An-Najah National University
Faculty of Graduate Studies

Molecular Genetic Assessment of Selected *Thyme* Species in Palestine Using Random Amplified Polymorphic DNA (RAPD)

By

Esra` Madhat Mas`od Solyman

Supervisor

Dr. Raed Alkowni

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This thesis was defended successfully on 16 / 6 / 2013 and approved by

Committee Defense Members	**signature**
1. Dr. Raed Alkowni (Supervisor)	.....
2. Dr. Ghaleb Adwan (Internal Examiner)	.....
3. Dr. Naim Iraki (External Examiner)	.....

Dedication

To my dear parents, sisters, brothers, and husband, for their patience and encouragement, with love and respect.

Acknowledgments

I would like to express my sincere special thanks and gratitude to my supervisor Dr. Raed Alkowni for his encouragement, guidance, patience, and help throughout this study.

Thank for faculty members of Graduate Studies and Biology department at An-Najah national University for their efforts and support during my Master program.

إقرار

أنا الموقع أدناه، مقدم الرسالة التي تحمل العنوان:

Molecular Genetic Assessment of Selected *Thyme* Species in Palestine Using Random Amplified Polymorphic DNA (RAPD).

أقر بأنّ ما شملت عليه هذه الرسالة إنّما هو نتاج جهدي الخاص، باستثناء ما تمّت الإشارة إليه حيثما ورد، وإنّ هذه الرسالة ككل، أو أيّ جزء منها لم يُقدّم من قبل لنيل أيّ درجة أو لقب علميّ لدى أيّ مؤسسة تعليمية أو بحثية أخرى.

Declaration

The work provided in this thesis, unless otherwise referenced . Is the researcher`s own work and has not been submitted from anywhere else, for any other degree or qualification.

Student`s name: اسم الطالب:

Signature: التوقيع:

Date: التاريخ:

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List of Abbreviations

RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment length Polymorphisms
PCR	Polymerase Chain Reaction
AFLP	Amplified Fragment Length Polymorphisms
WCSP	World Checklist of Selected Plant Families
EDTA	Ethylendiaminetetraacetic acid
SDS	Sodium Dodecyl Sulfate
TE	Tris –EDTA
TAE	Tris/Acetate/EDTA
PCIA	Phenol- chloroform–isoamyl-alcohol
SPSS	Statistical Product and Service Solutions

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Abstract

Traditionally, subjective methods based on the morphological features such as shape, color, texture, and odor are used for the discrimination of herbal medicines. However, these methods fail to provide an accurate for discrimination and authentication. The use of highly discriminatory methods for the identification and characterization of genotypes is essential for plant production and their appropriate use. The RAPD (Random amplified polymorphic DNA) method was used for the genetic fingerprinting of five Palestinian thymes: *Thymus syriacus*, *Thymus fruticosus*, *Thymus incanus*, *Thymus majorana*, and *Thymus capitatus*. Among the polymerase chain reaction (PCR)-based molecular techniques, random amplified polymorphic DNA (RAPD) is convenient in performance and does not require any information about the DNA sequence to be amplified. Due to its procedural simplicity, RAPD was used as molecular markers for taxonomic and systematic analyses of plants. After DNA extraction from thyme species, ten decamer primers were tested for their ability to amplify DNA from selected thyme species using RAPD-

PCR. The RAPD-PCR products were separated by gel electrophoresis, and then the data were analyzed.

Eight of these primers were found to produce fragments for the studied plant species (OPD-19, OPH-02, OPAN-08, PH-01, KFP-6, OPAE-07, OPJ-06 and OPG-66). Three of these primer (OPD-19, OPH-02 and OPAN-08) were polymorphic and generated 78.6% average polymorphism across five *Thyme* species. The primer OPD-19, and OPH-02 was capable of differentiating thyme species from each other. Pairwise similarity of banding pattern between the studied plant species ranged from 0.18 to 0.67 for the three primers (OPD-19, OPH-02, and OPAN-08).

In this study, genetic diversity of five Palestinian *Thyme* species was revealed successfully using RAPD markers that could be useful in *Thyme*-breeding programs Palestine. Also these markers could assist in the identification of components in herbal medicine complexes leading to significant improvement in quality control.

Chapter One

Introduction

1.1 General background

West Bank is one of the richest places in the world in plant diversity, where valleys and mountains are covered with a different numbers of plant species (Médail and Quézel, 1997). About 2600 species exist on this small Mediterranean area, out of which more than 700 species are cited in ethnobotanical data and therefore named as Palestinian medicinal plants (Abu-Lafi *et al.*, 2007). Medicinal plants have broad popularity use in Palestine as alternative medicines to artificially synthesized ones (Azaizeh *et al.*, 2006). Medicinal plants contain powerful natural and bioactive chemical constituents and at the same time they are cheaper than those pharmaceutical drugs (Halberstein, 2005). Recently due to extensive harvesting, modern agricultural practices, environmental threats and consumption changes, some of the most popularly used medicinal plants are started to decrease in number (Alkowni and Sawalha, 2012; Abou Auda, 2010) and they are expected to be considered as threatened species.

Therefore, appropriate measures for the preservation of plant species in Palestine are needed. Proper identification is important for the preservation of plants growing in this region. Traditionally, subjective methods based on the morphological features such as shape, color, texture, and odor were used for the discrimination of herbal medicines. However, these methods are difficult to apply for an accurate discrimination and authentication use (Arif *et al.*, 2010). The use of chromatographic techniques and marker

compounds to standardize botanical measures were also restricted since the medicines have variable sources and chemical complexity, which is affected by growth, storage conditions and harvest times (Joshi *et al.*, 2004).

To optimally manage genetic resources for improvement of the cultivars, and to maintain and restore biodiversity, knowledge of genetic diversity within species is necessary (Karp *et al.*, 1997). 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; Lynch and Milligan, 1994; and Alamdary *et al*., 2011).

