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Evaluation of Genetic Variation between *Thymus* Accessions Using Molecular Markers

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ABSTRACT

Thirteen accessions of Thyme medicinal plant belonging to Iranian endemic species of *Thymus daenansis, T. migricus, T. pubsence and T. fedtschenkoi* were studied in aspect of molecular marker. The genomic DNA extracted using modified medicinal CTAB protocol. Evaluation of DNA quality was examined with electrophoresis. 20 primers were used for PCR analysis and only 8 primer showed clear bands. Out of 97 bands 61% were polymorphic. The data were analyzed with NTSYS program and the dendrogram was drawn based on UPGMA. Cluster analysis based on RAPD data allowed separating thyme in two groups at genetic distance of 80%. Group 1 was formed by *T. daenensis*, *T. pubsence* and *T. migricus*. Group 2 was formed by *T. fedtschenkoi* and *T. daenensis* subs daenensis (No. 7). Finally investigation the genetic variation with RAPD markers indicated that this marker is suitable to determine the polymorphic loci and to estimate the genetic distance.
KEY WORDS: *Thymus*, Thyme, genetic diversity, RAPD.

INTRODUCTION

The genus *Thymus* consists of about 350 species of perennial, aromatic herbs and sub shrubs native to Europe and North Africa. Various types of thyme are used all over the globe as condiments, ornamentals and sources of essential oil. Thyme oil is among the world's top ten essential oils. Commercial supply of thyme originates mainly from European countries, but recently this increased demand has led to new winter-hardy, high yield cultivars being developed for commercial cultivation in Canada and North America **(**8). Its essential oil represents 0.7–6.5% of the dry weight of leaves and flowers (5). The main constituent of thyme oil is thymol (36-55%), which also determines the organoleptic properties of the oil. Properties of thyme oil are considered to be due to oil components such as thymol, carvacrol, p-cymene, linalool and [gamma]-terpinene. Thyme oil is used for flavoring foods and oral hygiene products, but is also used in perfumery to create spicy, leathery notes. Because of its high phenol content, thyme oil has germicidal and antiseptic properties (4). Thyme is enemy of poison. It is antispasm and pain. It eases flood blowing and invokes sexual activities and promotes consciousness and intelligence as well. It is useful for liver disorder. Meanwhile it is used in pulmonary infections, catarrh, bronchitis, angina, indigestion, stomach sore and inflation. It is eatable for remedy of whooping cough and other types of coughs diarrhea or digestive disorders (9).

Thyme plant has a partial or complete anti-fatuous, anti-phlegmasia effect, in addition to regulating digestion system, as well as anti bacterial effect on the main factor of gastric ulcer, *Helicobacter pylori* and other illness producing bacteria (9).

Thyme is propagated by seeds or by stern cuttings pollination is mainly affected by bees (2). Male sterile and male fertile plants have been identified in several populations leading to high levels of polymorphism (1).

In recent years, molecular makers have been applied to a wide number of genetic and breeding studies, including fingerprinting individuals and the positional cloning of important genes. One of the most extensively used molecular markers is RAPDs (15) which have been applied to address genetic diversity issues in plants (6 and 7). RAPD are specially suited to species with little molecular information due to the following attributes: 1) No previous knowledge of the genome is required. 2) Rapid results can be obtained when compared with alternatives such as RFLPs. 3) A universal set of primers which can be used for genomic analysis in any species is commercially available (12).

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The genetic diversity between 17 accessions of mint was examined using RAPD marker. Out of 70 primers, 31 provided reproducible results. High genetic diversity was found within mint accessions. The result indicated that RAPD as a molecular marker was useful for detecting the genetic variation (12).

The essential oil composition and genetic variability of six commercial of thyme were analyzed by GC-MS and RAPD. The comparison of the oil components concentration allowed separation of the cultivars into two groups. On the basis of the RAPD patterns, the cultivars could be divided into two clusters, which coincides with results obtained by oil GC-MS analysis, with a correlation coefficient of -0.779 (5)

Bagherzadeh (2009) used RAPD marker for evaluating genetic diversity among *T. vulgaris,*

T. serphyllum, T. carmanicus, T. transcaspicus and *Zataria multiflora*. The amplification profiles produced by 8 primers gave a total of 330 bands, out of which 300 were polymorphic. The results indicated that RAPD was a good marker for evaluating genetic diversity.

The purpose of the present study was to use RAPD markers to evaluate the genetic relationship between thirteen accessions of thyme medicinal plant belonging to Iranian endemic species of *Thymus daenansis, T. migricus, T. pubsence* and *T. fedtschenkoi.*

MATERIALS AND METHODS

Plant materials: 13 accessions of thyme namely *Thymus daenensis* subs daenensis, *T. daenensis* subs lancifolius, *T. migricus, T. pubsense* and *T. fedtschenkoi* were selected for RAPD analysis. These plants are known and endemic of Iran. The plant materials were obtained from Agricultural and Natural Research Center of Razavi-Khorasan (Tab. 1).

