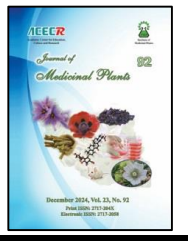
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Research Article

Interspecific hybridization in *Thymus* species: molecular and morphological characterization

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ABSTRACT

Background: Thyme is a famous medicinal plant from the Lamiaceae family, which is grown in the wild throughout Iran and has one of the world's top ten essential oils. **Objective:** In this study, four species of the *Thymus* genus including *T. lancifolius*, *T. daenensis*, *T. vulgaris*, *T. kotschyanus* were crossed. Out of about 2,000 crosses, 70 seeds were obtained. Molecular and morphological assessments were done on hybrid seeds. Parents and hybrids were compared in terms of some morphological traits. **Methods:** DNA from leaf samples of both parents and hybrids were extracted. SSR markers including 23 pairs of primers and ISSR markers with 20 primers were used for hybrid identification. Out of 23 primers used for SSR analysis, five primers yielded scorable amplified products for hybrids in their respective parental lines. **Results:** Stem length in hybrids increased, and internode length decreased. Primer LT12, with 66.66% identification, was introduced as the best primer for hybrid identification. Also, out of 19 ISSR primers, five primers confirmed hybrids and their parents. Primer ISCS43 with 100% ability to identify hybrids was introduced as the best primer. **Conclusion:** In general, all hybrids characterized by morphological characters were supported by the ISSR and SSR data. These results indicate that ISSR and SSR data will be valuable tools to verify hybrids.

1. Introduction

The genus of *Thymus* (Lamiaceae) consists of more than 250 species growing all over the world. It is an aromatic perennial plant that originated from the Mediterranean region. Thyme *Thymus* species is known as a medicinal plant due to its biological and pharmacological properties, including antioxidant, antimicrobial, and antifungal effects. The aerial parts are used as a tonic and herbal tea, as well as an anti-inflammatory, anti-parasitic, anti-tussive, and carminative. The essential oil of thyme is used for treating colds, diarrhea, digestive, and respiratory system disorders [1]. Eighteen species of *Thymus* are represented in Iranian flora and among these, four species (*T. persicus*, *T. daenensis*, *T. lancifolius*, and *T. marandensis*) are native to Iran. *T. daenensis* Celak is a

Abbreviations: SSR, Simple sequence repeat; ISSR, Inter simple sequence Repeat

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perennial dwarf bush in semi-arid regions of Iran. *T. daenensis* is known from other species with slender leaves [2]. *T. lancifolius* grows from 10 to 30 cm in height and is found in mountain areas at ranging from 1100 to 3200 m [3]. *T. kotschyanus* Boiss. & Hohen species is a perennial plant with a height of about 20 cm, small wooden shoots, dark, green, thin leaves. This species with white flowers grows in mountainous regions, and although it is dispersed almost worldwide, especially in the Mediterranean region. In Iran, the most considerable dispersion belongs to this species [4]. Thyme (*T. vulgaris* L.), is a plant with a high economic value in Europe, North America, and, North Africa. Now, this plant is cultivated in significant areas in Iran [5].

High levels of natural hybridization within and between *Thymus* species have been reported. This is probably because of the absence of incompatibility and an existing dimorphic breeding system, in which populations include female and hermaphroditic individuals [6, 7]. Hybridization has played a critical role in the evolution of many plants. It can create a hybrid with faster growth, greater size, more biomass, and greater reproductive progenies than its parents [8]. Morphological characters have limitations for detecting hybridization, and more data are needed to prove hybrid origin. Molecular markers potentially provide unlimited markers at the DNA level that can be applied for discrimination of individuals at different taxon levels. Also, DNA markers have been widely used to evaluate genetic diversity and to confirm hybrids and their parents [9, 10]. Among molecular markers, SSR and ISSR markers are preferred due to their high reproducibility and repeatability and ability to discriminate heterozygous from homozygous individuals. These markers are helpful in assessing hybrids and their parent's genetic identities [11,12].

Radosavljević *et al*. observed the highest level of genetic diversity and allelic richness in hybrids between *Salvia officinalis* L. and *S. fruticosa* Mill. using microsatellite markers [13]. Also, these hybrids mostly had higher values in different genetic parameters than their parents. Investigating genetic diversity and species relationship in interspecific crosses among *Mentha spicata* and *M. longifolia* using ISSR markers confirmed genomic similarity in the crosses between the parents [14]. Comparing hybrids of Origanum with their parents-approved ITS marker efficiency in defining their molecular relationship [15,16]. Some other molecular markers like RFLP and AFLP have been also used for discrimination hybrids e.g. mint hybrids [17, 18].

