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Morphological and Molecular Genetic Assessment Of Some Thymus Species

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This study aimed to determine the morphological and genetically assessment in five Thymus species: *Thymus vulgaris, Origanum vulgare, Thymus argenteus, Thymus citriodorus* and *Origanum syricum*. Morphological assessment for the five Thymus species were obtained based on some vegetative parameters including: Plant height, Number of branches, Leaves fresh & dry weights and Volatile Oil%. Molecular genetic variability was assessed based on (SCoT-PCR) and (ISSR-PCR) analysis. Growth parameters were illustrated among five Thymus species in all growth parameters were had significant differences. The SCoT-PCR analysis using 5 out of 10 primers tested, the results illustrated that SCoT primers produced 24 Polymorphic bands out of 39 amplified bands with polymorphic average 60.52%, also five ISSR primers out of 14 primers tested, which analysis were generated 14 polymorphic bands out of 23 amplified bands with polymorphic average 60.86%. As well as assessment of SCoT and ISSR molecular marker techniques succeeded in generating reproducible and reliable amplified bands and from obvious results, SCoT-PCR analysis was better than ISSR-PCR analysis in molecular genetics. On The other hand, results obtained from an UPGMA dendrograms resulted in two genetically distinct clusters were determined between Thymus species. This results were conducted that SCoT and ISSR analysis could be useful as tools for identifying Thymes species in breeding programs.

Keywords: Growth Parameters; ISSR; Molecular Genetics; SCoT; Thymus Species.

Thymus genus which belongs to the family Lamiaceae, includes several hundreds of species distributed over world[1], where Mediterranean basin is considered the main center of this herbal plant[2].

Traditionally, most of plants discriminated on morphological-basis; however, these methods still difficult to apply for an accurate discrimination and authentication use[3]. Thymus genus is usually used for flavoring agents, herbal tea, and medicine and the aerial parts and volatile constituents of thyme are used as a medicinal material[2,4]. reported that, many species in the genus Thymus were polyploidy and disploidy/aneuploidy and further complicate the determination of species

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boundaries and there were have hybrid vigor in the species, probably due to the absence of incompatibility and the presence of a dimorphic breeding program, in genetic populations comprise female and hermaphroditic individuals.[5] Reported that, Knowledge of genetic diversity within species is necessary for any improvement of cultivars, and biodiversity maintenance and restoration. DNA-based molecular markers, which are not affected by ecological stress have become increasingly important for aromatical biodiversity and genome of aromatical plants[6]. These molecular genetics can also be taxonomically to biodiversity for cytological studies to taxonomically to species and subspecies[7,8,9,10].

The start codon targeted (SCoT) polymorphism is a novel, simple and reliable SCoT based on the translation start codon11. Primers for start codon targeted were designed based on the con-served region surrounding the translation initiation codon, ATG. Using a single 18-mer primer as a forward & reversed primers in polymerase chain reaction,[11] designed thirty-six primers that were used successfully for plant identification and biodiversity analysis in many medicinal plants. Being characterized by lower recombination levels between its markers and the gene/trait.[12] Conducted that, This ISSR-PCR technique is rapid, simple, inexpensive and more reproducible than RAPD amplification of DNA.. ISSR used to study the genetic diversity of plants for examples; Nepeta[13], Thyme[14], Salvia[15], Mentha aquatica L.[16], Satureja[17], Salvia[18], Thymus[19], *Phlomis kurdica* and *Phlomis oppositiflora*[20] and Ocimum[21].

Increasingly, the hybridization approach has taken advantage of developments in molecular genetics in order to karyotype of interest in a way that considerably accelerates natural selection and this genotypes approach consist of choosing desired genotypes on the basis of molecular biology, or having prior knowledge of the genes that determine the morphological traits in a plant[22].

This work was aimed to study molecular genetics and morphological assessment among different species of this plant using morphological and SCoT and ISSR markers, with a view toward conservation of this endangered species.