1.2 The genus *Thymus*

The genus *Thymus* which belongs to the family *Lamiaceae*, includes several hundreds of species distributed over nearly the whole world (Akcin, 2006). The Mediterranean region can be considered as the center of this genus (Stahl-Biskup and Seaz, 2002).

Thymus L.; referred to as Za`ater in Palestine, is an aromatic plant with increasing importance in food processing, and it`s one of those used in folk

medicine. Volatile oil constituents of thyme are used as antiseptic, antioxidant, insecticidal, preservative and anaesthetic. Theses properties of thyme due to their biologically active substances, such as thymol, carvacrol, linalool, geraniol and other volatiles in the essential oil (Akcin, 2006). Recent studies have shown that *Thymus* species have strong antibacterial, antifungal, antiviral, antiparasitic, spasmolytic and antioxidant activities (Stahl-Biskup and Seaz, 2002).

Thyme is useful in food and aroma industries and nowadays in phytotherapy. The genus taxonomy appears quite complex (Zarzuelo and Crespo, 2002), while reports on DNA-based fingerprinting of *Thymus* species are promising.

1.3 Genetic markers

Genetic markers are any coding and non- coding DNA sequence that can be used to understand genetic events. For most purposes, the marker must be polymorphic. In the case of a gene used as a genetic marker, it is the variation that is of interest, not the gene function. (Cummings, 1997). Genetic marker could be divided into four types as following:

- Single-locus marker: marker that derives from a single locus in the genome, such as allozymes, most RFLPs and the typical microsatellite markers.

- Dominant marker: marker that is scored as present or absent (null) and thus does not allow identification of homologous alleles (i.e. dominant markers fail to distinguish AA from Aa genotypes).

- Co-dominant marker: marker that allows identification of homologous alleles and thus scoring of homozygote and heterozygote states. For many population genetic questions, co-dominant markers are clearly superior to dominant markers because they allow estimation of allele frequencies; and for a given level of analytical power, co-dominant markers require smaller sample sizes than dominant markers.

- Multilocus marker: marker that screens many loci in the genome, as in the random amplified polymorphic DNA polymerase chain reaction (RAPD–PCR), amplified fragment length polymorphism PCR (AFLP–PCR) and minisatellite DNA fingerprinting (Lynch and Milligan, 1994).

1.4 Molecular-typing approaches that have been used to detect variation among plants

New technological developments have expanded the range of DNA polymorphism assays for genetic mapping, marker- assisted plant breeding, genome fingerprinting and for investigating genetic relatedness. These

technologies include restriction fragment length polymorphisms (RFLP), random amplified polymorphic DNA markers (RAPD), and amplified fragment length polymorphisms (AFLP) (Powell *et al.*, 1996).

1.4.1 Restriction fragment length polymorphisms (RFLPs)

Sequence variation can be revealed by differences in DNA fragment lengths resulting from digestion with a restriction enzyme. These enzymes can recognize and cut DNA at a specific short sequence. The specific sequences are usually palindromic. The resulting DNA fragments are then separated based on their length, by a process known as agarose gel electrophoresis. The separated DNA fragments are then transferred to a membrane via the Southern blot. Hybridization of the membrane to a labeled DNA probe then determines the length of the fragments which are complementary to the probe. RFLP occurs when the length of a detected fragment varies between individuals. Each fragment length is considered an allele, and can be used in genetic analysis. Most RFLP markers are codominant and highly locus-specific (Ulrich *et al.*, 1999).

Since the early 1980's, RFLPs have been used successfully for a wide range of plant species. However, the RFLP assay is time consuming, labor intensive, requires relatively large amount of sample, and requires high molecular weight, un-degraded DNA (Powell *et al.*, 1996).

1.4.2 Amplified fragment length polymorphisms (AFLPs)

AFLP are polymerase chain reaction (PCR)-based markers for the rapid screening of genetic diversity. AFLP uses restriction enzymes to digest genomic DNA, but DNA should be highly purified and free of polymerase chain reaction (PCR) inhibitors, followed by ligation of adaptors to the sticky ends of the restriction fragments. The end sequences of each adapted fragment consist of the adaptor sequence and the remaining part of the restriction sequence. These known end sequences serve as priming sites in the subsequent AFLP–PCR. The amplified fragments are separated and visualized on denaturing polyacrylamide gels, either through autoradiography or fluorescence methodologies, or via automated capillary sequencing instruments (Vos *et al.*, 1995; Ulrich *et al.*, 1999)

The time and cost efficiency, reproducability and resolution of AFLPs are superior or equal to most of the other markers. The AFLP methods primarily generate dominant rather than co-dominant markers (Powell *et al.*, 1996).

The main disadvantage of AFLP–PCR is the difficulty in identifying homologous markers (alleles), rendering this method less useful for studies that require precise assignment of allelic states, such as heterozygosity analyses. However, this method is still in use due to the reliable, and high-resolution markers that it generates. AFLP markers can be generated for any organism with DNA, and no prior knowledge about the genomic

makeup of the organism is needed. Therefore, AFLPs have broad taxonomic applicability and have been used effectively in a variety of *taxa*, including bacteria, fungi, animals and plants (Ulrich *et al.*, 1999).

1.4.3 Random amplified polymorphic DNA (RAPD)

RAPD markers are generated by amplification of random DNA segments with short primers (usually about ten nucleotides length) of arbitrary nucleotide sequence. If two of such primers bind to target DNA in appropriate orientation and on opposite strands, an amplified product could be generated. In many instances, only a small number of primers are necessary to identify polymorphism within species (Williams *et al.* 1990). Indeed, as Mulcahy *et al.* (1995) reported, a single primer may often be sufficient to distinguish among all of the sampled varieties. In general, one primer may produce 5-10 discrete DNA fragments of size 30-3000bp that could be separated by agarose gel electrophoresis. Due to its procedural simplicity, the use of RAPD as molecular marker for taxonomic and systematic analyses of plants, as well as in plant breeding and the study of genetic relationships, has considerably increased (Arif *et al.*, 2010).