Microsatellites were used for genetic research in *Thymus* spp. These markers help to determine the genetic structure and variation within and between the species. Genetic diversity between and among populations in *Artemisia capillaries* [19], *Salvia miltiorrhiza* [20], and *Lamiophlomis rotate* [21] were assayed using ISSRs. In the genus of *Thymus*, ISSRs and RAPDs have been applied in genetic characterization [22, 23], while AFLPs have been used for checking the genetic diversity, relationship, and population structure [7] Hadian et al. used ISSR markers to assess genetic variability among wild individuals of *Satureja rechingeri* [24]. Morphological assessment of five populations of *T. burrescens* unraveled significant changes in leaf length and width, and the number of leaf secretion glands that could be considered in breeding programs [25]. High genetic diversity was observed among *T. loscosii* populations, which was due to the polyploidy of this species. It led to an increase in fertility and adaptability. Generally, polyploidy

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along with heterozygosity, genetic, and biochemical diversity has led to the success of thyme in its natural habitats [26, 27].

Interspecific hybridization between *Thymus* species due to the existence of male sterility in some species could help mint breeders to produce hybrid seeds without emasculation. Also, hybrid identification is an essential operation in the seed trade. For the precise assessment of hybridity or the genetic identity of seeds, some informative markers are needed. The objectives of this study were: 1) investigating controlled interspecific hybridization in some *Thymus* species, 2) using molecular markers for identification and distinguishing hybrids from their parents, and 3) evaluating the variability of *Thymus* species by morphological characters.

2. Material and methods

2.1. Plant Material

Field experiments were carried out in the spring and summer of 2020-2022 at the Research Station of the Department of Horticultural Sciences of the University of Tehran, located in Mohammad Shahr Karaj. The place of cultivation of plants in Karaj city is at latitude 36 degrees 19 minutes north and, longitude 59 degrees east 38 minutes north, and altitude 1320 meters above sea level. Seeds of four species was provided from the Research Institute of Forests and Rangelands, Iran. First studied Seeds of four species was supplied from the Research Institute of Forests and Rangelands, Iran. Planting the seeds was made based on traditional agronomic disciplines. Then two-year-old plants, based on morphological traits and growth characteristics of plants with superior characteristics, they were selected and were considered to do crosses. The herbarium of the Faculty of Agriculture, University of Tehran, also confirmed the authentication of the studied species based on botanical characteristics (Table 1).

2.2. Hybridization

Crossing was done in two procedures; first, male sterile plants were identified. The pots contained male sterile and hermaphrodite plants placed next to each other in an isolated area to facilitate natural open pollination by insects. Then, hermaphrodite plants were emasculated. For hand pollination, pollens were collected from each species and were poured on the stigmas of the emasculated flowers. The pollinated flowers were enclosed in paper bags to prevent pollen contamination. The seeds produced by the above methods were collected and then the hybrid seeds produced together with the mother seed (to compare the morphological growth characteristics under the same conditions) were cultivated in a seed tray containing a mixture of cocopeat and perlite. The continuation of the trays in the germinator was maintained at a temperature of 23 degrees and 24-hour lighting, and irrigation was done with high precision with a sprinkler. After the seeds germinated, the trays were kept in the greenhouse during the day to use the sunlight (considering that the light requirement of the plant was not met by germination). After the seedlings reached a size of about 10 cm, they were placed in the greenhouse for a week to become uniform with the air of the greenhouse, then they were transferred to larger pots and kept in the greenhouse for about a week. And then it was taken to the open space with a pot for one month and finally it was transferred to the farm after three months.

2.3. Morphological Assay

Both parents and hybrids were assayed for morphological traits in the flowering stage. The traits included stem length, internode length, number of branches, the content of chlorophyll (a, b, and SPAD reading), and carotenoids.

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2.4. Measurement of leaf chlorophyll and carotenoids

0.5 grams of leaves were poured into a Chinese mortar, then crushed well using liquid nitrogen. 20 ml of 80% acetone was added to the sample, and then, placed in a centrifuge model Sigma 3-16K (Germany) at a speed of 6000 rpm for 10 minutes. Transfer the upper separated extract from the centrifuge to the falcon tube and pour some of the sample inside the tube into the spectrophotometer cuvette of the Lambda EZ 201 model and then separately at wavelengths of 663 nm for chlorophyll a, 645 nm for chlorophyll b and 470 nm for carotenoids. The absorbance value was read by spectrophotometer. Finally, using the following formulas, the amount of chlorophyll a, b, and carotenoids was obtained in terms of milligrams per gram of fresh weight of the sample [28].

