Egyptian J. Desert Res., **69**, Special Issue, 91-106 (2019)

GENETIC DIVERSITY AMONG *THYMUS* SPP. USING RAPD AND ISSR MARKERS

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This investigation was carried out to assess the relationships among three *Thymus* species; two wild species (*Thymus capitatus* and *Thymus decussatus*) and a cultivar (*Thymus vulgaris*). Two molecular markers were used to determine the genetic relationships among them; random amplified polymorphic DNA (RAPD) and the inter-simple sequence repeats (ISSR) markers techniques. The results suggest that RAPD marker is the best choice for the evaluation of diversity and the genetic relationships between two wild *Thymus* species with high accuracy, which revealed 224 DNA bands detected across 15 primers with high polymorphism than ISSR, which revealed 336 DNA bands. The genetic relationship among *Thymus* species based on molecular data was developed using a dendrogram constructed by UPGMA cluster analysis. Conservationist may use the information of the present study to make effective decisions regarding the global protection and management of *Thymus* species in Egypt.

Keywords: *Thymus*, genetic relationships, RAPD-PCR, ISSR

The genus *Thymus* belongs to the family Lamiaceae and includes several hundreds of species distributed over the world (Akcin, 2006). The systematics of species remains difficult because of the interspecific hybridization, polyploidy and morphological similarities among species (Morales, 1996 and Tzakow and Constantinidis, 2005). The genus *Thymus* is known in several countries as a spice and food preservative, as well as a

The 1st Scientific Conference of Plant Genetic Resources Department, Ecology and Dry Lands Agriculture Division, Desert Research Center "Plant Genetic Resources and Sustainable Development Under Egyptian Desert Conditions" 13-16 November, 2019, Sharm El-Sheikh, South Sinai, Egypt

protective and curative remedy for many ailments. In the present study, RAPD and ISSR markers were used to characterize and determine the percentage of genetic diversity between three Egyptian species of *Thymus* and evaluate their genetic relationships. These methods are broadly used in plant population genetics and differentiation studies (Monteleone et al., 2006; Solouki et al., 2008 and Trindade et al., 2009). Generally, RAPD has allowed the resolution of complex taxonomic relationships (Casiva et al., 2002 and Ruana et al., 2004) and phylogenetic studies (Mariette et al., 2007). ISSR marker permits detection of polymorphisms in inter- microsatellite loci, using a primer designed from dinucleotide or trinucleotide simple sequence repeats. ISSR amplifies inter-microsatellite sequences at multiple loci throughout the genome (Li and Xia, 2005). 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 (Nybom and Weising, 2007). These markers can also be taxonomically useful, i.e. for phylogenetic studies to distinguish plant species and subspecies (Mulcahy et al., 1995; Baigi et al., 2009 and Alamdary et al., 2011). In this research study, RAPD and ISSR were used to assess the genetic diversity among selected *Thymus* species, two grown naturally in Egypt (*Thymus capitatus* L. and *Thymus decussatus* Benth.) and a cultivar (*Thymus vulgaris*).

MATERIALS AND METHODS

1. Plant Material Collection

All The plants species in this study were kindly identified by members from Department of Ecology and Range Management, Desert Research Center. The plant leaf samples were collected in spring 2018, kept frozen in -80°C. The frozen leaves of all plant species were ground into fine powder using liquid nitrogen. Samples of the first species; *Thymus capitatus* (a type of wild thyme) were collected from naturally grown rocky ridge habitats, especially wet sites distributed on North coast, Mersa Matruh governorate. Samples of the second species; *Thymus decussatus* (a type of wild thyme) was collected from Saint Katherine Protectorate, South Sinai governorate. The plant is a chasmophyte growing in rocky areas, especially wet sites. The samples of the last species; *Thymus vulgaris* (a cultivar plant) were collected from farmers in Kirdasa, Giza governorate. In addition to the thyme species; *Origanum vulgare* that was introduced to this study as a random outgroup (control). Leaves of each species were used as source of DNA in this research study.

2. Extraction and Purification of Genomic DNA

Genomic DNAs were extracted from young leaves of the three species of *Thymus* as well as *Origanum vulgare* by DNeasy Plant Mini Kit (Qiagen Inc., Cat.no.69104, USA), and was performed following the manufacturer's instruction.