MATERIALS AND METHODS

Genetic Resources

The seedlings of the five Thymus species were obtained from two countries, Egypt and Kingdome of Saudi Arabia, as a commonly known species showed in Table 1. Thymus plants were collected during two seasons of 2017/2018 and 2018/2019, at Vegetable and Medicinal and Aromatic Plants Research Departments, Dokki, Giza and Biotechnology Research Lab., H.R.I., A.R.C., Egypt. Two months old seedlings of five Thymus Sp. were obtained from the greenhouse of Vegetable and Medicinal and Aromatic Plants Research Dep., Egypt.

Vegetative Parameters

Plant height (cm), Number of branches, Leaves fresh & dry weights and Volatile Oil %.

Molecular Genetic Assessment

DNA Extraction

The DNA extraction of the five species of Thymus was performed as described by[23]. DNA extractions were checked by means of absorbance ratios a260:a280 through a UV-spectrophotometer where Deoxyribo Nucleic Acid is pure with a same ratio from 1.8 - 2.0. Moreover, using electrophoresis in 1% agarose gel with ethidium bromide.

SCoT and ISSR Analysis

Obtaining clear reproducible amplification products require a number of factors were included polymerase chain reaction temperature cycle profile and concentration which were optimized according to24 and 25 respectively, in the PCR reaction using 5 SCoT primers and 5 ISSR primers in molecular genetic analysis for the five Thymus species. ISSR primers procured from Bio Basic Company Canada. On the other hand, SCoT primers sequence were designed and derived from the investigations studies by[26] and[27. 11] and procured from Biobasic Company.

inter-simple sequence repeat and start codon targeted assays were performed as described by[24,25 and 28].

Gel Electrophoresis

PCR products were running throw mini agarose unit at 100 V for one 30min. and 1.5 % agarose gel using 100bp Ladder DNA marker to study the molecular variation between the Thymus species.

Statistically Analysis

RCBD was adopted for the present study data were Statistical analysis by the standard methods according to[29]. The new Least Significant Difference (LSD) test was used for comparison between means. The bands of DNA generated by each primer were counted and their molecular sizes were compared with those of the ISSR and SCoT assays. The bands scored from DNA profiles generated by each primer were pooled together. Then the presence or absence for each band of DNA was treated as a binary character in a data matrix (coded one & zero, respectively) to calculate genetic similarity and to construct dendrogram tree among the studied five Thymus Species. Calculation was achieved using Dice similarity coefficients30 as implemented in the computer program SPSS-10.

RESULTS AND DISCUSSION

Vegetative Composition Diameter

The vegetative parameters results were including plant height (cm), number of branches/ plant and leaves fresh & dry weights (g), of (Thymus species) seedlings in both two seasons are shown in Table (2).

Plant Height

Table (2) represented that plant height, *Origanum vulgare* was the highest plant in first season in the two cuts were as follow (31.39 and 35.37cm) and increased in the second season was cuts as follows (35.90 and 32.31cm) and this followed by *Thymus citriodorus, Thymus vulgaris* and *Origanum syricum*. While, the lowest in the Thymus Sp. in plant height was Thymus 3 which results in first season in the two cuts were as follows (6.82 and 8.91cm) and in the second season data in the tow cuts were as follows (9.62 and 9.19cm), respectively.

Branch Number

Table (2) results revealed that, the number of branches it was clear from that the greatest branches number were revealed by Thym.1in the first season: in two cuts were as follow (16 and 39) and in the second season: in the two cuts were as follow (39 and 42.67) and this results were followed by *Thymus argenteus, Thymus citriodorus* and *Origanum syricum*. While, the lowest number of branches were recorded in *Origanum vulgare* which were in the first season: in the two cuts as follow (3.67 and 4) and in the second season: in the two cuts ere as follow (4.33 and 5.33), respectively.

