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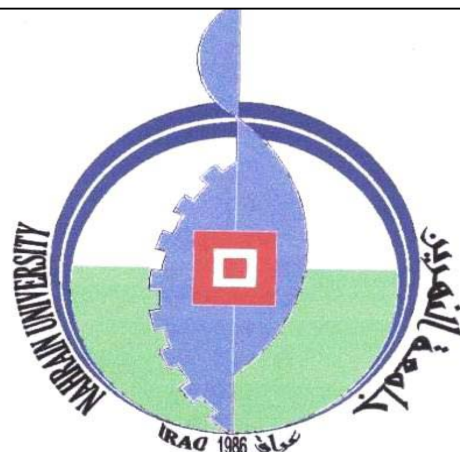

Molecular Differentiation Between Two Varieties of *Rosemarinus officinalis* Grown in North East Region of Iraq

A thesis
Submitted to the College of Science, Al-Nahrain University
in partial Fulfillment of the Requirements for the Degree of
Master of Science in Biotechnology

By
Taif Nazar Mahmood Al-Shakir
B.Sc., Biotechnology, College of Science, 2004
Al-Nahrain University

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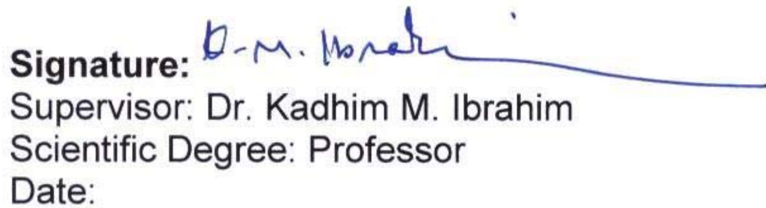

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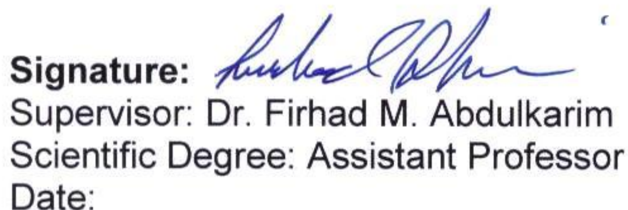

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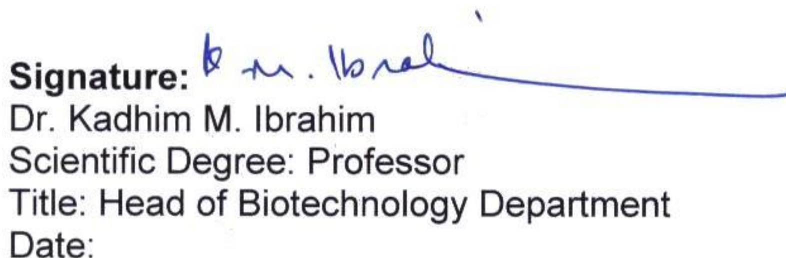

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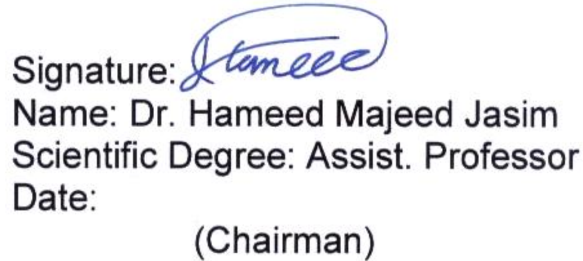

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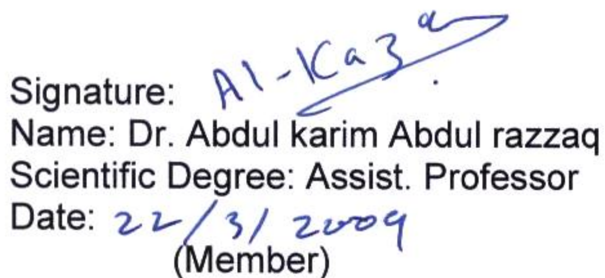

