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**ORIGINAL PAPER**

# Genetic diversity and relationships detected by FAME and RAPD analysis among *Thymus* species growing in eastern Anatolia region of Turkey

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## Abstract

*In this study, we examined phenotypic and genetic relationships between 15 Thymus species including T. canoviridis, T. haussknechtii, T. fallax, T. leucotrichus, T. pubescens, T. pseudopulegioides, T. sipyleus, T. convolutus, T. spathulifolius, T. cappadocicus, T. brachychilus, , T. kotschyanus, T. fedtschenkoi, T. migricus, T. transcaucasicus wildly growing in Eastern Anatolia region of Turkey by using Fatty acid (FAMEs) and RAPD profiles. According to the gas chromatography results, 16:0, 19:1:ω9t fatty acids were found in almost all analyzed thymus species. Also, C9 Dicarboxylic acid was observed in all species except for T. transcaucasicus, T. pubescens T. haussknechtii, T. convolutus, T. fallax. Moreover some fatty acids like 15:0 iso, C20 N alcohol, and C25 N alcohol were only observed in T. leucotrichus, T. haussknechtii, T. haussknechtii respectively (38, 28; 37, 40; 14, 78). On the other hand, RAPD analysis was carried out in 15 Thymus species. Twenty oligonucleotide primers were screened on Thymus, and among them, 7 primers showed clear polymorphic patterns. Cluster analysis of Thymus species was performed based on data from polymorphic RAPD bands, by using Jaccard's similarity coefficient. A similarity matrix showed that the highest genetic similarity (0.077) was between T. canoviridis and T. haussknechtii and the least (0.867) was between T. convolutus and T. transcaucasicus. In addition to this, three genetically distinct groups were determined between Thymus species.*

Keywords: *Thymus*; Genetic and Phenotypic Diversity; FAME; RAPD

## Introduction

The genus *Thymus* L. (Lamiaceae) consists of about 300 species of herbaceous perennials and subshrubs. This genus is mainly distributed over Mediterranean country, northern part of Africa and Southern Greenland [1]. *Thymus* is represented in Turkey flora by 38 species and 64 taxa, 20 of which are endemic [2]. Turkish name of the genus is "kekik".

*Thymus* species are commonly used as spices, herbal tea, insecticide and flavoring materials [3]. Also, *Thymus* have been most frequently used in traditional herbal medicine due to its antiseptic, carminative, expectorant, antispasmodic, antiinflamatory properties [4]. Recent studies have showed that this genus have strong antifungal, antibacterial and antioxidant activities [5,6,7]. Because of these medical features of this genus, *Thymus* is cultivated all over the world.

In spite of their economic importance, there is not much information available on the genetic relationship between T*hymus* species. Besides, earlier classification and evaluations of the genus *Thymus* were done primarily based on phenotypic expression of the plants such as color, shape and other morphological characters. However morphology and allozyme

variation have limited ability to distinguish genetically similar individuals, but this can be overcome by using molecular markers [8].

Fatty acid metil esters (FAMEs) analysis and PCR-based Random Amplified Polymorphic DNAs (RAPD) have been used to identify phenotypic and genetic variations between plant species since their applications does not need any prior information about target sequence on the genome [9]. RAPD data may provide a solution to the taxonomic problems within and among plant species. In addition, RAPD markers have been successfully employed to reveal relationship at the section level or above in several plant genera [10,11].

The aim of the current study was to evaluate the suitability of RAPD markers and FAMEs analysis to determine the relationships between 15 species of the genus *Thymus* growing in the eastern Anatolia region of Turkey.

## Materials and Methods

### Sample collection

Plant samples of 15 *Thymus* species including *T. canoviridis, T. haussknechtii, T. fallax, T. leucotrichus, T. pubescens, T. pseudopulegioides, T. sipyleus, T. convolutus*, *T. spathulifolius, T. cappadocicus, T. brachychilus, T. kotschyanus, T. fedtschenkoi, T. migricus, T. transcaucasicus* were collected at flowering stage from different locations in the vicinity located in the eastern Anatolia, Turkey. Collected plant materials were dried in shadow, and the leaves of plant were separated from the stem, and ground in a grinder with a 2mm in diameter mesh. Then, the powdered plant material was used for DNA extraction. The voucher specimen (A voucher specimen is any specimen, usually but not always a cadaver, that serves as a basis of study and is retained as a reference) has been deposited at the Herbarium of the Department of Biology, Atatürk University; Erzurum-Turkey (Table 1). Plants were collected around Erzurum in 2006-2007, and deposited at ATA *(*Atatürk University Herbarium).