Fresh and Dry Weight of Leaves /Plant

the results of leave fresh & dry weights in the five sp. of Thymus in the two seasons data were revealed in Table (2), *Thymus argenteus* results were recorded as the highest data in all Thymus sp. under study and results were as follow, in the first season : fresh weight of leaves/plant in the two cuts were as follow (422.4 and 519 gm) and in dry weight of leaves/plant were as follow in the two cuts (49.25 and 53.98gm). While, in the second season: fresh weight of leaves/plant in the two cuts were as follow (522.14 and 659.99 gm) and in dry weight of leaves/plant were as follow in the two cuts (52.34 and 65.20gm) and this results were followed by *Origanum vulgare, Thymus vulgaris* and *Thymus citriodorus*, respectively. On the other hand, *Origanum syricum* was the lowest in both fresh and dry weight of leaves/plant in the two seasons and the results were as follow, the first season: fresh weight of leaves/plant in the two cuts were as follow (23.74 and 27.51 gm) and in dry weight of leaves/plant were as follow in the two cuts (8.41 and 9.57gm). While, in the second

Table 1. The Thymus species numbers and the names of the five studied species

Cultivar Number	Thymus species	Common name	Origin
1	*Thymus vulgaris*	Balady	Egypt
2	*Origanum vulgare*	Syrian	Saudi Arabia
3	*Thymus argenteus*	Oregano	Egypt
4	*Thymus citriodorus*	Jordanian	Saudi Arabia
5	*Origanum syricum*	Gabaly	Saudi Arabia

Table 2. Vegetative parameters, plant height, branches number, fresh weight,dry weight and volatile oil % of five Thymus species through two cuts and two seasons.

	First cut					Second Cut				
	Plant height (cm)	Branches number	Fresh weight (g/plant)	Dry weight (g/plant)	Volatile oil %	Plant height (cm)	Branches number	Fresh weight (g/plant)	Dry weight (g/plant)	Volatile oil %
First season										
Thymus vulgaris	21.53	16.00	33.74	11.47	0.10	23.76	39.00	49.59	17.20	0.12
Origanum vulgare	31.39	3.67	36.42	12.39	0.27	35.37	4.00	51.04	14.28	0.29
Thymus argenteus	6.82	6.67	422.46	49.25	0.07	8.91	8.67	519.81	53.98	0.08
Thymus citriodorus	29.24	5.33	29.10	11.42	0.22	32.22	9.00	41.26	14.36	0.26
Origanum syricum	12.30	4.33	23.74	8.41	0.05	13.72	5.67	27.51	9.57	0.06
New L.S.D. (0.05) =	1.42	1.88	86.27	9.87	0.02	1.11	3.16	93.45	14.08	0.01
Second season										
Thymus vulgaris	26.76	39.00	36.51	12.85	0.12	24.47	42.67	52.34	18.79	0.14
Origanum vulgare	35.90	4.33	39.99	13.82	0.31	31.31	5.33	58.29	15.94	0.33
Thymus argenteus	9.62	8.33	522.14	52.34	0.11	9.19	8.67	659.99	56.20	0.13
Thymus citriodorus	32.76	7.33	36.10	13.08	0.27	30.48	9.33	43.92	12.36	0.29
Origanum syricum	14.25	5.00	29.36	11.13	0.07	12.73	6.67	30.40	11.07	0.12
New L.S.D. (0.05) =	0.70	2.63	81.85	9.90	0.02	1.42	1.17	114.93	12.81	0.03

season: fresh weight of leaves/plant in the two cuts were as follow (29.36 and 30.40 gm) and in dry weight of leaves/plant were as follow in the two cuts (11.13 and 11.07gm).

Volatile Oil %

The results were observed in Table (2) illustrated the largest amount of volatile oil % was in *Origanum vulgare* in both two seasons as follow, the first season: results in the two cut were as follow (0.27 and 0.29%) and in the second season: results in the two cuts were as follow: (0.31 and 0.33%) respectively, and this results were followed by *Thymus citriodorus, Thymus vulgaris* and *Thymus argenteus*. While, the lowest amount of volatile oil % was observed in *Origanum syricum* in both two seasons and the results as follow, the first season: the two cut were as follow (0.05 and 0.06%) and in the second season: results in the two cuts were as follow: (0.07 and 0.12%), respectively.

