 3). The highest and the lowest essential oil

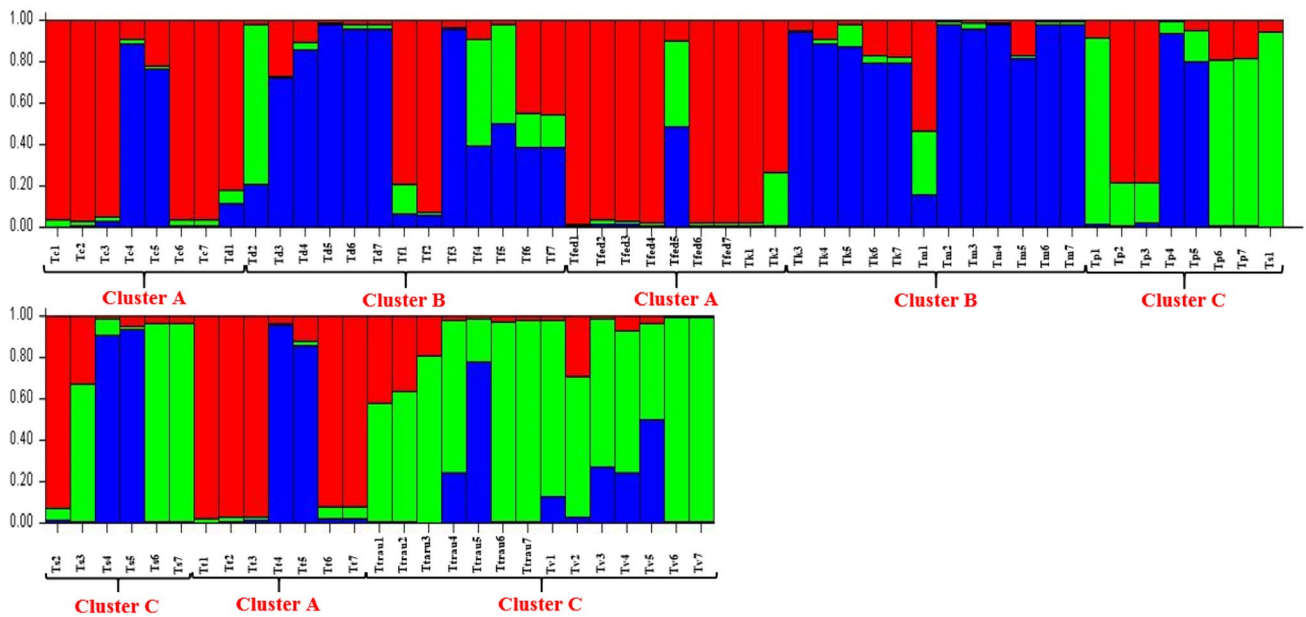
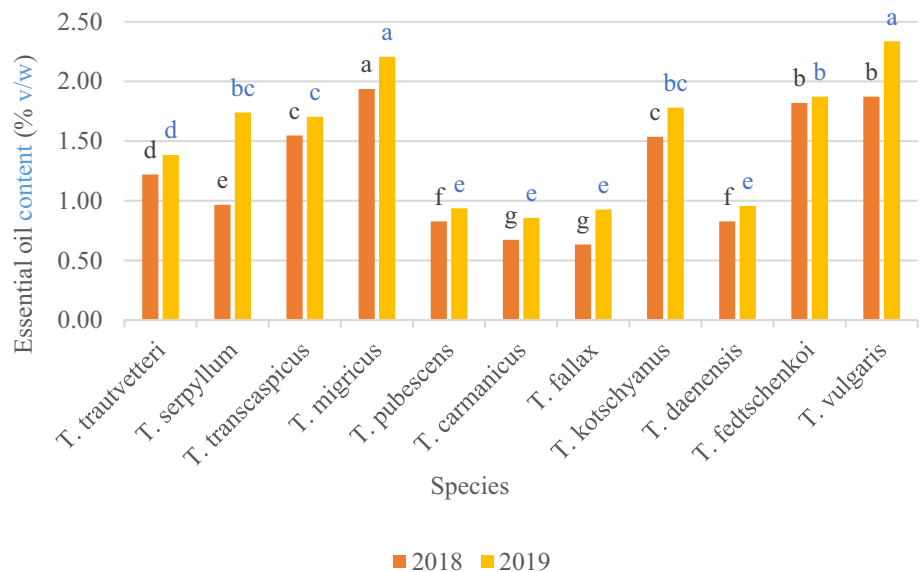