Tab 1: Genotypes used as plant materials

Number	Accession	Number	Accession
1	*Thymus pubsence*	8	*T. daenensis* subs daenensis
2	*T. migricus*	9	*T. daenensis* subs daenensis
3	*T. fedtschenkoi*	10	*T. daenensis* subs daenensis
4	*T. daenensis* subs lancifolius	11	*T. daenensis* subs daenensis
5	*T. daenensis* subs daenensis	12	*T. daenensis* subs daenensis
6	*T. daenensis* subs daenensis	13	*T. daenensis* subs daenensis
7	*T. daenensis* subs daenensis		

Isolation of genomic DNA: Equal amounts (0.5 g) of leaf tissue from each accession were combined to make pooled sample. Leaf material was placed in a porcelain mortar chilled with liquid nitrogen and was ground with a pestle to a fine powder. Total genomic DNA was extracted as described by Khanuja (1999). DNA was resuspended in TE buffer and stored at 4˚C.

PCR amplification: twenty arbitrary 10-mer primers (CinnaGen Co, Iran) were used for PCR amplification of the total genomic DNAs. PCR was performed based on the protocol of Williams *et al.* (1990) with minor modification in temperature and number of cycles. Amplification were carried out in 25 µl of reaction mixture containing 2.5 µl of PCR buffer, 2mM MgCl2, 0.4mM of dNTPs, 0.4µM of the primer, 1 unit of *Taq* DNA polymerase (CinnaGen Co, Iran) and 50 ng of DNA template. DNA amplification was performed in an Eppendorf thermocycler gradient programmed as follows: pre denaturation 3 min at 94˚C followed by 36 cycles, each of 1 min at 94˚C, 1 min at 36˚C, 2 min at 72˚C, followed by one final extension cycle of 10 min at 72˚C. The amplification products were electrophoresed in 1.2% agarose gels with 0.5X TBE buffer. Gels were stained with ethidium bromide and visualized under UV light and the size of amplified products was determined by comparison with lambda DNA digested with ECORI (size range from 1000 to 250) used as DNA size marker.

Data analysis: For molecular data all the accessions were scored for the presence of band (1) or its absence (0). Only those RAPD bands that appeared distinct in both of the replicate PCR reactions were recorded. The NTSYS –pc software ver. 2.02 was used to estimate genetic similarity with the Jaccard**'s** coefficient. The matrix of generated similarities was analyzed by the unweighed pair group method with the arithmetic averages (UPGMA), using the SAHN clustring module (14).

RESULTS AND SISCUSSION

Thirteen plants were examined for the extent of genetic variability by extracting a RAPD marker profile from 20 primers, but only 8 oligonucleotide primers (Tab. 2) were selected for the identification of thyme materials and evaluation of their relationships, of band patterns. Using the 8 primers, 97 RAPD bands were scored, ranging in size from 100 to 2500 bp. The number of amplification products per primer varied from 8 (OPP3) to 16 (DPA 9).

Tab. 2: Primers used for PCR amplification

Number	Primer code	Primer sequence
1	OPP 3	CTG ATA CGC C
2	OPS 17	TGG GGA CCA A
3	OPC 8	TGG ACC GGT G
4	OPJ 21	ACG AGG GAC T
5	OPA 9	GGG TAA CGG C
6	OPI 14	TGA CGG CGG T
7	OPF 5	CCG AAT TCC C
8	OPU 6	ACC TTT GCG G

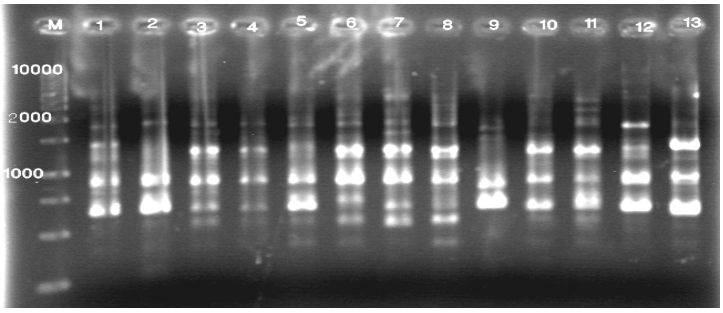

Fig. 1: RAPD profile of the thyme accessions amplified with OPF5 primer on 1.2% agarose gel. M: DNA ladder (lambda DNA digested with ECORI), number 1-13 are accessions of table 1.

61.86% of the bands were polymorphic that conformed the result of Bagherzadeh (2009) that reported 92% polymorphism were observed in thyme. An example of the patterns obtained by the RAPD analysis of thyme materials using OPF5 is shown in Fig. 1. We used UPGMA from NTSYS-PC program to produce RAPD marker-derived dendrogram. Cluster analysis based on RAPD data (Fig. 2) allowed separating thyme in two groups at genetic distance of 80%. Group 1 was formed by *T. daenensis*, *T. pubsence* and *T. migricus*. Group 2 was formed by *T. fedtschenkoi* and *T. daenensis* subs daenensis (No. 7). Group 1 could be separated in two subgroups. The first subgroup was formed by all accession of *T. daenensis* subs daenensis except No. 7 and 13. Whereas the other subgroup was formed by *T. bubsence*, *T. daenensis* subs lancifulious, *T. migricus* and *T. daenensis* (No. 13).