Molecular Genetics Assessment

This results of the genetic variability in five species of Thymus using SCoT-PCR and ISSR-PCR analysis. Where five SCoT primers out of ten tested primers were succeeded on the five different Thymus Species, and five ISSR primers out of fourteen tested primers generated reproducible amplified bands.

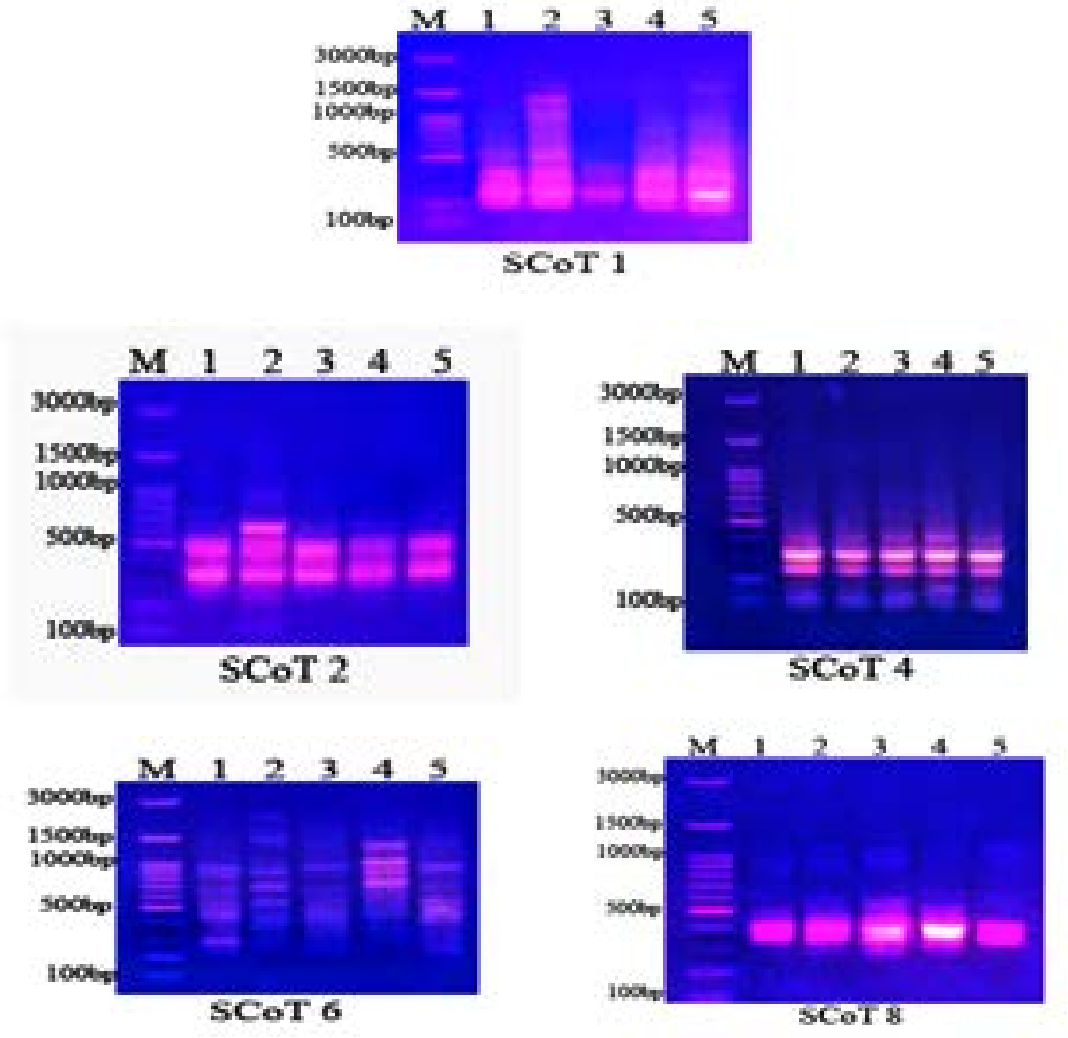

Fig. 1. SCoT-PCR Profile for five species of Thymus amplified with five primers for each analysis