Chl a value = [(11/23 * A663) – (2/04 * A645)]

Chl b value = [(20/13 * A645) – (4/19 * A663)]

Carotenoids amount = [1000 (A470) – 1/90 (A663) – 14/63 (A645) / 214]

2.5. DNA Extraction

Young leaves, 0.2 g, from parents and hybrid plants were sampled for genomic DNA extraction. The DNA extraction protocol was based on a modified CTAB method [29]. The quality and quantity of the isolated DNA were measured using both a nanodrop spectrophotometer and electrophoresis. The DNA was diluted to an operating concentration of 10 ng/µl.

2.6. ISSR-PCR Amplification

Nineteen ISSR primers were applied for PCR analysis (Table 2). The PCR reaction was performed in a total volume of 15 µl containing 7.5 µl of the Master Mix Red (Ampliqon Co.) including (dNTPs, MgCl2, Taq DNA polymerase), 3µl of distilled water, 2.5µl of total DNA (10 ng/µl), and 2µl of each primer. PCR amplifications were carried out with an initial denaturation for 5 min at 94 °C followed by 35 cycles with 50 s at 94 °C, annealing (at the temperature shown in Table 2) for 1 min, extension at 72 °C for 2.5 min, and finally an extension cycle of 5 min at 72 °C. Amplified fragments were electrophoresed on 1.2% (w/v) agarose gel using 10x TAE buffer at 80 V/cm for 150 min, then photographed using Gel Doc (BioDoc-1t TM System UPV).

2.7. SSR-PCR Amplification

Twenty-three SSR primers were used for PCR analysis (Table 3). The PCR reaction was carried out in 15µL reaction volumes containing 3.5µl genomic DNA sample, 1 µl of each microsatellite primer pair, 7.5 µl of the Master Mix Red (Ampliqon Co.) including (dNTPs, MgCl2, Taq DNA polymerase), 3µl of distilled water. For PCR amplification, samples were initially denatured at 94 °C for 3 min, 30 cycles with denaturation at 94 °C for 60 s, annealing (at the temperature shown in Table 3) for 60 s, extension reactions at 72 °C for 1 min, and finally an extension cycle of 10 min at 72 °C. PCR products were subjected to 2% agarose gel using 10x TAE buffer at 80 V/cm for 180 min and then observed using Gel Doc (BioDoc-1t TM System UPV). Finally, the five primers that showed a difference were selected to continue the experiment.

Table 1. Studied *Thymus* species and their Herbarium codes from the Faculty of Agriculture, University of Tehran

Species	Herbarium Code	Seed Bank Code
Thymus vulgaris	KAR 007067	33
Thymus daenensis	KAR 007068	1110
Thymus lancifolius	KAR 007069	7505
Thymus kotschyanus	KAR 007070	14216

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Table 2. List of ISSR primers used in the molecular study of *Thymus*

No.	Primer	Sequence (5´-3´)	Ta (°C)
1	ISCS7	TCTCTCTCTCTCTCTCTCC	60.1
2	ISCS10	ACACACACACACACACC	52
3	ISCS12	TTGTTGTTGTTGTTGTTGC	52.2
4	ISCS17	DBDBCACCACCACCACCAC	64.9
5	ISCS30	ACACACACACACACACYT	54.5
6	ISCS43	GAAGAAGAAGAAGAAGAA	54.3
7	ISCS47	CACACACACACACACARG	52
8	ISCS51	CACACACACACACACART	49.3
9	ISCS58	GAGAGAGAGAGAGAGAYC	58.2
10	ISCS64	GAGAGAGAGAGAGAGAC	49.3
11	ISCS65	GAGAGAGAGAGAGAGAA	52.5
12	ISCS69	CACACACACACACACAA	52.7
13	ISCS70	CACACACACACACACAG	58.2
14	ISCS87	AGAGAGAGAGAGAGAGYA	56.4
15	ISCS18	DBDBCCACCACCACCACCA	54.7
16	ISCS32	ACACACACACACACACYG	54.5
17	ISCS50	CACACACACACACACARC	59
18	ISCS73	GTGTGTGTGTGTGTGTT	49
19	ISCS77	T ACACACACACACACACT	49

Table 3. List of SSR primers used in the molecular study of *Thymus* [17]