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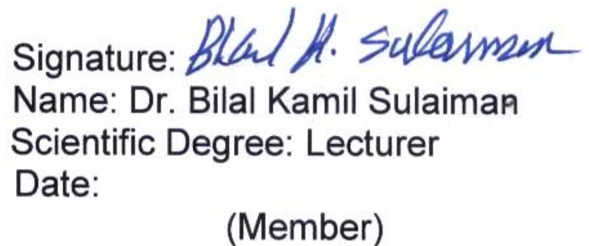

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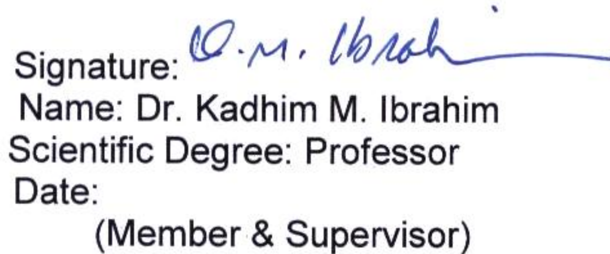

Signature:
Name: Dr. Kadhim M. Ibrahim
Scientific Degree: Professor
Date:
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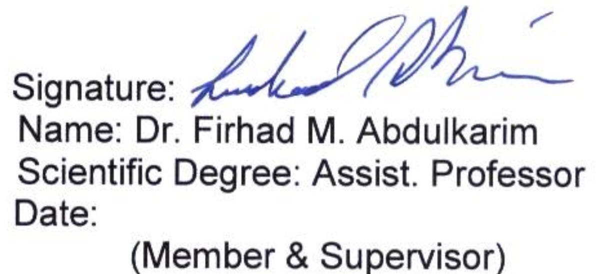

Signature:
Name: Dr. Firhad M. Abdulkarim
Scientific Degree: Assist. Professor
Date:
(Member & Supervisor)

I here by, certify upon the decision of the examining committee

Signature:
Name: Dr. Laith Abdul Aziz Al-Ani
Scientific Degree: Assist. Professor
Dean of the College of Science

Dedication

This Work is Dedicated To:

My Country Iraq

My Family

My Kid

Acknowledgment

This is to acknowledge, Allah for his merciful and support to complete this work, both my supervisors Dr.Kadhim M. Ibrahim for his kind follow up and supervision through out the whole research stages and his continuous visits to the northern region where this work was established, also Dr.Firhad M. Abdulkarim for his great supervision on the lab. Work and his ideas that led to these nice results in the Molecular Biology Part of this thesis.

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REGARDS

Taif N. Mahmood

Abstract

In an attempt to differentiate and study one of the most popular medicinal plants (*Rosemarinus officinalis* L.) grown in the North of Iraq, a number of experiments were carried out to differentiate between subspecies available in Kurdistan Province at the morphological and molecular level.

A survey and morphological study was conducted on these subspecies of rosemary in Erbil and Sulaimania governorates, it was found that there are morphological differences in leaves, flowers, shoot growth directions, number of branches, leaf size and shape, accordingly two subspecies were identified and marked as class A and class B of *R. officinalis* in north Iraq.

Subspecies A was characterized by a straight shoot growth, dark green leaves and whitish blue flowers while subspecies B have random growth, green leaves and white flowers.

In order to investigate the genetic variation, the DNA is isolated and purified from the two subspecies, and then electrophorized using agarose gel, chromosomal DNA appeared as a clear smear on gel for both A and B.

Six types of restriction enzymes were used to digest the genomic DNA of both subspecies A and B, namely *Eco*RI, *Eco*RV, *Bam*HI, *Hin*dIII, *Sal*I and *Sma*I.

Resulted genomic DNA was amplified using PCR technique for the Ribosomal DNA (rDNA) as the target sequence, of 668 bp which includes 18S, 5.8S and 26S RNA genes; separated by two ITS regions that represent the non-coding sequence of interest for subspecies identification.

It was found that there are obvious genomic differences in the rDNA of both subspecies, according to the recognition sites availability of restriction enzymes in template DNA, as there was cleavage sites for each one of *Eco*RV, *Bam*HI, *Hin*dIII and *Sal*I in rDNA sequence of subspecies A, while no one of the six restriction enzymes cleavage sites used is present in subspecies B.