### Extraction and analysis of FAMEs

Preparation and analysis of FAMEs from whole cell fatty acids from plant samples were performed according to the method described by manufacturing manual (Sherlock Microbial Identification System version 4.5, MIDI ,inc., Newark, DE). Plant leaves were powdered after lyophilization in liquid nitrogen. Approximately 40 mg of powdered cells from each samples was added to 1 ml 1.2 M NaOH in 50% aqueous methanol with 5 glass beads (3 mm dia) in a screw cap tube, then incubated at 100$^{o}$C for 30 min in a water bath. After the saponified samples were cooled at room temperature for 25 min, they were acidified and methylated by adding 2 ml 54% 6 N HCl in 46% aqueous methanol and incubated at 80$^{o}$C for 10 min in a water bath. After rapid cooling, methylated fatty acids were extracted with 1.25 ml 50% methyl-tert butyl ether (MTBE) in hexane. Each sample was mixed for 10 min and the bottom phase was removed with a Pasteur pipette. The top phase was washed with 3 ml 0.3 M NaOH. After mixing for 5 min then the top phase was removed for analysis. Following the base wash step, the extract (FAMEs) is cleaned in anhydrous sodium sulfate and then transferred into a GC sample vial for analysis.

FAMEs were separated by gas chromatography (HP6890, Hewlett Packard, Palo Alto, CA) with a fused-silica capillary column (25m by 0.2mm) with cross-linked 5% phenyl methyl silicone. The operating parameters for the study were set and controlled automatically by computer program. The chromatograms with peak retention times and areas were produced on the recording integrator and were electronically transferred to the computer for analysis, storage and report generation. Peak naming and column performance was achieved through

the use of Eukary calibration standard mix (Microbial ID 1201-A) containing nC9-nC30 saturated and 2&3 hydroxy fatty acids. Cellular fatty acids were identified on the basis of equivalent chain length data. FAME profiles of each plant species tested was identified by comparing the commercial databases (Eukary) with the MIS software package.

**DNA extraction**

Genomic DNA was extracted from powdered plant materials using a modified method described by Lin et al. [12]. Approximately 10-15 mg tissue samples from each plant species were snap frozen in liquid nitrogen in 2 ml Eppendorf tubes. 1000 µl DNA extraction buffer [100mM Tris-HCl (pH 8.0); 50mM EDTA (pH 8.0); 500mM NaCl; 2% SDS (w/v); 2% 2-mercaptoethanol (v/v); 1% PVP (w/v)] was added and mixed well. The mixture was incubated at 65$^{o}$C in a water bath for 40 min with intermittent shaking at 5 minute intervals. The mixture was centrifuged at 12 000xg for 15 min at 4$^{o}$C, the supernatant was transferred into a new 1.5 ml tube and mixed with equal volume of phenol:cloroform:isoamylalchol (25:24:1), and centrifuged. The supernatant was collected and mixed with 1/10 volume 10% CTAB-0.7M NaCl in a new tube. After centrifugation, the supernatant was collected and equal volume of cloroform:isoamylalchol (24:1) was added and mixed gently. The DNA was precipitated by the addition of 0.6 volume of freezer-cold isopropanol, left at -20$^{o}$C for 10min. The DNA was pelleted by centrifugation (12 000xg, 10min) and the isopropanol was poured off; the DNA was allowed to air-dry before being dissolved in 100 µl of TE buffer.

**RAPDs**

Samples were screened for RAPD variation using standard 20-base primers supplied by operon (Operon Technologies Inc., Alameda, CA, USA). Thirty µl of reaction cocktail was prepared as follows: 10x Buffer 3.0 µl, dNTPs (10mM) 1.2 µl, magnesium chloride (25mM) 1.2 µl, primer (5µM) 2.0 µl, Taq polymerase (5unit) 0.4 µl, water 19.2 µl sample DNA 3.0 µl (100ng/ µl). Twenty oligonucleotide primers were screened on *Thymus*, and amog them, 7 primers showed clear polymorphic patterns (Table 1).

The PCR reaction was performed as follow; the thermalcycler (Eppendorf Company) was programmed as 2 min at 95°C; 2cycles of 30 sec at 95°C, 1 minute at 37°C, 2 minute at 72°C; 2 cycles of 30 sec at 95°C, 1 minute at 35°C, 2 minutes at 72°C; 41 cycles of 30 sec at 94°C, 1 minute at 35°C, 2 minute at 72°C; followed by a final 5 minute extension at 72°C then brought down to 4$^{o}$C.

The PCR products (27 µl) were mixed with 6x gel loading buffer (3 µl ) and loaded onto an agarose (1.5% w/v) gel electrophoresis in 0.5XTBE (Tris-Borate- EDTA) buffer at 70 V for 150 min. The gel was stained in ethidium bromide solution (2 µl Etbr/100ml 1xTBE buffer) for 40 min and visualized under UV in Bio Doc Image Analysis System with Uvisoft analysis package (Cambridge, UK).