3. Estimation of DNA Concentration

The concentrations and quality of the genomic DNA samples were estimated using ND-2000 spectrophotometer (Nanodrop, USA). Finally, all the genomic DNA samples were diluted to a final concentration of 40 ng/µl with TE buffer (10 mM Tris-HC1, pH 8.0; 1 mM EDTA) and stored at -20°C for further use.

4. ISSR and RAPD Analysis

4.1. Primers Selection and ISSR-PCR and RAPD-PCR Reactions

Twenty ISSR and Fifteen RAPD primers were selected to be tested for the ability to generate DNA polymorphism among six samples of the three different *Thymus* species and one sample of *Origanum vulgare* as control (an outgroup). These primers were synthesized by Metabion Corp., Germany. The primers code and nucleotide sequences are presented table (1 and 2). The PCR amplification reactions were performed in 25 µl volume composed of 2.5 µl (1x) reaction buffer, 0.5 µl (0.2 mM) dNTPs, 2 µl (1.5 mM) $MgCl_2$, 1 µl (0.2 µM) primer, 1 µl (0.5 unit) *Taq* polymerase (Qiagen Ltd., Germany) and 50 ng of template DNA, in sterile distilled water.

5. ISSR and RAPD-Thermocycling Profile and Detection of the PCR Products

PCR amplification of the DNA was performed in a Perkin Elmer thermal cycler 9700. The temperature profile in the different cycles was as follows: an initial strand separation cycle at 94°C for 5 min, followed by 40 cycles comprised of a denaturation step at 94°C for 1 min, an annealing step at 45°C for 1 min and an extension step at 72°C for 1.5 min. The final cycle was a polymerization cycle for 7 min at 72°C.

PCR products were mixed with 5 µl gel loading dye and resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5 mg/ml) in 1 x TBE buffer at 120 volts. A 100 bp DNA ladder was used as molecular size standard. PCR products were visualized under UV light and documented using a ™XR+ Gel Documentation System (Bio-Rad).

Table (1). List of the twenty selected ISSR primers for polymorphic DNA generation of *Thymus* species.

No.	Primer	Sequence
1	UBC-824	$(TC)_8G$
2	UBC-826	$(AC)_8C$
3	UBC-827	$(AC)_8G$
4	UBC-834	$(AG)_8TT$
5	UBC-852	$(TC)_8AA$
6	UBC-868	$(GAA)_6$
7	UBC-876	$(GATA)_2(GACA)_2$
8	UBC-880	$(GGAGA)_3$
9	814A	$(CT)_8TG$
10	844A	$(CT)_8AC$
11	HB08	$(GA)_6GG$
12	HB10	$(GA)_6CC$
13	HB12	$(CAC)_3GC$
14	HB13	$(GAG)_3GC$
15	HB14	$(CTC)_3GC$
16	ISSR- 11	$(AC)_8YA$
17	ISSR- 12	$(AC)_8YC$
18	ISSR- 13	$(AG)_8YT$
19	ISSR- 14	$(CTC)_5TT$
20	ISSR- 15	$(CT)_8CG$

Adenine (A), Cytosine (C), Guanine (G), Thymine (T) and C or T (Y)

Table (2). List of the fifteen selected RAPD primers for polymorphic DNA generation of *Thymus* species.

No.	Primer	Sequence
1	**OPA-05**	5'-AGGGGTCTTG-3'
2	**OPA-10**	5'-GTGATCGCAG-3'
3	**OPB-15**	5'-GGAGGGTGTT-3'
4	**OPD-05**	5'-TGAGCGGACA-3'
5	**OPD-19**	5'-CTGGGGACTT-3'
6	**OPE-01**	5'-CCCAAGGTCC-3'
7	**OPE-20**	5'-AACGGTGACC-3'
8	**OPF-03**	5'-CCTGATCACC-3'
9	**OPF-14**	5'-TGCTGCAGGT-3'
10	**OPH-02**	5'-TCGGACGTGA-3'
11	**OPA-07**	5'-GAAACGGGTG-3'
12	**OPA-08**	5'-GTGACGTAGG-3'
13	**OPB-12**	5'-CCTTGACGCA-3'
14	**OPB-17**	5'-AGGGAACGAG-3'
15	**OPB-18**	5'-CCACAGCAGT-3'