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List of Abbreviations

Abbreviation	Term
°C	Degree Celsius
μl	Micro Litter
A	Adenine
A site	Aminoacyl Site
bp	Base Pair
C	Cytosine
dATP	Deoxy Adenine Tri Phosphate
dCTP	Deoxy Cytosine Tri Phosphate
DDH_2O	Double Distilled Water
dGTP	Deoxy Guanine Tri Phosphate
DNA	Deoxy Nucleic Acid
dnTP	Deoxy Nucleotide Tri Phosphate
dTTP	Deoxy Thymine Tri Phosphate
EDTA	Ethylene Diamine Tetraacetic Acid
E site	Exit Site
EF-G	Elongation Factor G
EF-Tu	Elongation Factor - Tu
fMet-tRNA	Methionine-Transfer RNA Complex
g	Gravity Factor
G	Guanine
GTP	Guanidine Tri Phosphate
IF	Initiation Factor
ITS1	Internal Transcribed Spacer 1
ITS2	Internal Transcribed Spacer 2
M	Molar
M/W	Molecular Weight
mg	Milli Gram
MgCl2	Magnesium Chloride
Min.	Minute
n	Any Nucleotide Base
NaCl	Sodium Chloride
NCBI	National Center of Biotechnology Information
OD	Optical Density
PCR	Polymerase Chain Reaction
Pmol	Pico mole
P site	Peptidyl Site
R. officinalis	*Rosmarinus officinalis*
rDNA	Ribosomal DNA
RF	Release Factor
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribo Nucleic Acid

RNP	Ribonucleoprotien Particles
rRNA	Ribosomal Ribo Nucleic Acid
rpm	Round Per Minute
r-Protein	Ribosomal Protein
SnoRNP	Small Nucleolar Ribonucleoproteins
T	Thymine
T(a)	Annealing Temperature
T(m)	Melting Temperature
tRNA	Transfer Ribo Nucleic Acid
USDA	United States Department of Agriculture

Chapter One

Introduction and Literature Review

1. Introduction

Rosemary (*Rosmarinus officinalis* L.) is a member of Lamiaceae family. Rosemary extract had been widely used as topical applications for wound-healing, antiaging and disease treatments. This plant produces flavonoid compounds with phenolic structures. Such phytochemicals are highly reactive with other compounds such as reactive oxygen species and biological macromolecules to neutralize free radicals or initiate biological effects (Leithy, *et al*., 2006).

R. officinalis or more commonly known as rosemary is well known as medicinal and aromatic plant. It was used for medicinal, culinary and cosmetic applications in the ancient civilizations (Al-Sereitia *et al*., 1999).

It has been cultivated since ancient days in England, Germany, France, Denmark and other Scandinavian countries, Central America, Venezuela and the Philippines (Tyler *et al*., 1976). The plant is native to the Mediterranean, Portugal, and northwestern Spain (Kowalchik and Hylton, 1987).

Botanists before late 19th century were only using characteristics that they could see (morphological features), and deciding which differences were important and which to ignore. This was purely speculative and based on the prejudices of individuals. As there can only be one truly genetic classification system (the one which reflects the actual route of evolution). The many different systems proposed cannot all have been right (Anonymous, 2000).

With the development of various analytical methods of high precision, and advances in molecular biology and genetic engineering, it is now possible to isolate compounds in extremely small quantities, study their chemical structure and therapeutic potentialities and then to alter the molecule to be suitable for production of novel and more selective new therapeutic agents (Al-Sereitia *et al*., 1999). RFLP analysis was also the basis for early methods

of Genetic fingerprinting, useful in the identification of samples retrieved from crime scenes, in the determination of paternity, and in the characterization of genetic diversity or breeding patterns in animal populations.

In this work each one of 18S, 5.8S and 26S RNA genes with two ITS regions of rDNA sequence were considered for *R. officinalis* species identification in the North of Iraq using PCR based RFLP techniques.

As a result of the importance of this locally grown plant as a potential source of phytochemicals, this research work was aiming to:

1- Conduct a survey in northern region of Iraq to detect morphological differences among the grown subspecies of *R. officinalis*, then making a comparison between the subspecies.

2- Study the genomic DNA of the subspecies by using PCR based RFLP technique (commonly known as DNA fingerprint) in order to differentiate between the subspecies at the molecular level.