**Data Analysis**

Each gel was analyzed by scoring the presence (1) or absence (0) of polymorphic bands in individual lanes. The NTSYS-pc software [13] was used to estimate genetic similarities with the Jaccard's coefficient. The matrix of generated similarities was analyzed by the unweighted pairgroup method with arithmetic average (UPGMA), using the SAHN clustering module. The cophenetic module was applied to compute a cophenetic value matrix using the UPGMA matrix. MXCOMP module was then used to compute the cophenetic correlation, i.e., to test the goodness of fit of the cluster analysis to the similarity matrix.

## Results and Discussion

Results of our RAPD analysis are summarized in Table 1. Seven of the 20 initial primers produced clear and reproducible polymorphic bands among the 15 *Thymus* species. Those Seven random primers generated a total of 80 RAPD bands: OPA- 2, OPA- 13, OPB- 10, OPH- 16, OPH- 17, OPH- 18, OPY- 7 produced 8, 12, 13, 7, 12, 8 and 17 polymorphic bands, respectively. The size of the amplicons range bettwen 700 bp to 5000 bp. Primers OPY- 7 gave the highest number of RAPD products (18). Primers OPA- 2, OPH-16, OPH- 18 gave the lowest number of RAPD products (8) (Table 1). OPY- 7 produced 94.4 % polymorphic bands. In total 96.25 % of the bands were polymorphic.

**Table 1** Primers used in RAPD reaction with the number of RAPD markers obtained, their sequence, the size of the fragments, and the percentage of polymorphic markers (P) for each primer

| Primer name | Sequence 5'→3' | Size (bp) Min-max | Polymorphic Bands | Monomorphic Bands | Total Bands | P(%) |
|---|---|---|---|---|---|---|
| OPA- 2 | TGCCGAGCTG | 700- 1800 | 8 | 0 | 8 | 100 |
| OPA- 13 | CAGCACCCAC | 1100- 3000 | 12 | 1 | 13 | 92.3 |
| OPB- 10 | CTGCTGGGAC | 800- 2500 | 13 | 0 | 13 | 100 |
| OPH- 16 | TCTCAGCTGG | 750- 2000 | 7 | 1 | 8 | 87.5 |
| OPH- 17 | CACTCTCCTC | 1000- 5000 | 12 | 0 | 12 | 100 |
| OPH- 18 | GAATCGGCCA | 750- 2000 | 8 | 0 | 8 | 100 |
| OPY- 7 | AGAGCCGTCA | 1100- 3000 | 17 | 1 | 18 | 94.4 |
| Total | | 700- 5000 | 77 | 3 | 80 | 96.25 |

The dendrogram realized from the RAPD markers grouped the 15 genotypes into 3 major clusters (Fig. 1). Cluster 1 was *T. canoviridis, T. haussknechtii, T. fallax, T. leucotrichus, T. pubescens, T. pseudopulegioides*, *T. sipyleus, T. convolutus, T. spathulifolius, T. cappadocicus, T. brachychilus, T. kotschyanus*. Clusters 2 consist of *T. fedtschenkoi, T. migricus*. Clusters 3 consist of *T. transcaucasicus*. The greatest similarity was observed between genotype *T. canoviridis* and *T. haussknechtii* (0.077), the greatest dissimilarity was observed between genotype *T. convolutus* and *T. transcaucasicus* (0. 867).