The standard RAPD technique (Williams *et al.*, 1990) utilizes short synthetic oligonucleotides (10 bases long) of random sequences as primers to amplify nanogram amounts of total genomic DNA under low annealing temperatures by PCR. Amplification products are generally separated on

agarose gel and stained with ethidium bromide. At an appropriate annealing temperature during the amplification process, oligonucleotide primers of random sequence bind several priming sites on the complementary sequences in the template genomic DNA and produce discrete DNA products provided that these priming sites are within an amplifiable distance of each other. The profile of the amplified DNA primarily depends on the nucleotide sequence homology existing between the template DNA and the oligonucleotide primer at the end of each of the amplified product. Nucleotide variation between different sets of template DNAs will result in the presence or absence of bands because of changes in the priming sites (Bardakci, 2001). RAPDs are dominant markers, but homologous alleles can sometimes be identified with the help of pedigrees (Ulrich *et al.*, 1999). RAPD technique can be used to determine taxonomic identity, assess kinship relationships, analyze mixed genome samples, and create specific probes. Main advantages of the RAPD technology include (i) suitability for work on anonymous genomes, (ii) applicability to problems where only limited quantities of DNA are available, (iii) efficiency and low expense (Hadrys, 1992).

1.4.4 Microsatellites

a simple sequence repeat (SSR) consisting of two to six, but usually two or three, nucleotides that are repeated many times in tandem and that show

high variation in repeat number between individuals. By developing PCR primers for the regions flanking a microsatellite repeat, microsatellite allele variation at this site can be screened through high-resolution electrophoresis of microsatellite PCR products. Development of a sufficient number of microsatellite primers requires considerable molecular skills (i.e. cloning and sequencing) and patience (involving a minimum of several months of work). Microsatellite primers developed for one species can rarely be used beyond the very closest relatives; practically, therefore, microsatellite primers need to be developed de novo for each new species. The analytical strengths of microsatellite markers are co-dominance and hypervariability (the typical microsatellite locus has more than two alleles, if not dozens) (Ulrich *et al.*, 1999).

A recently developed modification of SSR-based marker systems, i.e. ISSR (intersimple sequence repeat) analysis, circumvents this requirement for flanking sequence information, and thus has found wide applicability in a variety of plants. ISSR analysis involves PCR amplification of genomic DNA using a single primer that targets the repeat *per se*, with 1-3 bases that anchor the primer at 3′ or 5′ end. In addition to freedom from the necessity of obtaining flanking genomic sequence information, ISSR analysis is technically simpler than many other marker systems. The method provides highly reproducible results and generates abundant polymorphisms in many systems (Liu *et al*, 2001).

1.5 Aim of study

Beside acquiring knowledge and technology transfer, this research study aims to use one of the most popular molecular tools (RAPD technique) for DNA fingerprinting on medicinal aromatic plants. For that purpose, selected thyme species, grown naturally in Palestine, were used for identifying their genetic relationship using RAPD.

Chapter Two

Materials and Methods

2.1 Plant material collection

Five thyme species [*Thymus syriacus* Bioss synonym *of Majorana syriaca* (L.) Raf.; *Thymus fruticosus* (L.) Link synonym of *Micromeria fruticosa* (L.) Druce; *Thymus incanus* Sm synonym of *Calamintha incana* (Sm.) Boiss; *Thymus majorana* (L.) Kuntze synonym of *Origanum majorana*; *Thymus capitatus* (L.) Hoffmanns & Link synonym of Coridothymus *capitatus* (L.) Rchb.f.] were collected from different areas in north West Bank and potted under suitable growth conditions.

2.2 Taxonomical analysis of the collected plant species

The collected thyme species were classified based on their morphological characters and their botanical properties, since they are belonging to different species.

The morphological analysis of thyme species was carried on freshly collected samples from different locations in northern part of Palestine. The different parts of thyme species (stem, leaves, and flowers) were identified to determine their classification. Some of the morphological characters of selected thyme species, which were used for thymes identifications, are illustrated in table 2.1.

Table 2.1. Some characters of thyme species used for taxonomical analysis.

Species	**Botanical Characters for taxonomical analysis**
Thymus fruticosus (L.) Link 	Habitat: Hard rock outcrops Floresence: February, March, April, May, June. Petal or tepal color : white, Cream Stem: 40-70cm, square in cross section Leaf color and shape: silvery gray, egg shaped. Leaf arrangement : opposite (two leaves per node) Leaf type : entire Leaf or leaflet margin : smooth Life form: dwarf evergreen shrub
Thymus incanus Sm	Habitat : Batha, Phrygana Floresence : May, June, July, August, September, October Petal color : Purple, pink Stem: 40 – 60 cm, it is not square in cross section Leaf color: grey-green Leaf arrangement : opposite Leaf type : entire Leaf or leaflet margin : dentate or serrate

Life form: non-woody perennial

Thymus syriacus Bioss

Habitat : Mediterranean maquis , Batha, Phrygana, Hard rock outcrops

Floresence: April, May, June, July, August, September

Petal color : white

Stem: reaches a height of 40 cm, The stem is not square in cross section

Leaf arrangement: opposite

Leaf color and shape: Soft-gray-green, small, broad ovate, felty. The veins are prominent on lower side.

Leaf type : entire

Leaf or leaflet margin : smooth

Life form: woody perennial shrub

Thymus capitatus

Habitat: Batha, Phrygana, Dry, calcareous soils.

(L.) Lk. & Hoffm.

Floresence: May, June, July, August, September, October

Petal color : Purple, pink

Stem: growing to 30 cm, is square in cross section

Leaf arrangement : opposite (two leaves per node)

Leaf color and shape: fleshy, narrow green leaves

Leaf type : entire

Leaf or leaflet margin: smooth

Life form: shrub

Thymus majorana (L.) Kuntze

Habitat : Hard rock outcrops, Phrygana (Dry slopes and rocky places, occasionally in partial shade)

Floresence : June, July, August, September

Petal color : white

Stem: growing to 40 cm, it is square in cross section. Red-brown stem.