2. Literature Review

2.1. Plant Description

Rosemary is a member of the mint family (Lamiaceae Family), it has opposite, simple, entire, evergreen leaves up to two inches long and an eighth of an inch wide, the leaf margins are revolute and the leaves are shiny green on top and whitish beneath due to a dense collection of very fine hairs (Dirr, 1990).

The plant begins to flower in late winter and continues through spring. Flowers are normally blue, but cultivars can be found with pink or white blooms (Armitage, 1997).

Figure (1): (*Rosmarinus officinalis* L.) plant (Peterson, 2002).

2.2. Traditional uses of rosemary

Rosemary is one of the ancient plants, closely associated with love and marriage, birth and death. In England and Germany it is considered as a symbol of remembrance and is still used in bridal bouquets and in spring is placed in

the cradle of a newly born child to protect against evil influences and forces; it is also placed in books and among clothes to protect them from moths and to produce a pleasant odor (Leth, 1976).

Rosemary plant is cultivated for its aromatic oil which is called "rosemary oil". It is normally obtained by steam distillation of the fresh leaves and flowering tops of the plant. It is a colorless or pale yellow liquid having the characteristic odor of the plant, it is an ingredient for Eau-de-cologne, hair tonics, hair lotion, cold cream and others (Tyler *et al.*, 1976).

The leaves are used for flavoring foods as condiment, since the ancient days rosemary has been used in folk medicine for manifold conditions. Some of which may be enumerated as follows:

1-analgesic.
2-antirheumatic.
3-carminative.
4-diuretic.
5-expectorant.
6-antiepileptic
7-effects on human fertility

other uses are as a general tonic in case of excessive physical or intellectual works and in heart diseases, it is used also as an insecticide and herbicide. Externally, it is a rubefacient, and is used to stimulate the growth of hair and treatment of eczema of the scalp, boils and wounds (Hussain, 1979).

2.3. Propagation

It was reported that rosemary is difficult to grow from seed, so propagation is almost exclusively by cuttings. The recommended technique is striping leaves from the lower half of a four inch cutting, then dipping the stripped end in a rooting hormone (Debaggio, 1990). Thereafter cuttings are

inserted in a peat-based rooting medium. Rooting occurs within two weeks though Long (1998) noticed a delayed rooting time during the hottest part of the summer. Rooted cutting should be transplanted into a larger container. Pinching the new plant immediately after transplanting increases branching.

2.4. Pharmacology of Rosemarinic Acid

Rosemarinic acid is a naturally occurring non-steroidal antiinflammatory agent with novel properties which are as follows:

a) Effects of rosemarinic acid on cell mediators

Rosemarinic acid strongly inhibits the formation of 5-hydroxy-6, 8, 11, 14-ecosatetraenoic acid and leukotriene in human polymorphonuclear leucocytes, while the formation of prostaglandin is enhanced by cafiec acid and several of its derivatives including rosemarinic acid (Kimura *et al*., 1987).

b) Free radical scavenging activity of rosemarinic acid

Hydroalcoholic extracts of some medicinal plants with high amount of hydroxycinnamic derivative content (of which rosemarinic acid is present in more than 3-6% of the dry weight) were tested and showed significant antioxidative activities. The antioxidative activity was attributed partly to the high rosmarinic acid content of these plants (Lamaison *et al.*, 1990; Lamaison *et al*., 1991).

In another study concerning the use of spin trapping methods, rosemary extract was reported to have a scavenging effect on the active oxygen free radical in stimulated polymorphonuclear leucocyte system (Zhao *et al.*, 1998).

c) Effect of rosemarinic acid on the complement system

Activation of the complement system can be contributed to the inflammatory reaction in a number of ways, such as by increasing the vascular permeability and formation of oedema, stimulating chemotaxis of leucocytes, enhancing platelet activation and aggregation, enhancing prostaglandin

synthesis in macrophages, and by releasing lysosomal enzymes. Several drugs have shown to modulate the bactericidal activity of serum complement (Chakrabarty *et al.*, 1981; Chakrabarty *et al.*, 1985).

Rosemarinic acid was reported to produce an inhibitory activity when tested in three *in vivo* models in which complement activation plays a role (Englberger *et al*., 1988).