No.	Primer		Sequence	Tm (C°)
1	LT01	F	AGTATTTGTGCCGAGGGTTG	55.8
		R	ACAGGAAAGGGAGAGGGAGA	
2	LT02	F	GAGGAGGCAGGCAGAAGG	55.8
		R	TGTTAGGTGTCATCGGCTCAC	
3	LT03	F	CAAATCCAGCCCCAAATCA	55.7
		R	TTCCTCTTTCAGGTTCCATCAG	
4	LT04	F	CACGAGGCACACAAGCAC	55.8
		R	TTGAACAGAACCCATCTCCTTC	
5	LT05	F	GGAGCTGGAGAAAGAGAACA	57.3
		R	TGCAAGAAAAGCAAGCTACA	
6	LT06	F	CGCAATCCTCCCTCATAAAT	56.3
		R	GACCTTCTTCACGCTGGTG	
7	LT07	F	GGGGCTGTGGTGTTTCAT	55.8
		R	TTTCTCATCTGGGCTATCAAGA	
8	LT08	F	AAGCGTGAGAAGAGCAGCAC	55.8
		R	CCACCACAACAGGAGAGACC	
9	LT09	F	GAGCATCTCGAAGCGAAAGT	60.4
		R	CGGCATAAGCAACCTCTTTT	
10	LT10	F	AAGTTTGGGACGGAGTTAGT	58.7
		R	CTGAAGCACCTTTTGATTTG	
11	LT11	F	GATCCACCTCAATTTCAAGA	56.3
		R	TGTGCCTCCTTCTATTCATC	
12	LT12	F	GTAGGGATTGTCGCCGTTG	60.4
		R	CCTCCGCCATTTTCATTTCT	
13	LT13	F	GTGAAGTAACGCTTCCATGAGAG	56.3
		R	GAGTACAAAAGAGCTACAGATG	

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Table 3. List of SSR primers used in the molecular study of *Thymus* [17] (Continued)

No.	Primer	Sequence	Tm (C°)	No.
14	LT14	F	TTTGCGCAGATCTCAAGTGC	55.8
		R	AAGCGGTGACTGACGGAGAC	
15	LT15	F	GGATGATGCTGAGTTGGTGATAAG	60.8
		R	CCTGACACGCCACAAAAGTG	
16	LT16	F	GGGCATTAAAGCTAAGGAGCG	60.4
		R	CAGCCGATCACCTGTCCTTC	
17	LT17	F	CACACGCACTGGTGAGGTG	57.9
		R	TTCCCGCAGATCTCCAGAAC	
18	LT18	F	CCAAGAATGCCGATGTCAAAG	55.9
		R	CTCCACCTCCTAGTTTCTTGGC	
19	LT19	F	GAAAAGCGAAGCCGTTGAAG	53.2
		R	TGCTGAGCCTTTGCCCTTAG	
20	LT20	F	AGCCAAACTCGCTGCTTCTG	52.7
		R	GGTAAAAAGGGTAATAGACGTGG	
21	LT21	F	AAGATCGAAGGCATCGATCG	57.9
		R	GGTGAAAAATGAATACAGTGGGC	
22	LT22	F	CATCAAGTTCAATAATGCTGTG	56.6
		R	CAGATAGTTGCATCGAGGTTAG	
23	LT23	F	TCCCATCATTTTCCTCCGTC	54.7
		R	CCCCACTACAGCAGAAACCG	

2.8. Statistical Analysis

Morphological data were subjected to one-way analysis of variance (ANOVA) by the software of SPSS v.22. Means were compared using LSD test with a significance threshold of 0.01. Comparison between hybrids and parents was done by orthogonal contrast analysis. Heterosis and high parent heterosis estimation were done by the following formulas:

$$H_P = \frac{\overline{X}_F - \overline{X}_P}{\overline{X}_P} \times 100$$

$$H_{HP} = \frac{\overline{X}_F - \overline{X}_{HP}}{\overline{X}_{HP}} \times 100$$

Where H_P and H_{HP} were heterosis and high parent heterosis values, respectively. $\overline{X}_F$, $\overline{X}_{HP}$ and $\overline{X}_P$ were the mean values of hybrids, parents, and high parents, respectively.

The amplified bands were scored as 1 for the presence and 0 for the absence of a band. Then for identification of the hybrids and their parents, the SSR and ISSR profiles of hybrids were compared with their respective parents. The monomorphic and polymorphic bands were taken down. The monomorphic bands are those that exist in both the parents and may or may not be revealed in the hybrids. Polymorphic bands are those that are in either of the parents and are also expressed in the hybrids [30].