6. Data Analysis

DNA polymorphism generated by ISSR and RAPD-PCR were calculated based on the presence or absence of amplified bands were scored as present (1) or absent (0) across the *Thymus* species. The only clear major bands were subjected to scoring. The specific bands useful for identifying species were named with a primer number followed by the approximate size of the amplified fragment in base pairs. To construct a dendrogram describing the genetic relatedness of *Thymus* species, genetic distance was calculated based on the Jaccard coefficient (Jaccard, 1908), using the correlate module of SPSS software version 20, after making a pair wise comparison between them, relying on the proportion of shared bands that produced by each used primer. Jaccard's coefficients are common estimators of genetic identification, and were calculated as [Jaccard's coefficient = NAB/(NAB+NA+NB)]; where NAB is the number of bands shared by samples, NA represents amplified fragments in sample A, and NB represents fragments in sample B. Similarity matrix based on these indices were calculated. This was used using classify module of SPSS software version19.

RESULTS AND DISCUSSION

Molecular markers are efficient tools for identification and estimation of relatedness through DNA fingerprinting. In the present investigation, two types of molecular markers namely, ISSR and RAPD were employed to assess the genetic polymorphism within and among two wild species; *Thymus capitatus* and *Thymus decussatus* and the cultivar *Thymus vulgaris*.

1. Genetic Polymorphism as Detected by ISSR Markers

The twenty ISSR primers amplified a total of 336 bands in the set of six samples of the three *Thymus* species and *Origanum vulgare* (Fig. 1). DNA polymorphisms generated by ISSR were calculated based on the presence or absence of amplified fragments. The number of bands for each primer ranged from 11 for primer 844A to 25 for primer ISSR-12 with sequences of (CT) 8AC and 5'-ACACACACACACACACYC-3', respectively with an average of 16.8 at the species level. The percentage of polymorphic bands (PPB) was calculated based on Jaccard's coefficients to be ranged between 78 and 100% with an average of 96.76% (Table 3). Such high level of polymorphism is comparable to the results of some similar molecular researches on medicinal plants of Lamiaceae family (Trindade et al., 2009 and Agostini et al., 2010).