Leaf arrangement : opposite

Leaf color and shape: small and hairy green, tongue shaped

Leaf type : entire

Leaf or leaflet margin : smooth

Life form: non-woody perennial

After that, the putatively species had been identified, comparison with its corresponding species identified in the region, such as: *flora* of Palestine (Zohary, 1996), *flora* of Israel (Danin, 2006), and Traditional Arabic Palestinian Herbal Medicine (Ali-Shtayeh and Jamous, 2008) was ascertained. The classification and the scientific names of Thyme species were also determined and reviewed using plant list web site, WCSP (World Checklist of Selected Plant Families), PFAF (Plant For A future), wild flower of Israel. Also, Our classifications were confirmed by local thyme's experts.

Salvia officinalis was introduced to the study as random out group to ensure the validity of RAPD in recognizing the relationship among thymes but no other plants.

The leaves of each species were taken to be used in the research as source of DNA.

2.3 DNA Extraction

Genomic DNA from young leaves of each thyme species as well as *Salvia officinalis* (as out group) were isolated by using plant DNA miniprepration (Dellaporta *et al*., 1983) following the manufacturer protocol. Briefly, plant samples were ground using mortar and pestle. Ground tissues (50 mg) were mixed with 500 µl extraction buffer consisting of 500 mM NaCl, 100 mM

Tris–HCl pH 8.0, 50 mM ethylendiaminetetraacetic acid (EDTA), and 10 mM 2-mercaptoethanol. Then 33 µl of 20% SDS were added before the incubation of suspension at 65℃ for 10 min with shaking. After that, 160 µl of 5 M potassium acetate were added, then centrifuged for 10 minutes at 14000 rpm (MICRO 120, Hettich/Zentrifugen, Germany). Then 2 µl of RNAase were added to the supernatant and incubated at 37℃ for 10 minutes, followed by phenol- chloroform–isoamyl-alcohol (PCIA 25: 24:1) treatment. Then 500 µl PCIA were added to the supernatant and centrifuged for 10 minutes at 14000 rpm. The supernatant was transferred to a new tube. Cold isopropanol (2 volumes) was added and the mixture was incubated for 20 minutes at -20℃. DNA extracts were centrifuged for 10 minutes at 14000 rpm, washed with 500 µl 70% ethanol and pellet was dissolved in 60 µl TE buffer (10 mM Tris-HCl, pH 8; 1 mM EDTA), and conserved at -20℃ for later use.

2.4 PCR primers

Ten decamer primers were tested for their ability to amplify DNA from five thyme species. These primers were 60% - 70% G+C content and ordered from two biotechnological companies (Metabion Hy.labs, Ltd., Israel) and (Sigma-Aldrich, USA). The primers´ names and their sequences are listed in the Table 2.2.

Table 2.2 List of PCR primers.

No.	Primer	**Sequence** 5`- 3`	**Source**
1	PH-01	AACGCGCAAC	Arif *et al.*, 2010
2	KFP-6	TCCCGACCTC	Megendi *et al*., 2010
3	OPAE-07	GTGTC GTGG	Megendi *et al*., 2010
4	OPD-19	CTGGGGACTT	Sudré *et al*., 2011
5	OPAG-02	CTGAGGTCCT	Tonk *et al*., 2010
6	OPAN-08	AAGGCTGCTG	Tonk *et al*., 2010
7	OPB-12	CCTTGACGCA	Tonk *et al*., 2010
8	OPJ-06	TCGTTCCGCA	Tonk *et al*., 2010
9	OPG-06	GTGCCTAACC	Ben el Hadj Ali, *et al*., 2012
10	OPH-02	TCGGACGTGA	Chowdhury *et al*., 2002

2.5 RAPD-PCR conditions and agarose gel electrophoresis.

Polymerase chain reactions were performed to amplify 10µl of thymes DNA in 50µl PCR mixture, containing 4 µl of 5 µM primer (0.4 µM final concentration) (sigma-Aldrich and metabion hy.labs), 25µl one Taq quick load 2x master mix with standard buffer [1x contain: 40 mM Tris–HCl, pH: 8.9, 44 mM KCl, 3.6 mM MgCl2 an, 5% glycerol, 0.4 mM each dNTP, 25 unit/ml of Taq DNA polymerase, 0.06% IGEPAL CA630, 0.05%

Tween-20, xylene cyanol FF, and tartrazine)] (New England Biolabs, USA). Beside out group, another water- sample tube (without template) was included as a negative control in each experiment to check out for any contaminations. All the reaction mixtures and components were kept on ice all the time of preparations.

RAPD-PCR amplification was performed using thermal cycler (Biometra, An Anaylik Jena Company, Germany) with the following thermal conditions: preheating for 7 min at 94℃, initial DNA denaturation for 3 min at 94℃, followed by DNA amplification for 35 cycles, each consisting of 94℃/1min, 35℃ / 1 min, and 72℃ / 2 min. Final extension was carried out at 72℃ for 10 min, the amplified products were stored at 4℃ for later gel electrophoresis analysis.. All reaction mixtures were amplified using a concluding elongation for 10 min at 72℃. Figure 2.1 shows the main stages carried for RAPD-PCR .