The killing of *Escherichia coli* was inhibited by rosemarinic acid at a concentration of 2mM, but not that of *Staphylococcus aureus*. The inhibition of the killing was due to an impaired opsonization, caused by an adverse influence of rosemarinic acid on the complement activation. Direct effects of rosemarinic acid on the killing mechanisms of polymorphonuclear leucocytes were not observed (Verweij-van Vaght *et al*., 1987).

2.5. Classification of *R. officinalis* L.

Kingdom	Planta	Plants
Subkingdom	Trachiobionta	Vascular Plants
Superdivision	Spermatophyta	Seed Plants
Division	Magnoliophyta	Flowering Plants
Class	Magnoliopsida	Dicotyledons
Subclass	Asteridae	
Order	Lamiales	
Family	Lamiaceae	Mint Family
Genus	Rosmarinus L.	Rosemary
Species	*Rosmarinus officinalis* L.	Rosemary (USDA, 2008)

2.6. Plant Classification Systems

Classification requires:

- Plant material to be classified.
- Identifying features which can be used for grouping the plants.
- A logical way of ordering the resulting groups. (Baumgardt, 1982).

The huge variability in plant form provides a very diverse range of identification features or characters which can be used for grouping. One of the oldest and commonly used methods of grouping plants depends on physical characters, or morphology (Stace, 1989).

These characters are mostly visible with the naked eye or a hand lens and many are used, including:

- Size, shape, number and arrangement of flower parts.
- Arrangement of groups of flowers in an inflorescence.
- The way that anthers (pollen-containing structures) open.
- Leaf shape, texture, pattern of veins, arrangement on stem.
- Type and shape of fruit.
- Plant habit (tree, climbing annual, perennial etc.).
- Sap color.
- Smell.

These characters are also used in plant identification, as well as these easy-to-see features. Botanists also use other characters (Jeffrey, 1982). Many structural features are only visible with a microscope, for example the shape of pollen grains and their surface sculpturing. With the correct treatment and staining, the chromosomes within plant cells, plus other cellular structures which can be seen under a microscope including their shape and number as distinguishing characters (Mabberley, 1997).

Biochemistry is also useful as many chemicals are only found in certain groups of plants. In many cases this indicates that members of the group are closely related (Jeffrey, 1982).

2.7. Plant Classification According to Genes

It has been recently devised a new classification of flowering plant families, based entirely on differences between genes. Genes are long strings of instructions for making proteins the ‘building blocks’ of life. These instructions are coded by a four-letter alphabet (the DNA bases). Genes are passed down through generations, so if one of the ‘letters’ changes in a plant, its entire offspring will inherit that change (Anonymous, 2000).

These changes gradually accumulate, so they can be used to trace plant ancestry. Two species are more likely to be closely related if they show only a few differences in their gene sequences, than if the differences are larger (Mabberley, 1997).

Scientists chose three genes found in all plants, and 565 plant species to represent all the world’s flowering plant families. For each plant, the three genes were sequenced, and the sequences (long lists of the letters of the DNA bases) were compared using computer analysis. The result was a huge ‘family tree’ of plants with branches showing how species have separated into natural groups. This new classification of plant families represents evolutionary relationship better than any other before it (APG, 1998).

2.8. Agarose Gel Electrophoresis

Electrophoresis through agarose is of a big importance of the molecular biology and is used to separate, identify, and purify amplified DNA fragments. The technique is simple, rapid to perform, and capable of resolving fragments of DNA that can not be separated adequately by other procedures, such as density gradient centrifugation. Furthermore, the

location of DNA within the gel can be determined directly by staining with low concentrations of fluorescent intercalating dyes, such as ethidium bromide (Sambrook *et al*., 2003).

The following factors determine the rate of migration of DNA through agarose gels: (Sharpe, 1980)

- Molecular size of the DNA.
- Concentration of agarose.
- Conformation of the DNA
- Presence of ethidium bromide in the gel and electrophoresis buffer.
- Applied voltage.
- Type of agarose
- Electrophoresis buffer.

2.9. Polymerase Chain Reaction (PCR)

Cloning, DNA Sequencing, and PCR (polymerase chain reaction) underlies almost all of modern molecular biology. Of these three, the PCR is the oldest in theory and the most versatile in practice. The method was first proposed in the early 1970s as a strategy to lessen the labor involved in chemical synthesis of genes (Kleppe *et al*., 1971).