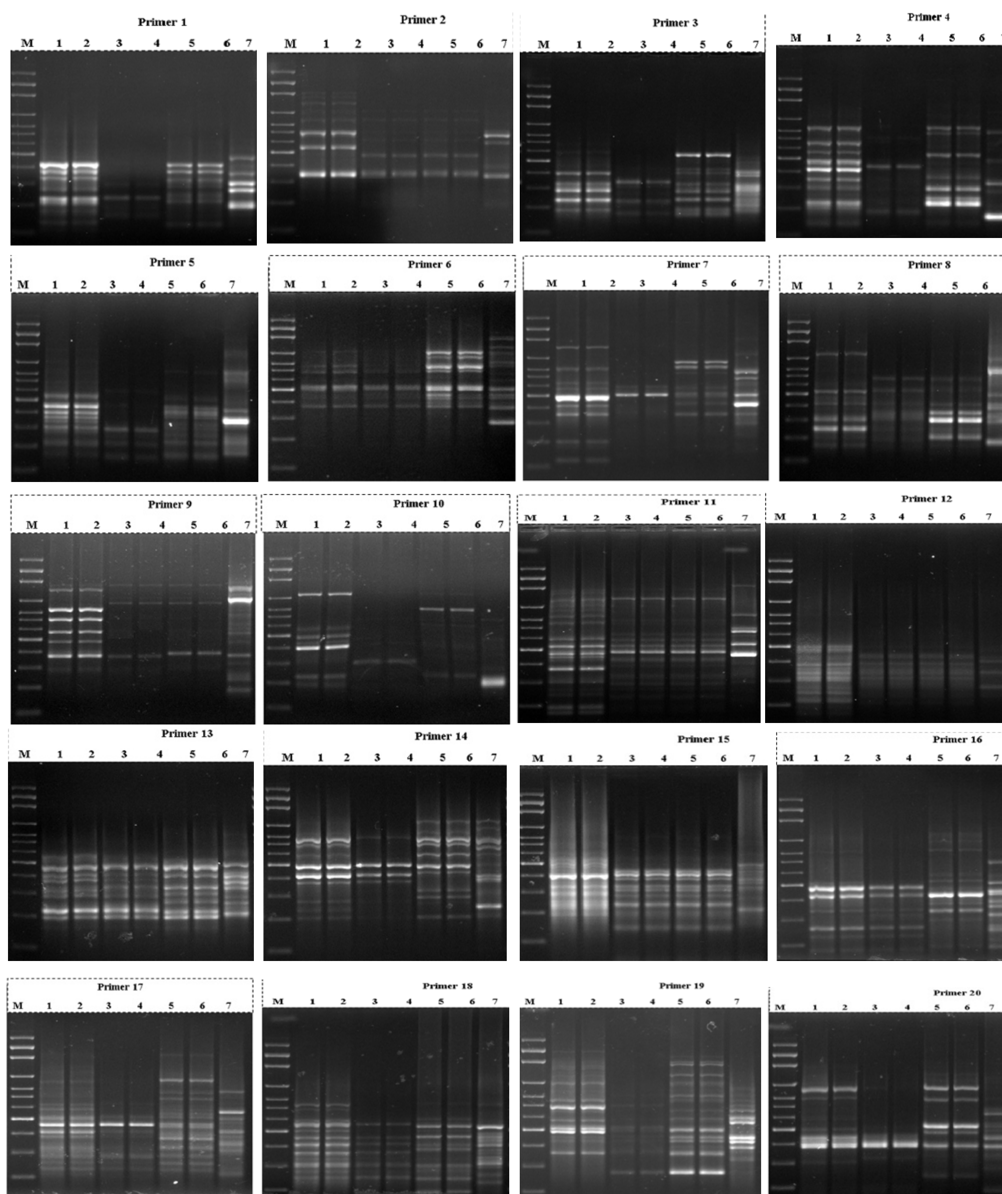

Fig. (1). Gel electrophoresis pattern of ISSR amplification using twenty primers. M: marker; (1 and 2) *Thymus vulgaris*, (3 and 4) *Thymus capitatus*, (5 and 6) *Thymus decussatus* and (7) *Origanum vulgare*.

Table (3). The polymorphism generated by twenty ISSR primers for *Thymus* species.

No.	Primer	Mono-morphic bands	Polymorphic (without Unique)	Unique bands	Polymorphic (with Unique)	Total number of bands	Polymorphism (%)	Mean of band frequency
1	UBC-824	0	10	5	15	15	100	0.4
2	UBC-826	1	9	5	14	15	93	0.4
3	UBC-827	1	10	4	14	15	93	0.4
4	UBC-834	0	16	2	18	18	100	0.4
5	UBC-852	2	13	1	14	16	88	0.5
6	UBC-868	3	7	8	15	18	83	0.5
7	UBC-876	2	9	4	13	15	87	0.5
8	UBC-880	2	10	3	13	15	87	0.5
9	814A	1	8	5	13	14	93	0.4
10	844A	0	9	2	11	11	100	0.4
11	HB08	1	19	4	23	24	96	0.5
12	HB10	2	10	1	11	13	85	0.5
13	HB12	3	9	3	12	15	80	0.5
14	HB13	3	9	4	13	16	81	0.6
15	HB14	2	11	2	13	15	87	0.6
16	ISSR- 11	1	12	8	20	21	95	0.4
17	ISSR- 12	1	17	7	24	25	96	0.4
18	ISSR- 13	4	11	3	14	18	78	0.6
19	ISSR- 14	0	19	4	23	23	100	0.4
20	ISSR- 15	1	10	3	13	14	93	0.4
Total		30	228	78	306	336	91	
Mean per primer		1.5	11.4	3.9	15.3	16.8	91	

Data of ISSR profiles scanned from the six *Thymus* samples with twenty reproducible primers were used to generate similarity coefficients with Jaccard measure using SPSS software version19 (Table 4). Pairwise similarity of banding patterns between the studied plant species were ranged from 51 to 100% for species. The maximum similarity (100%) was observed between *Thymus vulgaris* from different places. The lowest similarity value was observed between *Thymus vulgaris* and *Thymus capitatus* (51%). *Thymus capitatus* showed the closest relationship with *Thymus decussatus* as a wild type. *Origanum vulgare* as an outgroup, confirmed the reliability of the ISSR method and similarity analysis tests to show quite divergent from other *Thymus* species with similarity coefficient range from 30 to 42% as shown in the similarity matrix (Table 4). Based on the Jaccard coefficient, the obtained distance coefficients were used to construct a dendrogram using classify

module of SPSS software version19 (Fig. 2). The cluster tree analysis showed that *Thymus* species were divided into two main groups: the first group including *Thymus decussatus, Thymus vulgaris* and the second group including *Thymus capitatus*. However, *Origanum vulgare* was found to be quite divergent and did not fall in any of these two clusters.