Fig. 3 Genetic structure of 77 *Thymus* genotypes as inferred by STRUCTURE software with nine ISSR markers data set. Single vertical line represents an individual accessions and different colors represent genetic stocks/gene pools. Segments of each vertical line show extent of admixture in an individual (For interpretation of the refer-

ences to colour in the text, the reader is referred to the web version of this article). Tm: *T. migricus*; Tf: *T. fallax*; Tc: *T. carmanicus*; Tk: *T. kotschyanus*; Tp: *T. pubescens*; Td: *T. daenensis*; Trau: *T. trautvetteri*; Tt: *T. transcaspicus*; Tfed: *T. fedtschenkoi*; Tv: *T. vulgaris*; Ts: *T. serpyllum*

Fig. 4 Essential oil content of studied *Thymus* species



content were recorded for *T. migricus* (1.93%) and *T. carmanicus* (0.67%), respectively, with an average value of 1.25% (Table 3).

In the second agronomic year, inflorescence diameter ranged from 12.5 to 23.6 mm, with a means of 19.59 mm (Table 3). The highest number of floret in inflorescence was obtained in *T. serpyllum*, while the lowest one was measured in *T. migricus* (Table 3). Stem diameter varied from 0.65

to 1.32 mm, with an average value of 0.91 mm (Table 3). *T. serpyllum* was the shortest (13.4 cm), while *T. vulgaris* (21.1 cm) was the tallest species (Table 3). Fresh weight and dry weight ranged from 43.4 to 123.8 g/plant, and 18–92 g/plant, with average values of 74.50 and 40.36 g/plant (Table 3). *T. pubescens* had the earliest flowering time, while the latest time was recorded for *T. fallax* (181.3 days). Inflorescence length varied from 16.6 mm (*T. migricus*) to

Table 7 Analysis of variance for measured traits in *Thymus* species

Traits	Means square														
	df	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	X12	X13	X14
Source of variation															
Replication	2	0.8 ns	0.36 ns	0.72 ns	0.19 ns	0.005 ns	12.4*	2.07*	0.88 ns	0.03*	4.63 ns	19.14*	3.54 ns	0.97 ns	0.004 ns
Species	10	171.9**	197.09**	41.05**	39.91**	3.95**	67.6**	430.07**	142.3**	0.12**	45.4**	2273.9**	2561.39**	559.34**	1.55**
Species* Replication	20	1.4 ns	0.58 ns	1.05 ns	0.77 ns	0.13 ns	2.2 ns	1.19 ns	2.09 ns	0.007 ns	4.64 ns	4.84 ns	5.23 ns	2.57 ns	0.004 ns
Year	1	0.6 ns	1.51 ns	4.74*	49.03**	1.83*	19.1*	4.27*	47.05**	0.0006 ns	5.91 ns	79.97**	15,880.06**	7606.2**	1.099**
Year* Species	10	1.9 ns	1.39*	1.36 ns	3.77*	1.27**	2.8 ns	0.27 ns	11.09*	0.08**	4.42 ns	19.57*	70.25*	127.47**	0.062**
Year* Replication	2	0.6 ns	0.33 ns	1.01 ns	0.12 ns	0.004 ns	27.1*	0.74 ns	7.53*	0.03*	5.07 ns	4.04 ns	2.48 ns	0.79 ns	0.003 ns
Error	20	1.19	0.52	1.15	0.76	0.15	3.30	0.78	1.84	0.007	5.27	3.36	11.69	3.51	0.004
CV%	-	0.62	0.39	0.53	8.19	9.26	9.51	2.40	5.75	9.14	13.80	2.64	5.79	7.14	4.72

X1: Days to flowering, X2: days to 50% flowering, X3: days to 100% flowering, X4: leaf length (mm), X5: leaf width (mm), X6: inflorescence diameter (mm), X7: canopy extension(cm), X8: inflorescence length (mm), X9: stem diameter (mm), X10: Plant height (cm), X11: number of floret in inflorescence, X12: fresh weight (g/plant), X13: dry weight (g/plant), X14: essential oil content (% v/w)

ns: non-significant

*Significant at 5% level of probability

**Significant at 1% level of probability

31.1 mm (*T. carmanicus*) (Table 3). *T. pubescens* and *T. fallax* exhibited the highest and the lowest canopy extension, respectively. Leaf length ranged from 5.8 (*T. vulgaris*) to 12.4 mm (*T. serpyllum*) with an average value of 8.93 (Table 3). *T. vulgaris* had the highest essential oil content (2.33%), while *T. fallax* recorded the least (0.93%) essential oil content. Finally, the highest days to 100% flowering was observed in *T. carmanicus* (204.1 days), while the lowest belonged to *T. kotschyanus* (195.7 days).