3. Results

3.1. Hybridization

In this study, about 2000 crosses were done among the four *Thymus* species, and out of six possible interspecific crosses, three crosses were led to producing live and healthy seeds. From all successful crosses, only 70 seeds were produced (Table 4). It needs to be noted that both forward and reverse crosses were made by the parent species.

3.2. Morphological Assay

Morphological data from the hybrids and the parents showed significant differences for the investigated traits. The results of mean comparisons related to parents and their hybrids were done based on the LSD test for the phenotypic attributes (Table 5). The highest stem length belonged to *T. vulgaris* and the hybrid of *T. vulgaris* × *T. kotschyanus*. The stem length of

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the hybrids may be affected by *T. vulgaris*, as a parent. The species of *T. kotschyanus* had the lowest stem length, while there was no significant difference between the three other parental species. Between parental species, *T. kotschyanus* and *T. lancifolius* had higher internode length rather than other parents. The hybrid of *T. kotschyanus* × *T. vulgaris* had the highest internode length among the hybrids. The lowest internode lengths belonged to the hybrid of *T. kotschyanus*× *T. lancifolius*, which showed significant differences with other hybrids and parents. The hybrids of *T. vulgaris*× *T. daenensis* and *T. kotschyanus* ×*T. lancifolius* revealed the maximum and the minimum number of lateral branches, respectively.

T. daenensis showed the highest content of chlorophyll (a,b) and carotenoids. The content of chlorophyll (a, b) and carotenoids in all hybrids was intermediate. However, in the hybrid of *T. lancifolius*× *T. vulgaris*, chlorophyll b was higher than in parents. *T. vulgaris* × *T. kotschyanus* hybrid showed an increase in the content of chlorophyll (a, b) and carotenoids rather than in its parents. In general, SPAD reading was higher in hybrids except for *T. kotschyanus*. The color of the epiderm was various from light green to dark green when visually assayed.

Table 4. All successful crosses between *Thymus* species

Cross	No. of seeds	Cross	No. of seeds
T.lancifolius × *T. kotschyanus*	25	*T. vulgaris*× *T. daenensis*	38
T. daenensis × *T. kotschyanus*	-	*T. lancifolius* × *T .daenensis*	-
T. vulgaris× *T. kotschyanus*	8	*T. lancifolius* ×*T. vulgaris*	-

Table 5. Mean comparisons in the studied *Thymus* genotypes for morphological traits

Hybrids and parents	Stem length (mm)	Number of lateral branches	Internode length (mm)	Chlorophyll a (mg/ml)	Chlorophyll b (mg/ml)	Carotenoids (mg/ml)	SPAD
T. vulgaris × *T. daenensis*	16.02^{a}	13.75^{a}	0.96^{b}	12.11^{e}	5.25^{bc}	5.35^{d}	40.29^{b}
T. lancifolius × *T. kotschyanus*	10.08^{a}	8.04^{c}	0.64^{c}	15.30^{bc}	6.11^{bc}	6.59^{bc}	49.19^{a}
T. vulgaris× *T. kotschyanus*	17.87^{a}	13.75^{ab}	1.25^{ab}	17.46^{ab}	7.05^{b}	7.78^{b}	36.38^{bc}
T. vulgaris	20.50^{a}	10.50^{ab}	1.45^{a}	7.68^{de}	2.70^{c}	3.57^{cd}	7^{e}
T. lancifolius	9^{b}	12.5^{abc}	1.45^{a}	9.01^{cde}	2.99^{c}	7.11^{bcd}	17.40^{de}
T. daenensis	9.5^{b}	8.5^{bc}	1.25^{ab}	24.66^{a}	19.99^{a}	12.8^{a}	20.30^{ce}
T. kotschyanus	8.5^{b}	10.5^{abc}	1.10^{b}	16.38^{abc}	$5.78b^{c}$	4.04^{cd}	37.8^{abc}

Different letters represent statistically different values for $P \leq 0.01$ (LSD test)

3.3. Heterosis

According to the contrasts of hybrids versus parents, significant differences were found for the studied traits (Table 6). The hybrid of *T. vulgaris*× *T. daenensis* and its parents had a significant difference for all the studied traits. These differences are also expressed as positive heterosis for the number of lateral branches, chlorophyll (a,b), carotenoids, and SPAD, and negative heterosis for the internode length. Significant contrast of the hybrid of *T. vulgaris*× *T. kotschyanus* versus its parents for the measured traits, showed positive heterosis for the number of lateral branches, chlorophyll (a,b), and carotenoids. Positive heterosis in *T. lancifolius* × *T. kotschyanus* hybrid for stem length, chlorophyll (a,b), carotenoids, and SPAD, and

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negative heterosis for the number of lateral branches and the internode length were found.