Table (4). Genetic similarity (Jaccard`s) for *Thymus* species and *Origanum vulgare* as an outgroup by ISSR pattern generated by Twenty ISSR primers

Species	*T. vulgaris*	*T. vulgaris*	*T. capitatus*	*T. capitatus*	*T. decussatus*	*T. decussatus*	*O. vulgare*
T. vulgaris	100						
T. vulgaris	100	100					
T. capitatus	51	52	100				
T. capitatus	51	52	100	100			
T. decussatus	64	63	61	61	100		
T. decussatus	64	64	60	60	99	100	
O. vulgare	41	42	30	30	39	38	100

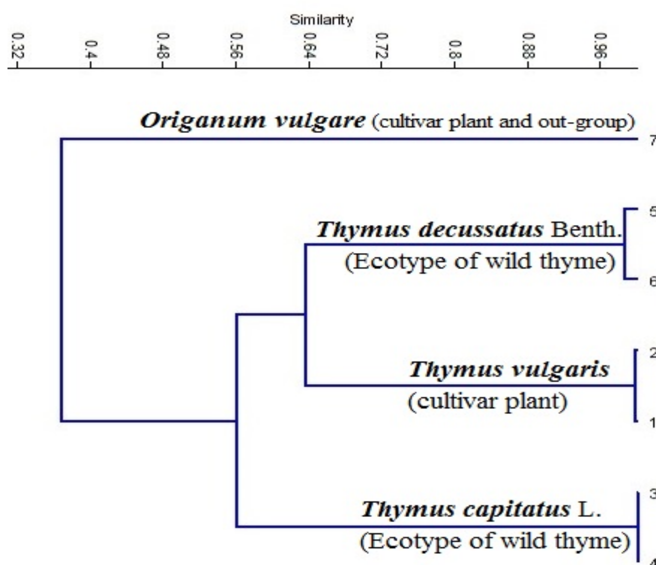

Fig. (2). The dendrogram cluster tree was generated for *Thymus* species where *Origanum vulgare* was used as an outgroup based on ISSR. (1 and 2) *Thymus vulgaris*, (3 and 4) *Thymus capitatus*, (5 and 6) *Thymus decussatus* and (7) *Origanum vulgare*.

2. Genetic Polymorphism as Detected by RAPD Markers

The data obtained in the present study regarding RAPD molecular marker studies on the six samples of *Thymus* species reveal that RAPD marker is a good choice for assessing the genetic diversity and relationship in Thymus species with polymorphism levels enough to establish informative fingerprints with a few markers.

The fifteen primers used for all populations generated 224 bands in the set of the six samples of *Thymus* species and *Origanum vulgare* (Fig. 3). The bands ranged in size from 200 to 2000 bp. The number of bands produced by primers varied from 9 for primers OPE-01, OPH-02 and OPA-07 to 20 for primer OPE-20, with an average of 14.9 at the species level. The percentage of polymorphic loci per primer varied from 80% (OPE-20 and OPB-18) to 100% (OPA-05, OPB-15, OPD-05, OPH-02, OPA-07 and OPA-08) (Table 5). Primers differed in their ability to distinguish individuals within and among populations. The highest number of RAPD fragments generated using the fifteen primers may be used as genotype-specific markers, when arranged in a descending order as follows, primer OPE-20 (four markers), primers OPA-10, OPF-14, OPB-12 and OPB-18 (three markers), OPF-3 (two marker) and OPD-19, OPE-01 and OPB-17 (one marker), while the remaining primers had no genotype-specific markers. Thus, *Thymus* species could be identified by genotype-specific RAPD markers.