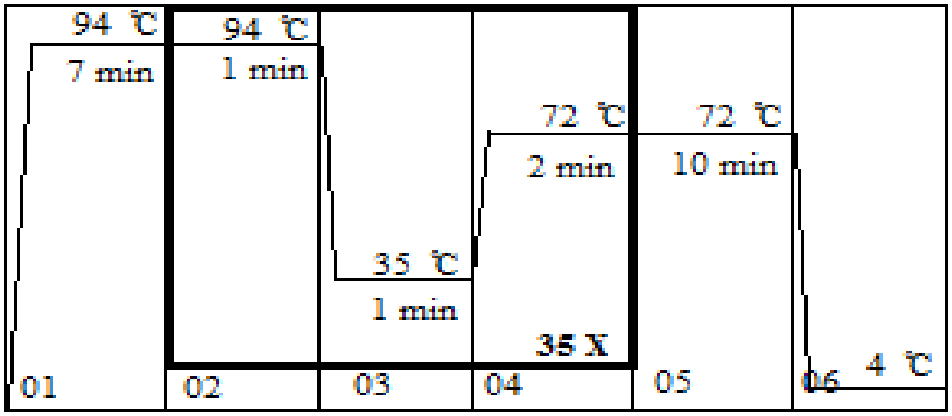


Figure 2.1. This graph illustrates PCR stages and number of cycles used in RAPD.

The RAPD-PCR products were separated by gel electrophoresis in 1.5% (w/v) agarose buffered with 1xTAE (pH 8), and stained with ethidium bromide. Bands were visualized under UV transilluminater (UVP ltd, USA), and photographed by digital camera (Canon, Japan). PCR Marker [50-2000 bp] was used as molecular weight indicator (Sigma, USA).

2.6 Data analysis

RAPD-PCR-amplified fragments were scored as present (1) or absent (0) across the 5 thyme species. Only clear and 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. Polymorphisms were calculated based on the presence or absence of bands.

Using the correlate module of SPSS software version19, genetic distance based on the Jaccard coefficient (Jaccard, 1908) was calculated after making a pairwise comparison between five thyme species relying on the proportion of shared bands produced by each used primers.

Jaccard’s coefficients, which are common estimator of genetic identity and calculated as follows:

$$\text{Jaccard's coefficient} = N_{AB}/(N_{AB}+N_A+N_B)$$

Where N_{AB} is the number of bands shared by samples, N_A represents amplified fragments in sample A, and N_B represents fragments in sample B. Similarity matrices based on these indices were calculated.

Chapter Three

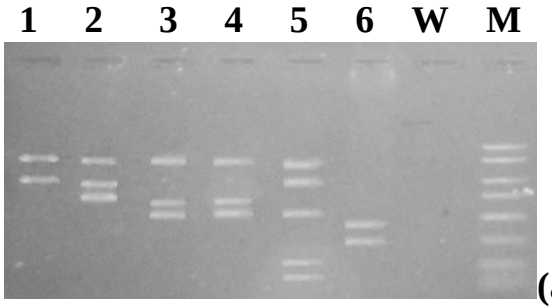
Results

3.1 Genetic polymorphism among *Thyme* species

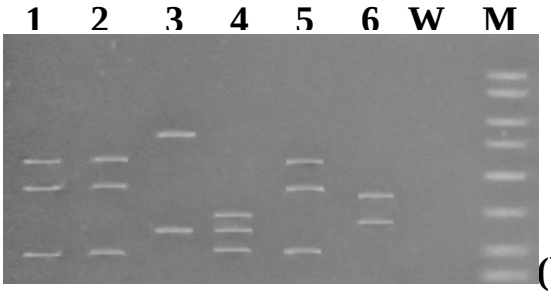
Thyme species were screened simultaneously by RAPD using ten random primers (Table 2.1) separately, including the outgroup (*Salvia officinalis)* and water sample as a negative control. Eight of these primers (OPD-19, OPH-02, OPAN-08, PH-01, KFP-6, OPAE-07, OPJ-06, OPG-06) were found to produce fragments for the studied plant species but not for water control; ensuring their reliability besides their sensitivity and specificity. These primers were also further tested twice to verify their reproducibility and consistency of RAPD banding patterns for each tested species (Fig. 3.1).

The obtained results from RAPD analysis revealed that five of these primers were producing single band for each thyme species while the other three primers were able to produce consistent patterns.

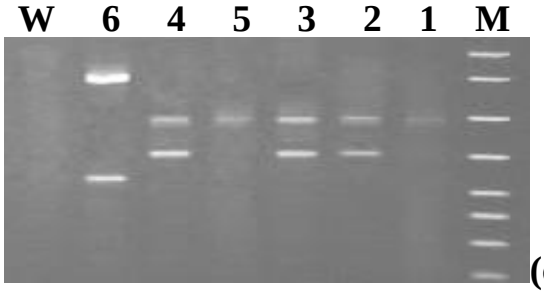
The RAPD profiles using all samples simultaneously generated amplicon products ranging from 50 to 1500 bp. For the selected thyme species, the maximum numbers of well-defined bands were observed with primer OPD-19, resulting in a total of 16 amplified products, of which 5 were monomorphic and 11 were polymorphic (Figure 3.1 a). All the fourteenths amplified DNA products obtained by OPH-02 primer were polymorphic (Figure 3.1 b). Primer OPAN-08 generated only 3 polymorphic bands out of eight (Figure 3.1 c)



(a)



(b)



(c)

Figure 3.1 Agarose gel electrophoresis analysis showing RAPD amplifications profiles of primers: OPD-19; OPH-02; and OPAN-08, respectively as shown in figures (a); (b); and (c). The following letter (M) referres to PCR Marker [50-2000 bp]; (W): Water negative control; and the numbers (1) to *Thymus syriacus*; (2): *Thymus majorana;* (3): *Thymus incanus*; (4): *Thymus capitatus; (*5): *Thymus fruticosus*; and (6)*:* an outgroup *[Salvia officinalis]*.

The Percentage of Polymorphisms were calculated by dividing *Polymorphic Bands* out of *Total Bands,* multiplied with 100% for each primer (OPD-19, OPH-02, OPAN-08) as shown in Table 3.1. The average polymorphism across five *Thyme* species was found to be 78.6%.

Table 3.1 DNA amplified bands and generated polymorphism in selected *Thyme* species using 3 RAPD markers.