SCoT and ISSR Analysis Assessment

Molecular genetic data produced by SCoT and ISSR analysis were shown in Figs (1 and 2) and Tables (3 and 4). These data showed that, in SCoT results, primer (SCoT-6) was resulted in the highest number of amplified bands and primer (SCoT-4) was represented the lowest number of amplified bands compared with other SCoT primers. On the other hand, in ISSR data, primer 44B resulted in the highest number of amplified bands and primer (HB-14) showed the lowest number of amplified bands in all ISSR primers.

On the other hand, SCoT primers except SCoT 4 and SCoT-8 generated 10 unique bands out of 39 amplified bands and ISSR primers except (44B, HB-10 and HB-14) generated 4 unique bands out of 23 amplified bands, May be these unique bands were useful as unique markers as explained by31 in cymbopogon; 32 in canolla; 33 in tomato and 34 in pumpkin.

Also, Table 5 showed that five species of Thymus, (*Thymus vulgaris, Origanum vulgare, Thymus argenteus, Thymus citriodorus* and *Origanum syricum*) characterized by five SCoT primers and five ISSR primers data, 23 polymorphic bands from 38 amplified bands were produced by SCoT primers with polymorphic average 60.52%. While, 14 polymorphic bands from 23 amplified

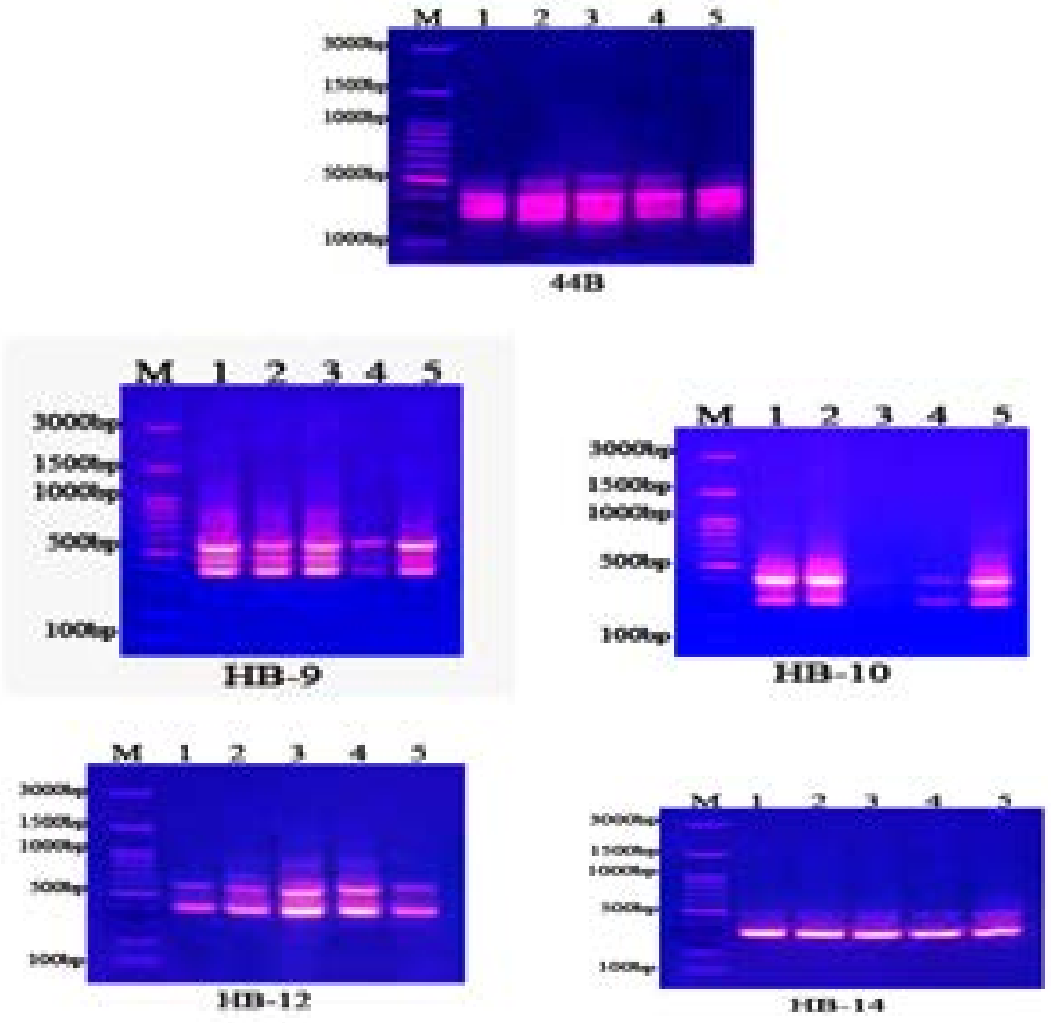

Fig. 2. ISSR-PCR Profile for five species of Thymus amplified with five primers for each analysis

Table 3. Molecular genetic data produced from amplified banding patterns of SCoT technique

Primer Name	Sequence (5→‘)	Molecular size range	Total Amplified Band	Monomorphic Band	Polymorphic band	Unique Band	Polymorphic %
SCoT 1	CAA CAATGGCTACCACCC	180:1470	8	3	5	2	62.50%
SCoT 2	CAACAATGGCTACCACCC	135:920	7	3	4	1	50%