Table (6) shows similarity indices between the six samples of the three *Thymus* species. The highest value (100%) was observed between *Thymus vulgaris* from different places, which indicate that these two populations are closely related to each other according to geographical distribution. On the other hand, the lowest similarity value (60%) was observed between *Thymus decussatus* and the other species, indicating that they were distantly related. Based on Jaccard coefficient, the obtained distance coefficients were used to construct a dendrogram using classify module of SPSS software version 20 (Fig. 4). The cluster tree analysis showed that *Thymus* species were divided into two main groups: the first group including *Thymus capitatus, Thymus vulgaris* and the second group including *Thymus decussatus*. However, *Origanum vulgare* was found to be quite divergent and did not fall in any of these two clusters.

The results based on RAPD markers revealed a low level of variation within populations and high differentiation among them. The analysis of population genetic variation with RAPDs could be hampered by a loss of a part of genetic information (Khalil and Li, 2012 and Sunar et al., 2009).

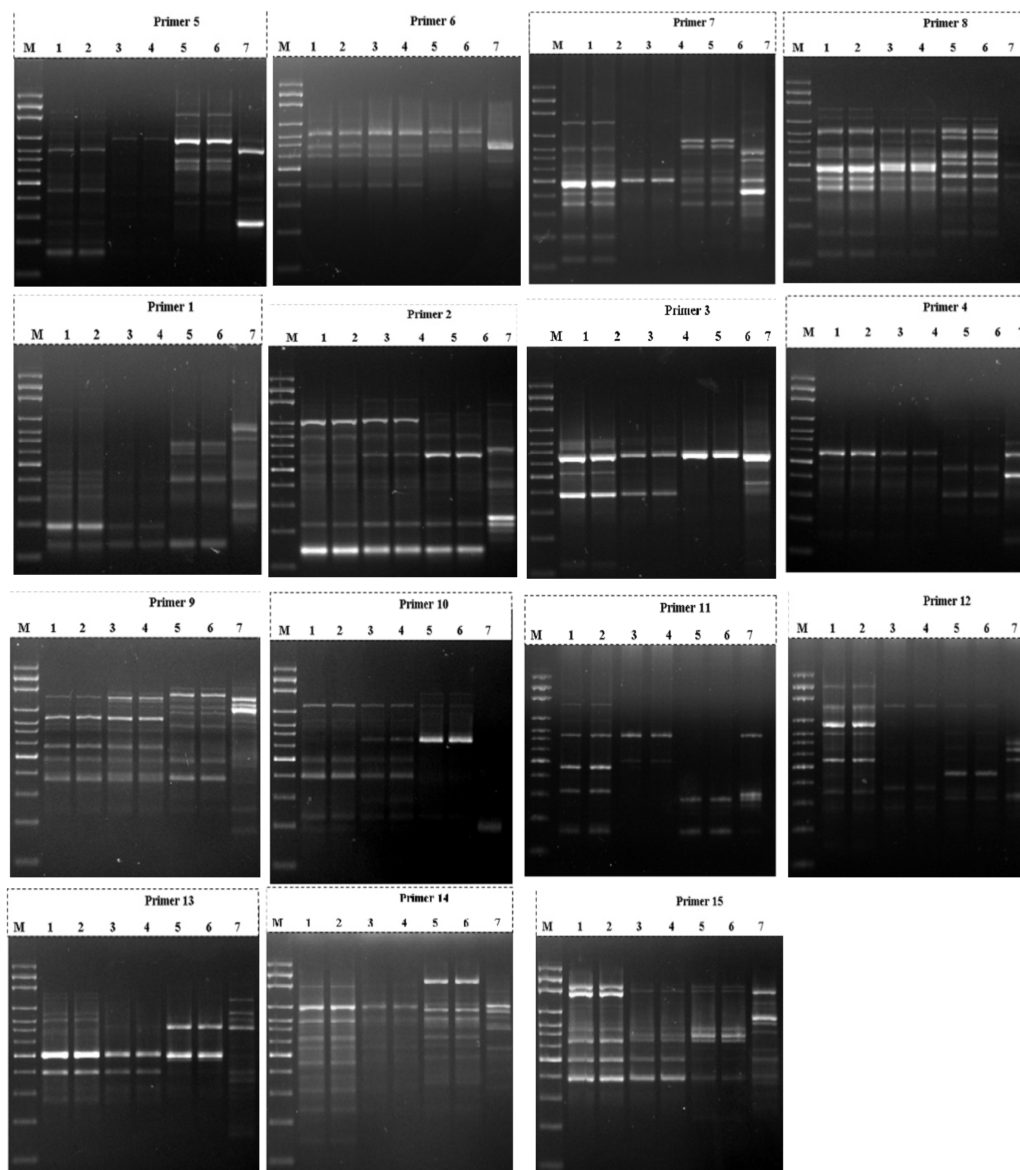