The technique was independently conceived 15 years later, given its present name, and put into practice by Kary Mullis and coworkers at Cetus Corporation, who described *in vitro* amplification of single-copy mammalian genes using the Klenow fragment of *Escherichia coli* DNA polymerase I (Saiki *et al.,* 1985; Mullis *et al*., 1986; Mullis and Faloona, 1987).

PCR is an iterative process, consisting of three stages: denaturation of the template by heat, annealing of the oligonucleotide primers to the

single stranded target sequences, and extension of the annealed primers by a thermostable DNA polymerase (Figure 2).

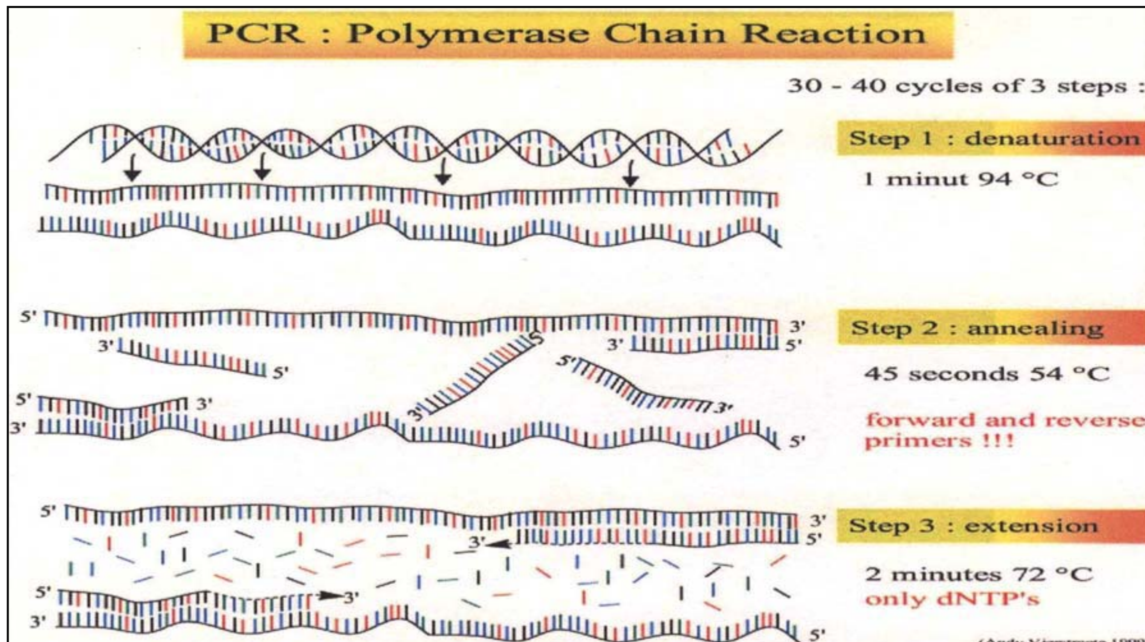

Figure (2): PCR basic steps for DNA amplification (Mas *et al.*, 2001).

Accordingly, these steps are repeated in a 30-40 cycles using PCR thermal cycler that result in a huge amount of the amplified DNA fragment (Figure 3).