SCoT 4	CAACAATGGCTACCACCT	135:48001	5	4	1	-	20%
SCoT 6	CAACAATGGCTACCACGC	193:1063	12	2	10	7	83.33%
SCoT 8	CAACAATGGCTACCACGT	195:587	7	1	4	-	14.28%
Total			38	15	24	10	60.52%

Table 4. Molecular genetic data produced from amplified banding patterns of ISSR technique

Primer Name	Sequence (5→3‘)	Molecular size range	Total Amplified Band	Monomorphic Band	Polymorphic band	Unique Band	Polymorphic %
44B	$(CT)_8GC$	150:560	6	3	3	-	50%
HB-09	$(GT)_6GC$	380:760	5	1	4	3	80%
HB-10	$(GA)_6CC$	300:560	4	1	3	-	75%
HB-12	$(CAC)_3GC$	300:840	5	1	4	1	80%
HB-14	$(CT)_3GC$	380:480	3	3	-	-	-
Total			23	9	14	4	60.86%

bands with polymorphic average 60.86% were generated by ISSR primers. On the other hand, in the combined results there were 37 polymorphic bands from total 61 amplified bands with total polymorphic average 60.65%. These obtained data indicates that SCoT-PCR and ISSR-PCR techniques were succeeded in differentiate between five Thymus species studied.

Molecular Distance of Combination of SCoT and ISSR Analysis

On the other hand, Table (6) illustrated that, results of molecular distance (MD) matrix between all five species of Thymus studied based on SCoT and ISSRs combined results.

Molecular distances based on SCoT analysis data were ranged from 0.633 (between *Thymus vulgaris* and *Thymus citriodorus* species) to 0.843 (between *Thymus vulgaris* and *Origanum vulgare* species) was lower than molecular distance based on ISSR ranged from 0.603 (between *Thymus vulgaris* and *Thymus citriodorus* species) to 0.942 (between *Thymus vulgaris* and *Origanum vulgare* species). While in molecular distance combination data were ranged from 0.164 to 0.404 among the same genotypes obtained by SCoT analysis.