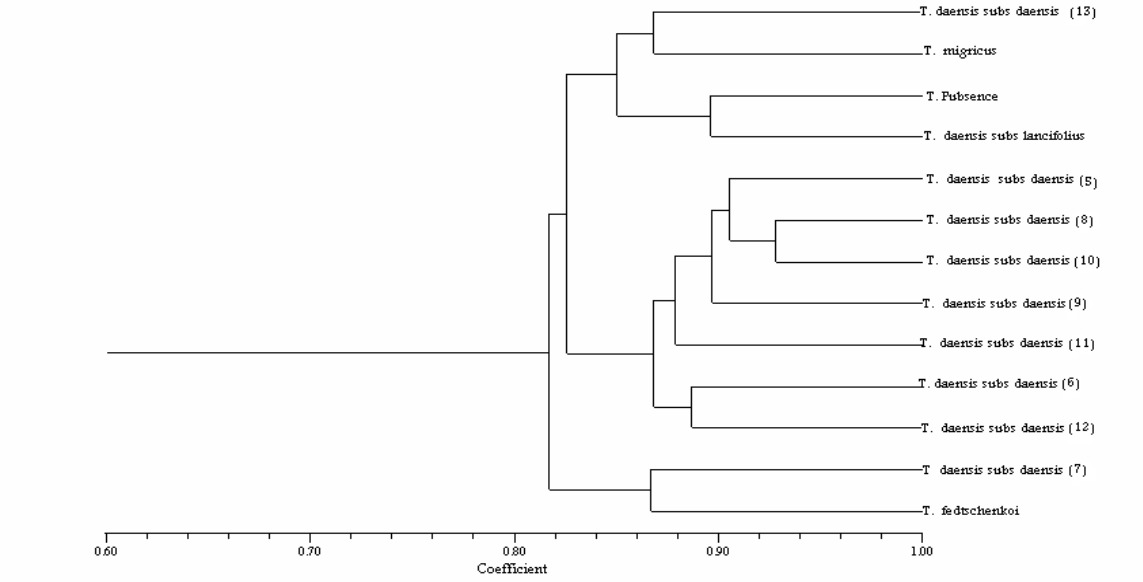

Fig. 2: UPGMA Dendrogram constructed on RAPD data

Nei ´s (1978) gene variation average was 0.2087. Bagherzadeh (2009) reported, there was 22% variation between thyme and it is due to one province dispersal of thyme in Iran. So when variation decreases, disease increases and quality of essential oil decreases (8). *T. daenensis* (No. 8 and 10) were in the lowest genetic distance and showed the most genetic similarity, so it is suitable for gene transformation. *T. migricus* and *T. daenensis* (No. 11) were in the most genetic distance and the lowest genetic similarity and can use them for desirable hybridization because of their heterosis (Tab. 3).

Tab. 3 – Similarity matrix for 13 thyme accessions

	N1	N2	N3	N4	N5	N6	N7	N8	N9	N10	N11	N12	N13
N1	1												
N2	0.8615	1											
N3	0.830	0.806	1										
N4	0.896	0.822	0.846	1									
N5	0.802	0.808	0.825	0.861	1								
N6	0.845	0.819	0.790	0.813	0.882	1							
N7	0.802	0.808	0.867	0.817	0.826	0.794	1						
N8	0.787	0.793	0.811	0.824	0.896	0.885	0.841	1					
N9	0.821	0.796	0.785	0.837	0.887	0.875	0.802	0.879	1				
N10	0833	0. 793	0.813	0.864	0.914	0.890	0.852	0.928	0.928	1			
N11	0.769	0.7596	0.794	0.830	0.867	0.837	0.811	0.855	0.888	0.903	1		
N12	0.896	0.854	0.809	0.873	869	0.887	0.811	0.842	0.892	0.885	0.824	1	
N13	0.868	0.8307	0.852	0.854	0.825	0.806	0.839	0.840	0.814	0.854	0.808	0.885	1

Echeverigaray *et al.* (2001) showed that RAPD is suitable for identifying genetic variation in thyme. So it seems that the RAPD method is an effective tool for genetic analysis of thyme germplasm.

Conclusion

Investigation the genetic variation with RAPD markers indicated that this marker is suitable to determine the polymorphic loci and to estimate the genetic distance for thyme. Dendrogram based on RAPD data allowed separating thyme in two groups. Also there was %20 variation between thyme and is due to one province dispersal of thyme in Iran. *T. daenensis* (No. 8 and No. 10) were in the lowest distance and it is suitable for gene transformation. *T. daenensis* (No. 11) and *T. migricus* were in the most genetic distance and can use them for desirable hybridization.

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