No	Primer Code	Total Produced Bands	Total Common Bands	Polymorphic Bands	% of Polymorphism
1	OPD-19	16	7	6	85.7
2	OPH-02	14	6	6	100
3	OPAN-08	8	2	1	50
Total		38	15	13	
Mean		12.7	5	4.3	78.6

3.2 Primers capable of differentiating species

Our study showed that the optimized RAPD procedure was able to distinguish clearly among the five different *Thyme* species that subjected to this study. After analyzing the data, primer OPD-19 were found useful in differentiating between any two *Thyme* species except *Thymus incanus* and *Thymus capitatus*. It was observed that OPD-19 primer gave 2 unique bands for *Thymus fruticosus*. Primer OPH-02 was found to be very useful for distinguishing *Thymus incanus* from any other four *Thyme* species with a single specific band. In the other hand, Primer OPH-02 generated one specific band for *Thymus capitatus*, which was absent in the other species; thus enabling to distinguish between *Thymus capitatus* and any other *Thyme* species used in this study. However, Primer OPAN-08 did not show

discriminatory bands for all species, as similar banding patterns were observed for *Thymus majorana* , *Thymus incanus*, and *Thymus capitatus*, and also similar band for *Thymus capitatus*, and *Thymus syriacus*.

3.3 Genetic relationship between *Thyme* species

Data of RAPD profiles scanned from the five *Thyme* species with 3 reproducible primers was used to generate similarity coefficients with Jaccard measure using SPSS software version19 which illustrated in Table 3.2.

Pairwise similarity of banding pattern between the studied plant species ranged from 0.18 to 0.67 for the three primers (OPD-19, OPH-02, OPAN-08).

The maximum pairwise similarity values (0.67) were observed between *Thymus syriacus* versus *Thymus majorana*; *Thymus syriacus* versus *Thymus capitatus*; and *Thymus fruticosus* versus *Thymus incanus*, suggesting them to be most closely related. The lowest pairwise similarity value among thyme species was observed between *Thymus syriacus* and *Thymus incanus* (0.18) indicating them as genetically most diverse.

Table 3.2 Similarity matrix for the five *Thymus* species by Jaccard's coefficient based on RAPD bands generated by primers OPD-19, OPH-02, and OPAN-08.

Thymus species	*T. syriacus*	*T. majorana*	*T. incanus*	*T. fruticosus*	*T. capitatus*	*S. officinalis*
T. syriacus	1.00					
T. majorana	**0.67**	1.00				
T. incanus	0.18	0.33	1.00			
T. fruticosus	0.27	0.42	**0.67**	1.00		
T. capitatus	**0.67**	0.50	0.23	0.31	1.00	
S. officinalis	0.08	0.07	0.00	0.07	0.06	1.00

Thymus majorana showed closest relationship with *Thymus capitatus*, *Thymus fruticosus*, and *Thymus incanus* having similarity values of 0.50, 0.42 and 0.33, respectively. *Salvia officinalis* as an outgroup, confirmed the reliability of the RAPD method and similarity analysis tests to show quite divergent from other thymes species with similarity coefficient range from 0.00- 0.08 as shown in similarity matrix Table 3.2.

The obtained distance coefficients (based on the Jaccard coefficient) were used to construct a dendrogram using classify module of SPSS software version19, as shown in figure 3.2.