Better parents' heterosis ranged from 135.75 to -58.33% for *T. vulgaris* × *T. kotschyanus*. The highest heterosis for the number of lateral branches, chlorophyll (a,b), and carotenoids was observed in *T. vulgaris* × *T. kotschyanus* by 30.95, 127.07,135.75 and 92.53% respectively. This hybrid showed the lowest SPAD by -3.75%. Better parents' heterosis for *T. lancifolius* × *T. kotschyanus* ranged from 54.34 to -55.32. This hybrid showed the maximum heterosis for stem length by 12.07% and the minimum heterosis for the number of lateral branches, chlorophyll (a,b), and internode length by -35.65, -6.59,5.76 and -55.32, respectively.

T. vulgaris× *T. daenensis* hybrid revealed the highest SPAD percentage and the lowest percentage for stem length and carotenoids.

3.4. Molecular Assessment

3.4.1. SSR-PCR Amplification

Markers that revealed bands common in hybrid and both of its parents were suitable markers to confirm the hybrid is of its respective parents. Out of 23 SSR primers, eight primers were suitable to confirm hybridization. The best one was primer number 12, with 66.66% identification ability. Out of 20 ISSR markers, 6 primers were polymorphic among the hybrids and their parents. The primer number 43 with 100% confirmation ability was the best compared with others (Table 6).

The SSR primers of 7 and 12 could validate the hybridization of *T. kotschyanus* and *T. vulgaris* with 50 and 66.66% confirmation, respectively. The SSR primers of 12 and 22 were efficiently confirmed *T. kotschyanus*× *T. lancifolius* hybrid with 11.79 and 11.72% hybrid, respectively. In the hybrid of *T. vulgaris*× *T.daenensis* hybrid, the primers of 7, 19, 11, and 12 showed 11.58, 26.99, 50, and 15.38% confirmation ability.

3.4.2. ISSR-PCR Amplification

In the ISSR marker, primers numbers 10, 43, and 47 were suitable for hybrid confirmation of *T. kotschyanus*× *T. lancifolius* with 59.09, 100, and 27.27% confirmation ability respectively. *T. vulgaris*× *T. daenensis* hybrid was confirmed with primers number 47, 51, and 69 with 16.66, 27.77, and 50% confirmation ability, respectively (Table 7, Fig. 1, 2).

Table 6. Contrasts of hybrid vs. its parents and heterosis in *Thymus* crosses

Traits	Contrasts	Means	Std. Error	T	Heterosis	High parents heterosis	
Stem length	Parents vs. Da × Vul	2.05	1.09	0.79^{ns}	10.53	Da	-
						Vul	-21.81
	Parents vs. La × Ko	2.67	1.55	1.71^{ns}	15.27	La	-
						Ko	12.07
	Parents vs. Ko × Vul	6.75	2.29	$2.93^{\times}$	23.27	Vul	-
						Ko	-12.8
Number of lateral branches	Parents vs. Da × Vul	8.51	1.52	$5.59^{\times\times}$	31.01	Da	-
						Vul	31.01
	Parents vs. La × Ko	-6.91	1.23	$-5.57^{\times\times}$	-30.05	La	-
						Ko	-35.65
	Parents vs. Ko × Vul	6.5	1.61	$4.02^{\times\times}$	30.95	Vul	30.95
						Ko	30.95

ns: non-significant ×: significant at 0.05 probability level ××: significant at 0.01 probability level
Da: daenensis Vul: vulgaris La: lancifolius Ko: kotschyanus

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Table 6. Contrasts of hybrid vs. its parents and heterosis in *Thymus* crosses (Continued)

