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 growth parameters including: Plant height, Number of branches, Leaves fresh weight, Leaves dry weight 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 Markers; SCoT; Thymus Species.

Thymus genus which belongs to the family Lamiaceae, includes several hundreds of species distributed over world¹, where Mediterranean basin is considered the main center of this herbal plant².

Traditionally, most of plants discriminated on morphological-basis; however, these methods still difficult to apply for an accurate discrimination and authentication use³. 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 material²-⁴. reported that, Polyploidy and diploidy/aneuploidy within many species in the genus Thymus further complicate the determination of species boundaries
and there were high levels of natural hybridization within and between the species, probably due to the absence of incompatibility and the presence of a dimorphic breeding system, gynodioecy, in which populations comprise female and hermaphroditic individuals. 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 environmental conditions, have become increasingly important for surveying genetic diversity and genotype identification of medicinal plants. These markers can also be taxonomically useful, i.e. for phylogenetic studies to distinguish plant species and subspecies.

The start codon targeted (SCoT) polymorphism is a novel, simple and reliable gene targeted marker technique based on the translation start codon. Primers for SCoT marker analysis were designed based on the conserved region surrounding the translation initiation codon, ATG. Using a single 18-mer primer as a forward and reverse primer in the PCR, designed thirty-six primers that were used successfully for cultivar identification and genetic diversity analysis in many crops. Being characterized by lower recombination levels between its markers and the gene/trait. 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, Thyme, Salvia, Mentha aquatica, Satureja, Salvia, Thymus, Phlomis kurdica and Phlomis oppositiflora and Ocimum.

Increasingly, the plant breeding approach has taken advantage of developments in molecular biology in order to genotype the species of interest in a way that considerably accelerates their selection and this types of approach consist of choosing desired genotypes on the basis of molecular markers, or having prior knowledge of the genes that determine the formation of a particular trait in a plant.

The aim of this study is to assess the 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**

**Plant Materials and Growth Conditions**

The seedlings of the five Thymus species were obtained from two countries, Egypt and Kingdom 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 Lab. Horticulture Research Institute, Agricultural Research Centre, Egypt. Two months old seedlings of five Thymus Sp. were obtained from the Experimental Farm of Medicinal and Aromatic Plants Research Department, El-Kanater El-Khairia, Egypt.

**Growth Parameters**

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

**Molecular Genetic Assessment**

**DNA Extraction**

The DNA extraction of the five species of Thymus was performed as described by. DNA quality was checked by means of absorbance ratios A260/A280 through a UV-spectrophotometer where DNA is pure with a ratio A260/A280 from 1.8- 2.0. Moreover, using electrophoresis in 1% agarose gel with ethidium bromide, a qualitative check for DNA samples was done.

**SCoT and ISSR Analysis**

Obtaining clear reproducible amplification products require a number of factors were included PCR temperature cycle profile and concentration of each of (template DNA, primer, MgCl2 and Taq polymerase) which were optimized according to 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 were designed from consensus sequence derived from the previous studies by and 11 and procured from Biobasic Company.

ISSR and SCoT assays were performed as described by 24,25 and 28.

**Gel Electrophoresis**

PCR products were run at 100 V for one 30min. on 1.5 % agarose gel using 100bp Ladder
DNA marker to detect polymorphism between five Thymus species under study.

**Statistical Analysis**

A randomized complete block design was adopted for the present investigation data were statistically analyzed by the standard methods according to\(^9\). The new L.S.D. test was used for comparison between means. The DNA bands generated by each primer were counted and their molecular sizes were compared with those of the DNA markers. The bands scored from DNA profiles generated by each primer were pooled together. Then the presence or absence of each DNA band was treated as a binary character in a data matrix (coded 1 and 0, respectively) to calculate genetic similarity and to construct dendrogram tree among the studied five Thymus Species. Calculation was achieved using Dice similarity coefficients\(^30\) as implemented in the computer program SPSS-10.