Previously data represented the important of SCoT-PCR technique in molecular genetic assessment in Thymus species in comparison with ISSR-PCR technique. These results were in agreement with Nepeta[13], Thyme[14], Mentha aquatica L.[16], Satureja[17], Salvia[18] and Thymus[19].

Dendrogram Analysis of Combination Between SCoT and ISSR Analysis

Fig. 3. illustrated Dendrogram tree of SCoT and ISSR analysis combination data were divided the five Thymus Species into two main clusters: The first cluster contained two Thymus sp. (*Thymus argenteus* and *Thymus citriodorus*) and the second cluster was divided into two sub-clusters: the first sub-cluster included *Origanum syricum* only. On the other hand, the second sub-cluster included the other species (*Thymus vulgaris* and *Origanum vulgare*).

Table 5. Polymorphic, Monomorphic, Unique bands and Polymorphic percentage generated by the (ISSR and SCoT) analysis

Primer Name	Total Amplified Band	Monomorphic Band	Polymorphic band	Unique Band	Polymorphic %
SCoT	38	15	23	4	60.52%
ISSR	23	9	14	4	60.86%
Total	61	24	37	8	60.65%

Table 6. Molecular distances (MD) between five Thymus Species based on Dice dissimilarity index for SCoT &ISSR and combined data

MD		*Thymus vulgaris*	*Origanum vulgare*	*Thymus argenteus*	*Thymus citriodorus*	*Origanum syricum*
Origanum vulgare	ISSR	0.942				
	SCoT	0.843				
	Comb	0.872				
Thymus argenteus	ISSR	0.743	0.813			
	SCoT	0.752	0.732			
	Comb	0.733	0.753			
Thymus citriodorus	ISSR	0.603	0.684	0.813		
	SCoT	0.633	0.702	0.732		
	Comb	0.602	0.680	0.763		
Origanum syricum	ISSR	0.902	0.853	0.684	0.661	
	SCoT	0.753	0.733	0.761	0.771	
	Comb	0.822	0.792	0.721	0.721	

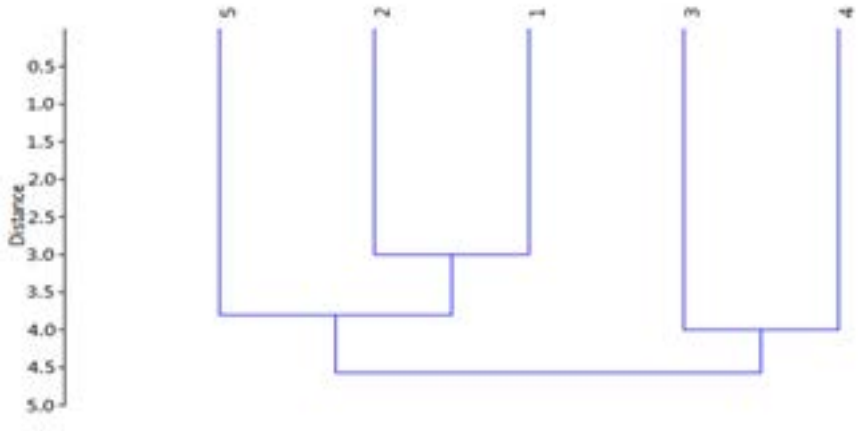

Fig. 3. Dendrogram tree for combination data of SCoT and ISSR analysis for the five Thymus species

This results were conducted that SCoT and ISSR analysis could be defined as tools for identifying Thymes species in breeding programs and combination data from SCoT and ISSR analysis were suitable for the genetic relationships evaluation between the five Thymus species and this results were in agreement with genetic analysis has been conducted by[35,36,37,] Salvia[18] and Thymus[19] *Phlomis kurdica* and *Phlomis oppositiflora*[20] and Ocimum[21,38]. Revealed that, by using of ISSR-PCR technique of some accessions of *Thymus daenensis*, was obtained two geographically diverse groups were generated by dendrogram.

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