Traits	Contrasts	Means	Std. Error	T	Heterosis	High parents heterosis	
Internode length	Parents vs. Da×Vul	-0.41	0.15	-2.67$^{\times}$	-24.11	Da	-22.59
						Vul	-
	Parents vs. La×Ko	-1.6	0.11	-14.04$^{\times\times}$	-55.32	La	-55.32
						Ko	-55.32
	Parents vs. Ko×Vul	-0.5	0.2	-0.23^{ns}	-1.96	Vul	-13.79
						Ko	-
Chlorophyll a	Parents vs. Da×Vul	-8.13	1.71	-4.$^{74\times\times}$	45.03	Da	-50.89
						Vul	-
	Parents vs. La×Ko	5.2	2.12	2.44$^{\times}$	20.5	La	-
						Ko	-6.59
	Parents vs. Ko×Vul	18.22	2.58	7.05$^{\times\times}$	109.1	Vul	-
						Ko	127.07
Chlorophyll b	Parents vs. Da×Vul	-12.2	0.79	-15.26$^{\times\times}$	84.28	Da	-73.72
						Vul	-
	Parents vs. La×Ko	3.45	1.08	3.17$^{\times\times}$	39.4	La	-
						Ko	5.76
	Parents vs. Ko×Vul	8.4	2.18	3.85$^{\times\times}$	147.39	Vul	135.75
						Ko	-
Carotenoids	Parents vs. Da×Vul	-5.66	0.77	-7.26$^{\times\times}$	40.66	Da	-58.15
						Vul	-
	Parents vs. La×Ko	2.03	0.96	2.10$^{\times}$	18.21	La	-
						Ko	-7.29
	Parents vs. Ko×Vul	7.95	1.51	5.23$^{\times\times}$	104.49	Vul	135.75
						Ko	-
SPAD	Parents vs. Da×Vul	53.27	2.96	17.99$^{\times\times}$	38.68	Da	-
						Vul	98.46
	Parents vs. La×Ko	43.18	5.13	8.40$^{\times\times}$	78.22	La	30.13
						Ko	-
	Parents vs. Ko×Vul	14.66	8.94	1.64^{ns}	25.24	Vul	-3.75
						Ko	-

ns: non-significant ×: significant at 0.05 probability level ××: significant at 0.01 probability level
Da: daenensis Vul: vulgaris La: lancifolius Ko: kotschyanus

Table 7. Hybrid confirmation of SSR and ISSR markers in interspecific hybrids of Thyme

Hybrid	No. SSR marker	Hybrid confirmation %	Hybrid	No. ISSR marker	Hybrid confirmation %
Vul×Ko	7	50	Ko×La	10	59.09
Vul×Ko	12	66.66	Ko×La	43	100
Ko×La	12	11.79	Ko×La	47	27.27
Ko×La	22	11.72	Da×Vul	47	16.66
Da×Vul	7	11.58	Da×Vul	51	27.77
Da×Vul	19	26.99	Da×Vul	69	50
Da×Vul	11	50			
Da×Vul	12	15.38			

Da: daenensis Vul: vulgaris La: lancifolius Ko: kotschyanus

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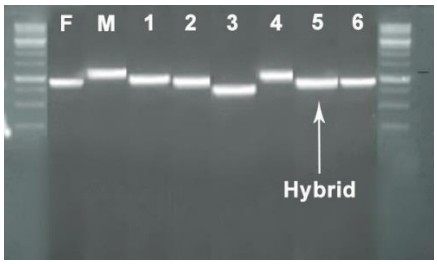


Fig. 1. SSR (LT12) profile of thymus hybrids and their parents; F: Female parent (*T. vulgaris*); M: Male parent (*T. kotschyanus*), Lanes 1-6: hybrid individuals (*T. kotschyanus* × *T. vulgaris*)

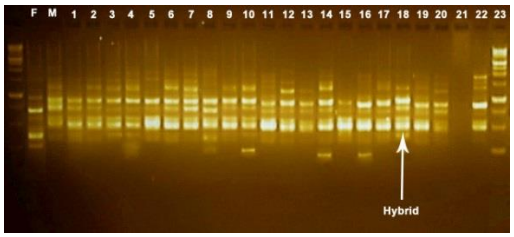


Fig. 2. ISSR (ISCS47) profile of thymus hybrids and their parents; F: Female parent (*T. lancifolius*); M: Male parent (*T. kotschyanus*), Lanes 1-23: hybrid individuals (*T. kotschyanus* ×*T. lancifolius*)