**RESULTS AND DISCUSSION**

**Growth Composition Diameter**

The growth parameters results were including Plant height (cm), number of branches/plant and fresh and dry weights of leaves/plant (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**

The data in Table (2) revealed that, the number of branches it was clear from that the greatest branches number were revealed by Thym.1 in 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 as follow (4.33 and 5.33), respectively.

**Fresh and Dry Weight of Leaves/Plant**

Results of fresh weight of leaves/plant and dry weight of leaves/plant 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 seasons 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.

### 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.

|                | Plant height (cm) | Branches number | First cut Fresh weight (g/plant) | Dry weight (g/plant) | Volatile oil % | Plant height (cm) | Branches number | Second Cut 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           |
| Origanumsyricum | 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           |
| Origanumsyricum | 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           |
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 9.57 gm). While, in the second 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 11.07 gm).

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

![SCoT-PCR Profile for five species of Thymus amplified with five primers for each analysis](image-url)
fourteen tested primers generated reproducible amplified bands.

**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

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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 \((5\rightarrow3')\) | 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             | 135:48001            | 5                    | 4                | 1                | -           | 20%          |
| SCoT 4      | CAACAATGGCTACCACCT             | 193:1063             | 12                   | 2                | 10               | 7           | 83.33%       |
| SCoT 6      | CAACAATGGCTACCACGT             | 195:587              | 7                    | 1                | 4                | -           | 14.28%       |
| SCoT 8      | CAACAATGGCTACCACGT             | 180:1470             | 8                    | 3                | 5                | 2           | 62.50%       |
| Total       |                                | 38                   | 15                   | 24               | 10               |             | 60.52%       |

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

| Primer Name | Sequence \((5\rightarrow3')\) | Molecular size range | Total Amplified Band | Monomorphic Band | Polymorphic Band | Unique Band | Polymorphic % |
|-------------|--------------------------------|----------------------|----------------------|------------------|------------------|-------------|--------------|
| 44B         | (CT)\(_3\)GC                  | 150:560              | 6                    | 3                | 3                | -           | 50%          |
| HB-09       | (GT)\(_3\)GC                  | 380:760              | 5                    | 1                | 4                | 3           | 80%          |
| HB-10       | (GA)\(_3\)CC                  | 300:560              | 4                    | 1                | 3                | -           | 75%          |
| HB-12       | (CAC)\(_3\)GC                 | 300:840              | 5                    | 1                | 4                | 1           | 80%          |
| HB-14       | (CT)\(_5\)GC                  | 380:480              | 3                    | 3                | -                | -           | -            |
| Total       |                                | 23                   | 9                    | 14               | 4                |             | 60.86%       |
SCoT primers with polymorphic average 60.52%. While, 14 polymorphic bands from 23 amplified 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 (5) illustrated that, results of molecular distance (MD) matrix between all five species of Thymus studied based on SCoT and ISSRs combined results. Molecular distances (MD) based on SCoT markers 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 range of MD 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 combined data were ranged from 0.164 to 0.404 among the same genotypes were defined by SCoT technique.

Previsouly 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 Nepeta13, Thyme14, Mentha aquatica L.16, Satureja17, Salvia18 and Thymus19.

### Dendrogram Analysis of Combination Between SCoT and ISSR Analysis

Fig. 3 represented dendrogram from UPGMA method using Dice-dissimilarity index. Dendrogram 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

#### 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            |
included Origanum syricum only. While the second sub-cluster included the two other species (Thymus vulgaris and Origanum vulgare).

This results were conducted that SCoT and ISSR analysis could be useful as tools for identifying Thymes species in breeding programs and this combination data of SCoT and ISSR analysis were suitable for evaluating the genetic relationships among five species of Thymus and this results were in agreement with genetic analysis has been conducted by35,36,37, Salvia18 and Thymus19 Phlomis kurdica and Phlomis oppositiflora20 and Ocimum21,38. 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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