PCA and cluster analysis for morphological traits

The clustering patterns of the *Thymus* species according to their morphological characters gained from Ward's method for both agronomic years are presented in Fig. 5. Results of the two-year data classified the species into three clusters. The first cluster (Cluster I) was comprised of *T. trautvetteri*, *T. kotschyanus*, *T. transcaspicus*, and *T. fedtschenkoi*. Cluster II included *T. fallax*, *T. carmanicus*, *T. daenensis*, *T. pubescens*, *T. serpyllum*. *T. migricus* and *T. vulgaris* with the highest essential oil content in comparison with other species in both agronomic years were grouped in cluster II, and overall, they had relatively low essential oil content than other species.

PCA confirmed the results of clustering in most cases (Fig. 6). The first three PCs showed the highest variation. PC1 explained 30.82% of the total variation and showed positive correlations, with the number of floret in inflorescence, and leaf width, inflorescence diameter, inflorescence length though with negative ones with plant height, fresh weight, dry weight, and essential oil content. PC2 explained 23.49% of the total variance and had positive correlations with days to flowering, days to 50% flowering, days to 100% flowering, and leaf width but negative correlations with canopy extension, number of floret in inflorescence, dry weight, and essential oil content. PC3 revealed leaf width, stem diameter, and essential oil content as positive components. Finally, canopy extension and plant height as negative compounds that account for 10.41% of the total variance.

The introduction of new species with high essential oil content and elite morphological traits is one of the major goals in the breeding of medicinal plants. Selection of high essential oil content, high genetic distance as well as similar ploidy level is a prerequisite for interspecific hybridization in *Thymus* species. The studied *T. vulgaris* and *T. carmanicus* were tetraploid (data are not shown). Moreover, these species were extreme in some critical breeding traits such as essential oil content and plant height, fresh weight, and dry weight, and consequently, they can be suggested for further breeding strategies in thyme. High variation among the studied species could be due to processes such as gene mutation, number of effective individuals, recombination of genes and chromosomes, and the genetic structure of species. High admixture in the species

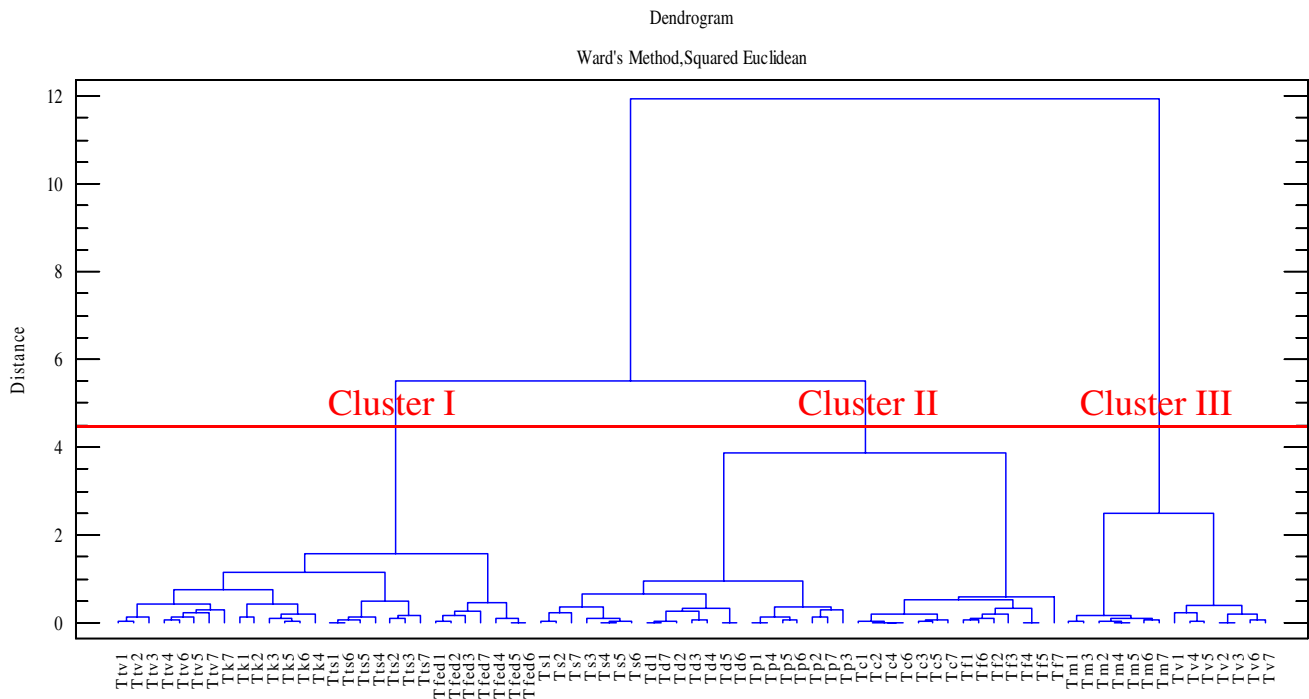
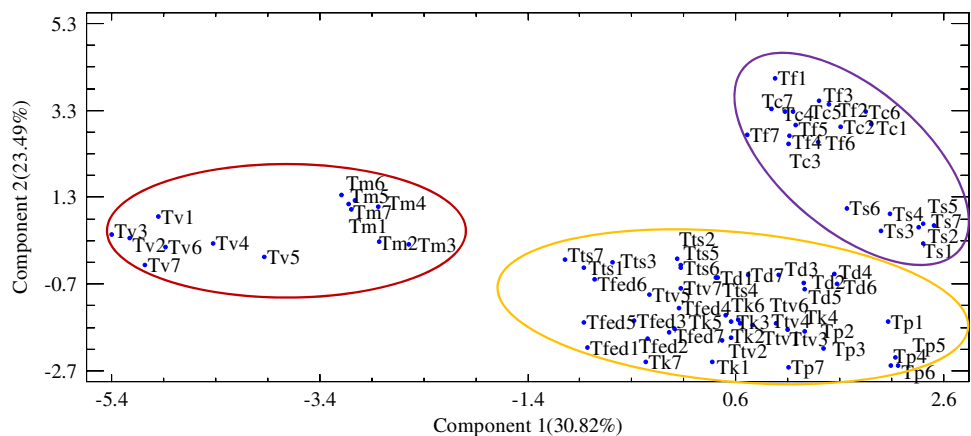