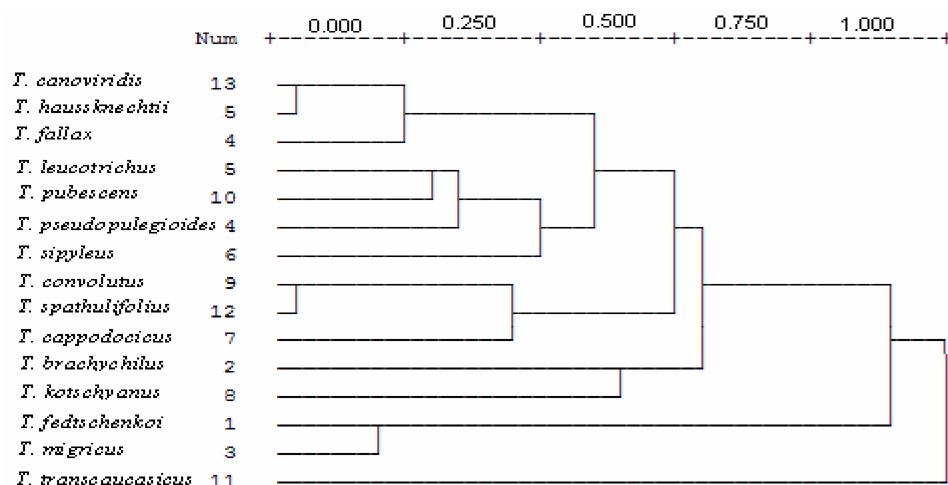


**Figure 1.** UPGM dendrogram showing the relationship of *Thymus* taxa

The *Thymus* species tested in this study were separated based on the presence and composition of 13 FAs found in all *Thymus* species (Table 2). Two fatty acids; 16:0 and 19:1:ω9t were mostly detected in all *Thymus* species. The relative proportions of the fatty acid 16:0, were the highest (89.80%) in all *Thymus* species. 15:0ISO, C20 N alcohol, C25 N alcohol were only observed in *T. leucotrichus, T.haussknechtii, T.haussknechtii* respectively (38,28; 37,40; 14,78). Only 16:0; C9 Dicarboxylic acid; 18:1: ω9c and 19:1: ω 9t fatty acids were determined in *T. spathulifolius, T. sipyleus, T. migricus, T.canoviridis, T. pseudopulegioides, T. fedtschenkoi.*

**Table 2** Composition of fatty acids in the *Thymus* taxa

| Fatty acids | *T. kotschyanus* | *T. spathulifolius* | *T. cappadocicus* | *T. leucotrichus* | *T. brachychilus* | *T. transcaucasicus* | *T. pubescens* | *T. sipyleus* | *T.haussknechtii* | *T. convolutus* | *T. migricus* | *T. fallax* | *T.canoviridis* | *T. pseudopulegioides* | *T. fedtschenkoi* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 15:0ISO | - | - | - | 38.28 | - | - | - | - | - | - | - | - | - | - | |
| 16:0 | 40.67 | 34.23 | 13.84 | 10.39 | 70.52 | 14.51 | 43.83 | 36.13 | - | 89.80 | 37.04 | 50.41 | 45.06 | 40.89 | 50.24 |
| 21:0 | - | - | 34.15 | - | - | - | - | - | - | - | - | - | - | | |
| 13:1 w5c | - | - | - | 9.08 | 6.56 | - | - | - | - | - | - | - | - | | |
| C15 N Alcohol | - | - | - | 10.79 | - | - | - | - | - | - | - | - | - | | |
| 16:1 w7t | - | - | 10.92 | 13.82 | - | - | - | - | - | - | - | - | - | | |
| C9Dicarboxylic acid | 19.89 | 14.98 | 6.04 | 5.61 | 9.13 | - | - | 10.67 | - | - | 13.23 | - | 17.04 | 13.75 | 18.59 |
| C21 primary alcohol | - | - | 17.97 | - | - | - | - | - | - | - | - | - | - | | |
| C20 Nalcohol | - | - | - | - | - | - | - | - | 37.40 | - | - | - | - | | |
| 18:1:ω9c | - | 27.35 | - | - | - | 85.49 | 29.39 | 29.78 | - | - | 17.18 | 19.93 | 19.19 | 21.76 | 14.18 |
| 25:0 2OH | - | - | - | - | - | - | - | - | 15.74 | 10.20 | - | - | - | | |
| 19:1:w9t | 39.44 | 22.64 | 17.08 | 12.04 | 13.79 | - | 26.78 | 23.42 | 31.08 | - | 32.54 | 29.66 | 18.70 | 23.60 | 16.99 |
| C25 N alcohol | - | - | - | - | - | - | - | - | 14.78 | - | - | - | - | | |

This paper constitutes the first application of RAPD markers and fatty acid profiles for the study of genetic relationship among species of *Thymus* growing in eastern Anatolia of Turkey. Both FAME and RAPD profiles have been used to study biochemical and genetic diversity in several plant species such as accessions of *Triticum* [14], *Cicer* [15], *Persimmon* [16], *Tripsacum* [17], *Vicia* [18], *Astragalus* [19], Salvia [20] showing that there are some level of similarities between both techniques which support our findings. RAPD markers have been used to determine genetic relationship at the species and subspecies categories. It is particularly useful for resolving relationship between closely related species and populations of genetically variable species [21].

## Conclusions

In conclusion, the results demonstrated that FAME and RAPD analyses are useful for evaluation biochemical and genetic diversity between *Thymus* species. This data provides a scientific basis for future studies on *Thymus*. A further study is necessary to determine the sequences of the polymorphic RAPD bands for each *Thymus* species tested, and used for their identification and characterization studies in the future. Additional phylogenetic studies using chloroplast or mitochondrial gene sequences or nuclear genes like Internal Transcribed Spacer (ITS) sequences of ribosomal DNA can be helpful to reevaluate the systematic positions of different *Thymus* species.

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