4. Discussion

The Lamiaceae family's genus Thymus shows a remarkable range of ploidy levels, including diploids, tetraploids, and hexaploids, which makes it more difficult to classify species systematically because of the frequent occurrence of hybridization and the morphological similarities between them [31]. The genetic relationships among Thymus species have been elucidated primarily by molecular markers like SSR and ISSR. For example, Karaca *et al*. reported this new set of microsatellite markers for investigating genetic diversity, relationship, population structure, and phylogenetic studies, and natural hybridization in *Thymus* spp. [32, 33]. The major aim of hybrid breeding is the exploitation of heterosis. Heterosis for agronomic traits for hybrids in all crops is a consequence of enhanced growth vigor [34]. Other studies also showed that hybrids' performance was much superior to that of the parents [35, 36, 37, 38]. Due of their significant polymorphism and reproducibility, SSR markers are generally recognized for their effectiveness in hybrid identification. In order to confirm the existence of hybrids through unique genetic profiles, Narendrula and Nkongolo's study, for example, showed that SSR markers could efficiently evaluate genetic variation in hybrid populations of *Picea mariana* and *Picea rubens* [39]. Likewise, Lin et al. demonstrated the value of ISSR markers in identifying interspecific hybridization in plants by using them to detect hybrids between *Phyllostachys* species [40]. SSR and ISSR markers are more reliable in verifying hybrid status because they can target distinct genomic regions, which allows them to capture a wider range of genetic variation than other markers like RAPD [39, 40]. Additionally, the research conducted by Shasany et al. demonstrated the high reproducibility of RAPD markers in detecting intraspecific and interspecific hybrids in Mentha species, supporting the idea that molecular markers can be reliable instruments for hybrid confirmation [41]. The findings indicate that these markers not only confirmed hybrid status but also provided crucial insights into inheritance patterns, which

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are vital for conservation and breeding efforts. To clarify the genetic relationships between *Thymus* species, molecular techniques like DNA barcoding and microsatellite analysis have been used. For example, taxonomically complex groups within the genus have been successfully analyzed using DNA barcoding, which has shown notable genetic differentiation between species such as *Thymus vulgaris* and *Thymus herbabarona* [6,42]. The use of SSR markers for hybrid identification has also been demonstrated in *Brassica juncea* L.[43], *Acacia* spp. [44] and *Phalaris canariensis* L.[44]. Furthermore, a history of natural hybridization has been indicated by microsatellite markers that show high gene flow and genetic differentiation among different *Thymus species* [33]. RadosavljeviĆ et al. also used microsatellite markers to assess of population structure, genetic diversity, geographic differentiation, and ecotypic divergence of *Salvia officinalis* [13]. Furthermore, the confirmation of interspecific hybrids is strengthened when morphological traits and molecular data are combined. As an illustration, Conceição et al. demonstrated how molecular methods like RAPD, where morphological characteristics were also evaluated to validate hybrid identity, were crucial in verifying interspecific hybrids among wild passionflower species [45]. Morphological traits are essential for identifying interspecific hybrids in addition to molecular analyses. Usually, hybrids combine characteristics from both parent species, such as differences in flower morphology, leaf shape, and essential oil composition. According to morphological research, hybrids can have novel traits that neither parent possesses, as well as intermediate traits, making conventional taxonomic classifications more complex [46]. Between parents, the highest chlorophyll content was for *T. kotschyanus*, and the lowest belonged to *T. vulgaris*. There were the same results in *Hyoscyamus niger* [47]; *Platanus acerifolia* [48] showed high ploidy levels resulting in darker green leaves. The primary aim of hybrid breeding is the exploitation of heterosis. Heterosis for agronomic traits for hybrids in all crops is a consequence of enhanced growth vigor [33]. Other studies also showed that hybrids' performance was much superior to the parents [34, 37]. Since the physical characteristics of *Thymus* species can reveal their hybrid origins, morphological characterization is a valuable addition to molecular data. For instance, the composition of essential oils, which differs significantly between species, has been connected to hybridization processes, indicating that interspecific hybrids might have distinct chemotypes [49].

5. Conclusion

In conclusion, the complex genetic landscape of *Thymus* species is highlighted by the confirmation of interspecific hybridization using SSR markers in conjunction with morphological characterization. The use of molecular and morphological tools not only aids in identifying hybrids but also deepens our understanding of the evolutionary processes that shape the diversity of *Thymus* species. In this research, all species have been successfully hybridized as determined morphologically and also by using molecular markers generated by ISSR and SSR. *T. vulgaris*× *T. kotschyanus* hybrid showed the best traits and heterosis in most studied traits, which makes it a good candidate for breeding programs. *T. vulgaris* × *T. daenensis* was the second suggestion for experimental hybridization programs.

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Author contributions

F. A. and M. Sh. R., carried out the laboratory work and composed the manuscript; M. Sh and V. N. supervised the project, reviewed and edited the draft.

Conflict of interest

The authors declare that there is no conflict of interest.

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