Fig. 5 Cluster of 77 studied *Thymus* genotypes based on morphological traits. Td: *T. daenensis*, Tm: *T. migricus*, Tv: *T. vulgaris*, Tf: *T. fallax*, Ttv: *T. trautvetteri*, Ts: *T. serpyllum*, Tts: *T. transcaspicus*, Tp: *T. pubescens*, T. fed: *T. fedtschenkoi*, Tk: *T. kotschyanus*, Tc: *T. carmanicus*

Fig. 6 PCA of morphological traits in 11 studied *Thymus* species. Td: *T. daenensis*, Tm: *T. migricus*, Tv: *T. vulgaris*, Tf: *T. fallax*, Ttv: *T. trautvetteri*, Ts: *T. serpyllum*, Tts: *T. transcaspicus*, Tp: *T. pubescens*, T. fed: *T. fedtschenkoi*, Tk: *T. kotschyanus*, Tc: *T. carmanicus*



can be interpreted by different factors such as pollination efficiency. In this study, significant morphological variation was observed among the *Thymus* species during two agronomic years. Thus, the results can help the researchers to begin new breeding programs in the studied species. *T. migricus* and *T. pubescens* were diploid (data are not shown). These species are important for future breeding programs in terms of essential oil content and genetic distance. Furthermore, thymol has been considered as the major component of *Thymus* species. In this regard, based on a previous research on chemical components in most of the studied species (Tohidi et al. 2017); it can be suggested that these two selected species viz. *T. migricus* (high

thymol content) and *T. pubescens* (low thymol content) are suitable for further breeding programs in this genus.

Conclusion

In this research, the genetic structure and variation of 11 *Thymus* species were analyzed using ISSR and morphological markers. The results of the study revealed the relatively high efficiency of ISSR markers for distinguishing among the different *Thymus* species from various samples. Furthermore, relatively high gene flow and differentiation were

observed among the species. The highest genetic distance was obtained between *T. vulgaris* and *T. fedtschenkoi*, and the highest genetic similarity was between *T. serpyllum* and *T. pubescens*. *T. transcaspicus* and *T. trautvetteri* showed the highest and lowest gene diversity, respectively. Based on the results, the high admixture of samples was observed among the species. The results of the morphological analysis indicated significant variations in morphological characteristics, as well as the essential oil content of the studied species. *T. vulgaris* and *T. migricus* were selected as the best species in terms of essential oil content in the two agronomic years. Finally, based on the genetic distances, essential oil content, and morphological traits among the studied species, *T. vulgaris*, and *T. carmanicus* may be suggested for further breeding programs after complimentary assessments.

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Compliance with ethical standards

Ethical statement The manuscript has not been previously published, is not currently submitted for review to any other journal, and will not be submitted elsewhere before one decision is made.

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