Fig. (3). RAPD amplification profiles obtained using fifteen primers analyzed in 1.5% agarose gel electrophoreses. M: marker; (1 and 2) *Thymus vulgaris*, (3 and 4) *Thymus capitatus*, (5 and 6) *Thymus decussatus* and (7) *Origanum vulgare*.

Table (5). The polymorphism generated by fifteen RAPD primers for *Thymus* species.

No.	Primer	Monomorphic bands	Polymorphic (without unique)	Unique bands	Polymorphic (with unique)	Total number of bands	Polymorphism (%)	Mean of band frequency
1	OPA-05	0	10	6	16	16	100	0.3
2	OPA-10	3	8	8	16	19	84	0.5
3	OPB-15	0	7	4	11	11	100	0.3
4	OPD-05	0	7	6	13	13	100	0.4
5	OPD-19	1	14	3	17	18	94	0.4
6	OPE-01	1	6	3	9	9	90	0.5
7	OPE-20	4	12	4	16	20	80	0.6
8	OPF-03	2	17	2	19	21	90	0.6
9	OPF-14	3	10	3	13	16	81	0.6
10	OPH-02	0	9	0	9	9	100	0.6
11	OPA-07	0	9	0	9	9	100	0.4
12	OPA-08	0	13	2	15	15	100	0.4
13	OPB-12	3	13	3	16	19	84	0.5
14	OPB-17	1	10	3	13	14	93	0.5
15	OPB-18	3	8	4	12	15	80	0.5
Total		21	153	51	204	224		
Mean per primer		1.4	10.2	3.4	13.6	14.93		

Table (6). Genetic similarity (Jaccard`s) among *Thymus* species and *Origanum vulgare* (an outgroup) by RAPD pattern generated by fifteen RAPD primers

Species	***T. vulgaris***	***T. vulgaris***	***T. capitatus***	***T. capitatus***	***T. decussatus***	***T. decussatus***	***O. vulgare***
T. vulgaris	100						
T. vulgaris	100	100					
T. capitatus	70	68	100				
T. capitatus	68	60	99	100			
T. decussatus	60	60	60	60	100		
T. decussatus	60	64	60	60	100	100	
O. vulgare	38	38	32	30	36	36	100