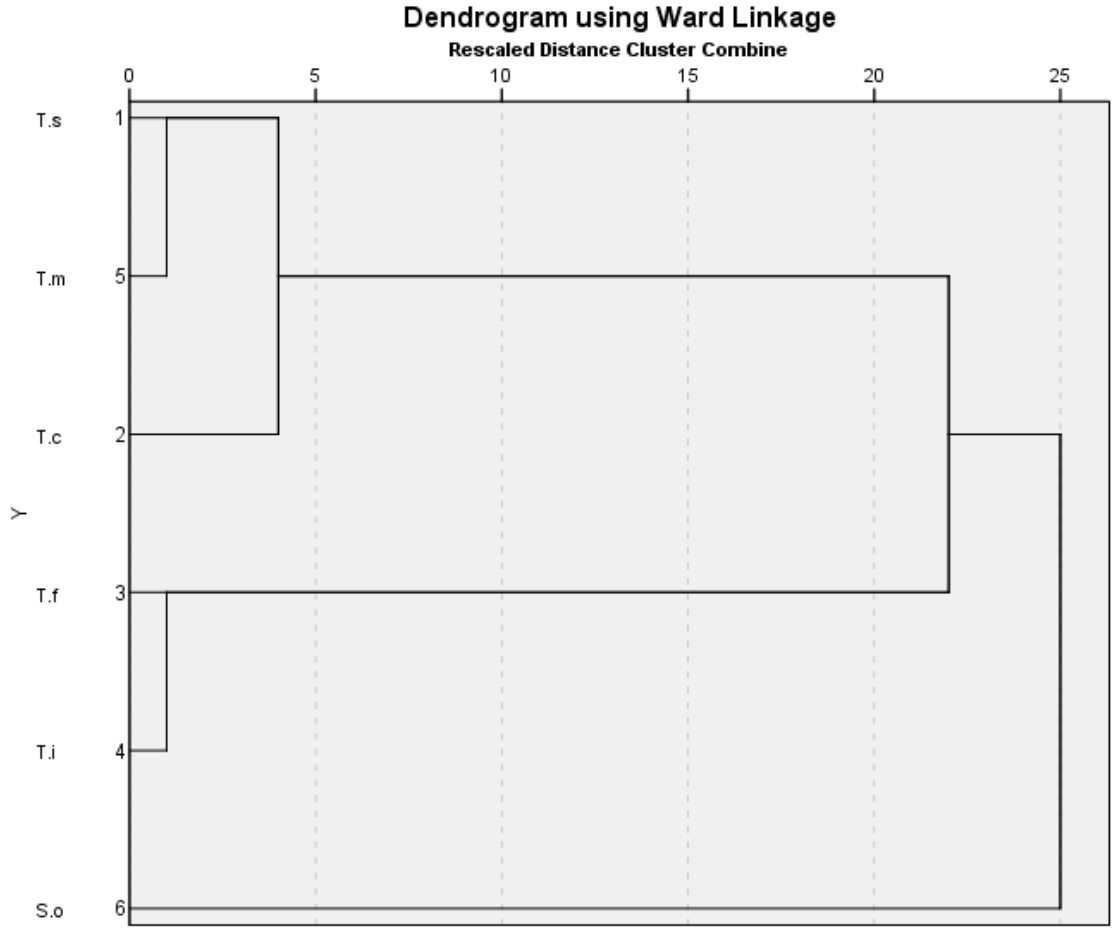


Figure 3.2. The cluster tree dendrogram generated by using classify module of SPSS software version19, for five thyme species where the abbreviation letters refer to as, T.s *: Thymus syriacus*, T.f: *Thymus fruticosus*, T.i: *Thymus incanus*, T.m: *Thymus majorana* , and T.c: *Thymus capitatus.* S.o*: Salvia officinalis* was used as an outgroup.

The cluster tree analysis showed that thyme species were divided into 2 main groups: *Thymus syriacus*, *Thymus majorana* , and *Thymus capitatus* in one group, and *Thymus fruticosus*, and *Thymus incanus* in another group. However, *Salvia officinalis* was found to be quite divergent and did not fall

in any of the major clusters as illustrated in Figure 3.2. It was separated from the above groups at a similarity coefficient of 0.08 as shown in similarity matrix in Table 3.2

Chapter four

Discussion

In this study, five Thyme species were identified and characterized using one of the molecular tools, which revealed genetic relationship among the selected thyme species. To the best of our knowledge, no molecular tool like RAPD has been reported in Palestine yet on Thymes.

Nearly 78.6% of the bands were polymorphic that confirmed the result obtained from other research studies (Bagherzadeh, 2009; Ben el Hadj Ali *et al.*, 2012; Pluhár *et al*., 2012; Khalil *et al*., 2012 and Alamdary *et al*., 2011) where the reported polymorphism percentage in thymes as 92%, 88%, 80.8%, 78.73% and 62% respectively. The relatively high level of polymorphism among the five thyme species (78.6%) revealed in this study, might suggest the possibility of the chosen primers screened with RAPD markers were generated on most genetic loci polymorphic among the species (Tonk *et al.*, 2010).

The primers OPD-19, OPH-02, and OPAN-08 produced 7, 6, and 2 bands in accordance with other RAPD research studies. For examples, the KFP-6 primer produces 6 bands when used in studying genetic diversity between cultivated and non-cultivated *Moringa oleífera* Lam. provenances assessed by RAPD markers using 96 individuals (Megendi *et al*., 2010), and the OPD- 19 primer produce 2 bands when used in molecular characterization of 27 wild genotypes of hawthorn (*Crataegus azarolus* L.); in the same study also, the number of polymorphic band per primer was 4.82 (Dumireih *et al.*, 2010).

Limited number of RAPD bands were quite normal as seen in different studies. Using RAPD Technique in genetic variability study of selected Turkish oregano (*Origanum onites* L.) clones, primers OPAG-18, OPAG-06, OPAC-12, OPAG-02, and OPG-07 were able to produce 3, 5, 5, 6, and 6 polymorphic bands from fourteen Turkish oregano clones (Tonk *et al*., 2010). In other RAPD analysis of *Thymus* species growing in eastern Anatolia region of Turkey, primers OPA-2, OPH- 16, and OPH- 18 were also able to produce totally 8 bands (Sunar *et al.*, 2009). In other research studies, determination of genetic variation and relationship in *Thymus vulgaris* populations in Syria by RAPD markers, 27 primers were used which were able to produce 198 total bands, with mean of 7.3 bands for each primer (Khalil *et al*., 2012). Comparing with this research study 12.7 bands was the mean for each of the 3 used primers, which considered good enough to reveal the genetic relationships among selected thymes. Furthermore, it was reported that the number of amplification products per primer could be varied from 8 (OPP3) to 16 (DPA 9) when used in RAPD studies for 13 accessions of thymes species (Alamdary *et al*., 2011).

So far, RAPD primers (OPD-19 and OPH-02) were able to distinguish clearly the five different *Thyme* species that subjected to this study, while sequence-based analysis failed to distinguish between them in other studies due to significant similarity found between their DNA sequences in the amplified region. In addition to that, RAPD primers were able to distinguish *taxa* below the species level (Choo *et al.*, 2009), since RAPD

analysis reflects both coding and non-coding regions of the genome (Vanijajiva *et al.*, 2005). So it would not be surprisingly the use of RAPD to detect the genetic diverse among different plant species in Palestinian figs, faba beans, wheat, …etc, using the same procedure with same or different primers that used in this study (Al-Fares and Abu-Qaoud, 2012; Basheer salimia *et al*., 2012)

Unexpectedly, Primers (PH-01, KFP-6, OPAE-07, OPJ-06, and OPG-66) were able to generate a single band for *Thymus incanus, Thymus capitatus, Thymus syriacus, Thymus fruticosus,* and *Thymus majorana* respectively. These results will be promising for future fingerprinting studies on thyme species and variants. Also could be the future research work for specific detection of species after testing them on wide varieties of thymes and other medicinal plants. Similar results had been reported when RAPD technique used to study genetic divergence among *Dimorphandra* spp. accessions where the primer OPAE-09 produced only single polymorphic band (Sudré *et al*., 2011), also Primer OPC-6 generated specific bands, only one specific band to *Tinospora cordifolia*; *Emblica officinalis*, and *Tribulus terestris* (Shinde *et al*., 2007).

The coefficient value of 0.67 which was found between thyme species (*Thymus syriacus* versus *Thymus majorana*, *Thymus syriacus* versus *Thymus capitatus*; and *Thymus fruticosus* versus *Thymus incanus*), was within the range of those obtained in previous studies: among *Thymus*

capitatus and *Thymus algeriensis* accessions (Ben el hadj ali *et al.*, 2012), among *Thymus vulgaris* cultivares (Echeverrigaray *et al.,* 2001), and between 13 accessions of *T. migricus* and *T. daenensis* (Alamdary *et al.*, 2011).

Information on genetic relatedness and diversity of available breeding genotypes increases the success of plant-breeding programs. In this study, genetic diversity of five Palestinian *Thyme* species was revealed successfully using RAPD markers, so this study will be valuable for any *Thyme*-breeding program could be constructed in Palestine.

RAPD procedure was able to distinguish clearly the selected different *Thyme* species subjected to this study. These species-specific RAPD markers could potentially be used for identifying *Thyme* species from any mixed population, similar approach has been successfully used for molecular diagnosis of species and cultivars by many workers (Sosinski and Doucher, 1996; Yamamoto and Duich, 1994). Also these markers could be used as a method of choice for identifying components for herbal medicine complex since RAPD technique had been used for determination of different components presented in herbal formulation (Cheng *et al.*, 1997; Cheng *et al.*, 1987). So, these will contribute significantly in quality control.

RAPD provides relatively quick results, with less time-consuming and low expensives (Arif and Khan, 2009), beside it gives information about

genomic variability below the species level (Williams *et al.*, 1990). Adding to that, the required RAPD-PCR chemicals and primers are readily available nowadays in the market.

With RAPD, it was able to identify the plant species without the need of laboratories with expensive sequencing instruments. Therefore, our findings provide guidance for identification of *Thyme* species, and help in their subsequent management and utilization in sustainable ways to combat human and natural pressures on these valuable natural resources.

The use of RAPD markers had enabled discrimination of 5 *Thyme* species and can be used successfully for any selection and improvement of such species cultivars in any future studies. These results can be further used to manipulate genetic determinants of horticulturally important traits and to characterize the basis of productivity of Thyme. Though RAPD markers proved to be a useful tool in germplasm characterization and diversity analysis of thymes, and can be used beside other molecular markers as AFLP, ISSR and SSRs.

It is worth to mention that the RAPD bands pattern obtained in this research were reproducible under the lab and experimental conditions. Nevertheless, further studies for finer molecular analysis of medicinal plants genotyping's to overcome the discrepancies left unresolved by RAPDs were advisable.

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جامعة النجاح الوطنية
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التقييم الجيني الجزيئي لأنواع مختارة من الزعتر في فلسطين باستخدام تقنية المضاعفة العشوائية متعددة الاشكال للحمض النووي DNA (RAPD)

إعداد
إسراء مدحت مسعود سليمان

إشراف
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قدمت هذه الأطروحة استكمالا لمتطلبات درجة الماجستير في العلوم الحياتية بكلية الدراسات العليا في جامعة النجاح الوطنية في نابلس، فلسطين.

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إشراف

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الملخص

تقليديا، الطرق الموضوعية استندت إلى الميزات الشكلية مثل: الشكل، واللون، والملمس، والرائحة للتمييز بين النباتات الطبية. ومع ذلك، هذه الأساليب صعبة التطبيق بدقة للتمييز والتوثيق. ان استخدام أساليب عالية التمييز لتحديد وتوصيف الأنماط الجينية أمر ضروري للإنتاج النباتي والاستخدام الانسب لهم. لقد تم استخدام تقنية المضاعفة العشوائية متعددة الاشكال للحمض النووي DNA (RAPD) لأخذ البصمات الجينية لخمس أنواع من الزعتر الفلسطيني: *Thymus syriacus*, *Thymus fruticosus*, *Thymus incanus*, *Thymus majorana*, and *Thymus capitatus*. من بين التقنيات الجزيئية القائمة على تفاعل البلمرة سلسلة (PCR) DNA، تقنية (RAPD) التي تعتبر مريحة في الأداء ولا تتطلب أي معلومات عن متسلسلة الحمض النووي ليتم تضخيمها. نظرا لبساطتها الإجرائية، فإن تقنية RAPD تستخدم كسمات جزيئية للتصنيف والتحليلات المنهجية للنباتات. فبعد استخراج الحمض النووي من الأنواع الخمسة للزعتر، تم اختبار 10 " نواشئ عشرية" لقدرتها على تضخيم الحمض النووي من أنواع الزعتر الخمسة باستخدام RAPD-PCR. ثم تم فصل منتجات RAPD-PCR بواسطة الهجرة الكهربائية في الهلام، ليتم تحليل البيانات بعد ذلك.

لقد تم العثور على ثمانية من هذه النواشئ قادرة على إنتاج قطع من أنواع النباتات الخاضعة للدراسة، وهم (OPD-19، OPH-02، OPAN-08، PH-01، KFP-6، OPAE-07،

OPJ-06، OPG-66)، ثلاثة من تلك النواشئ (OPD-19, OPH-02, OPAN-08) كانت متعدد الأشكال بمتوسط 78.6% لجميع أنواع الزعتر الخمسة. لقد كانا النواشئ OPD-19، و OPH-02 قادران على التمييز بين أنواع الزعتر الخاضعة للدراسة. وقد تراوح "التماثل الزوجي" في نمط النطاقات بين الأنواع النباتية التي درست من 0,18 الى 0,67 لثلاث نواشئ وهي (OPD-19، OPH-02، OPAN-08).

في هذه الدراسة، تم الكشف عن التنوع الوراثي لخمسة أنواع من الزعتر الفلسطيني بنجاح باستخدام تقنية RAPD ، لذلك سوف تكون هذه الدراسة قيمة لبناء أي برنامج اكثار للزعتر في فلسطين. وكذلك تمكن أيضا من استخدام هذه العلامة لتحديد طرق للكشف عن مكونات الأدوية العشبية المخلوطة، مما سيسهم إلى حد كبير في مراقبة الجودة.