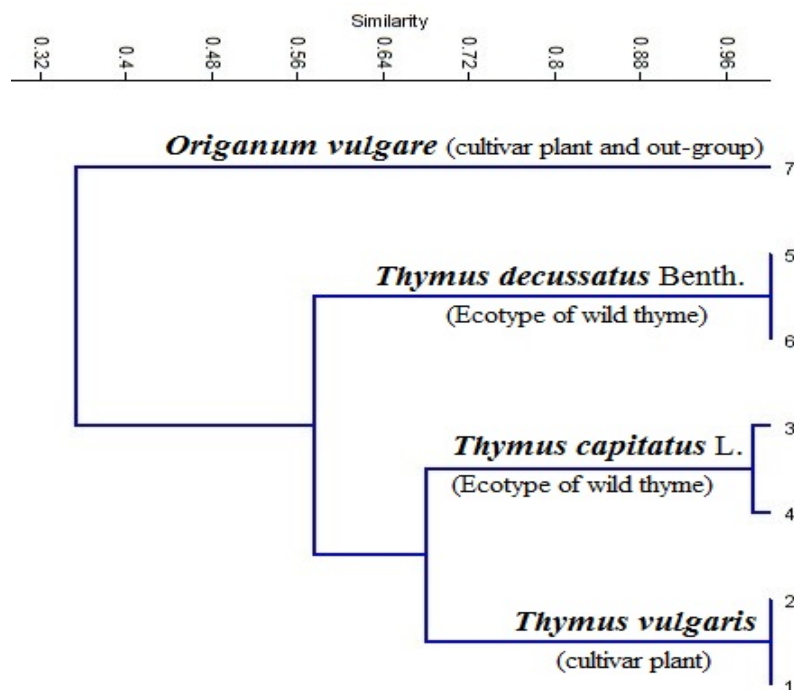

Fig. (4). The dendrogram cluster tree was generated for *Thymus* species, where *Origanum vulgare* was used as an outgroup based on RAPD. (1 and 2) *Thymus vulgaris*, (3 and 4) *Thymus capitatus*, (5 and 6) *Thymus decussatus* and (7) *Origanum vulgare*.

CONCLUSION

In this study, molecular tools could be much reliable for measuring the genetic relationship among *Thymus* species. Using twenty ISSR primers and fifteen of RAPD were found to generate relatively high level of DNA polymorphism among the three *Thymus* species. The results obtained in this study, might suggest that these chosen primers, which was screened in ISSR and RAPD tests were able to generate polymorphic bands on most genetic diverse loci among the *Thymus* species as proved by Tonk et al. (2010).

These results were considered to be promising for future specific DNA fingerprinting studies on *Thymus* species variants; suggesting to researchers to use them for specific detection of these species or expanding that work on other medicinal plants.

In general, ISSR and RAPD procedures were able to clearly distinguish the different selected *Thymus* species subjected to this study and also, could be potentially used for identifying *Thymus* species from any mixed

populations. A similar approach had been successfully used for molecular diagnosis of several species and cultivars by many other researchers (Sosinski and Doucher, 1996). Also, these markers could be used as a method of choice for identifying components for herbal medicine complex, since ISSR and RAPD techniques had been used for the determination of different components presented in herbal formulation. So, these will contribute significantly in quality control and give information about genomic variability below the species level (Williams et al., 1990), they provide relatively quick results, less time-consuming and low expensive (Arif et al., 2010).

These results can be further used to manipulate genetic determinants of horticulturally important traits and to characterize the basis of productivity of *Thymus*. ISSR and RAPD markers were proved to be useful tools in germplasm characterization and diversity analysis of *Thymus*, and can be used beside other molecular markers as AFLP and SSRs. Therefore, these findings provide guidance for identification of *Thymus* species, and help in their subsequent management and utilization in sustainable ways to combat human and natural pressures on these valuable natural resources. Finally, the genetic variation data would be very useful for improvement of *Thymus* species through conventional breeding programs as well as molecular breeding approaches such as marker assisted selection.

ACKNOWLEDGEMENT

The authors would like to express their special gratitude to Prof. Dr. Fareda Elsaied, Prof. of Genetics, Desert Research Center for the financial support and providing facilities and encouraging throughout. The authors also extend their gratitude to Academy of Scientific Research and Technology (ASRT) – Technology and Scientific Cooperation between Egyptian and Moroccan program. Project node no. is 10-2-11 (Valorization and conservation of medicinal and aromatic plants using biotechnology techniques: a case of wild endemic Thyme in Egypt and Morocco).

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التنوع الوراثي بين أنواع الزعتر باستخدام علامات RAPD وISSR

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تم إجراء هذا البحث لتقييم العلاقات الوراثية بين ثلاثـة أنـواع من الزعتر في مصر، نوعان بريان والثالث منزرع، وذلك باستخدام العلامات الجزيئية حيث تم استخدام تقنـيتي RAPDوISSR لتحديـد العلاقات الوراثية فيما بينها. تشير النتائج إلى أن استخدام علامة RAPD هي الخيار الأفضل لتقييم التنـوع والعلاقات الوراثيـة بين أثنـين من أنـواع الزعتر البري بدقة عاليـة والتي كشفت عن ٢٢٤ حزمة تم تحديـدها من خلال ١٥ من البادئات العشوائية مقارنةً بـ ISSR التي كشفت عن ٣٣٦ حزمة من خلال ٢٠ من البادئات. استنادًا إلى التحليلات الجزيئية باستخدام شجرة القرابة قد تساعد هذه الدراسة في إتخاذ قرارات فعالة فيما يتعلق بحماية وحفظ أنواع الزعتر وخاصة البري على مستوى العالم وفي مصر.