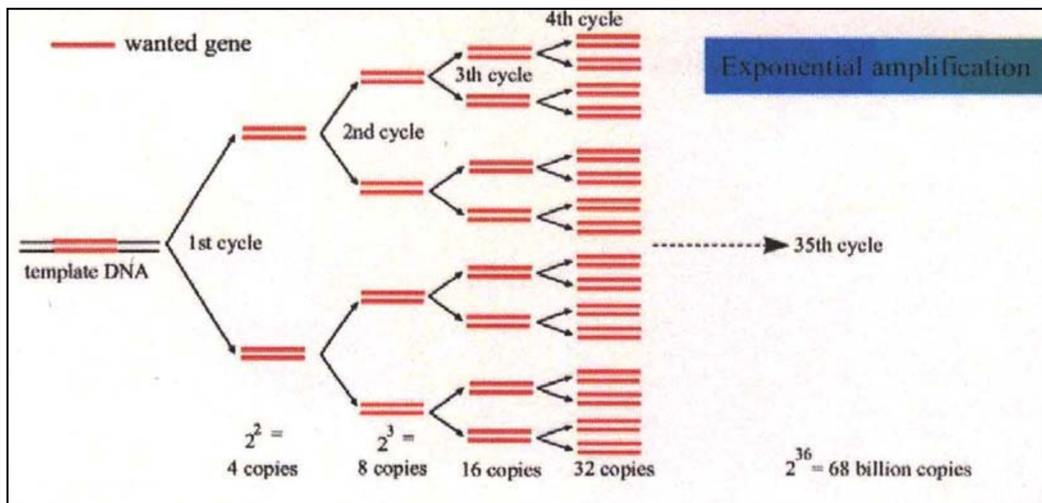

Figure (3): DNA amplification after 30-40 cycles (Mas *et al.*, 2001).

The ribosomal DNA (rDNA) found in the nuclear genome of *R. officinalis* consists of three conserved RNA genes transcribed as a single united code for the 18S, 5.8S and 26S RNA genes that are highly conserved and primers to these regions have been designated. Between these coding regions, the Internal Transcribed Spacer Regions (ITS1 and ITS2) separate the three RNA genes (NCBI, 2008).

The chief goal of primer design is specificity, which is achieved only when each member of a primer pair anneals in a stable fashion to its target sequence in the template DNA. As a rule of thumb, the longer an oligonucleotide, the higher its specificity for a particular target (Nei and Li, 1979).

2.10. Restriction Fragment Length Polymorphism (RFLP)

Restriction fragment length polymorphism is a variation in the DNA sequence of a genome that can be detected by breaking the DNA into pieces with restriction enzymes and analyzing the size of the resulting fragments by gel electrophoresis. It is the sequence that makes DNA from different sources, and RFLP analysis is a technique that can identify some differences in sequence (when they occur in a restriction site). Though DNA sequencing techniques can characterize DNA very thoroughly, RFLP analysis was developed first and was cheap enough to see wide application. Analysis of RFLP variation was an important tool in genome mapping, localization of genetic disease genes, determination of risk for a disease, genetic fingerprinting, and paternity testing (Saiki *et al*., 1985).

2.11. Applications of PCR Based RFLP Technique

Analysis of PCR-RFLP variation in genomes was vital tool in genome mapping and genetic disease analysis. If researchers were trying

to initially determine the chromosomal location of a particular disease gene, they would analyze the DNA of members of a family afflicted by the disease, and look for RFLP alleles that show a similar pattern of inheritance as that of the disease. Once a disease gene was localized, RFLP analysis of other families could reveal who was at risk for the disease, or who was likely to be carriers of the mutant gene (Nei and Li, 1979).

PCR based RFLP technique was used on sunflower plant for species identification by Rieseberg *et al*. (1999).

2.12. Ribosomes Function and Structure

In all organisms, ribosomes form the core of the translation machinery. Translation is a key step in gene expression, converting the genetic information encoded in messenger RNAs into contiguous chains of amino acids (polypeptides or proteins) with structural and catalytic properties (Denis *et al*., 2001).

Ribosomes have two main functions; decoding the message and the formation of peptide bonds. These two activities reside in two large ribonucleoprotein particles (RNPs) of unequal size, the ribosomal subunits. Each subunit is made of one or more ribosomal RNAs (rRNAs) and many ribosomal proteins (r-proteins) (Daugeron and Linder, 2001).

The small subunit (30S in bacteria and archaea, 40S in eukaryotes) has the decoding function, whereas the large subunit (50S in bacteria and archaea, 60S in eukaryotes) catalyses the formation of peptide bonds, referred to as the peptidyl-transferase activity. The bacterial (and archaeal) small subunit contains the 16S rRNA and 21 r-proteins (*Escherichia coli*), whereas the eukaryotic small subunit contains the 18S rRNA and 32 r-

proteins. The bacterial large subunit contains the 5S and 23S rRNAs and 34 r-proteins, with the eukaryotic large subunit containing the 5S, 5.8S and 25S/28S rRNAs and 46 r-proteins (Denis *et al*., 2001).

Each subunit has three binding sites for tRNA, designated the A (aminoacyl), which accepts the incoming aminoacylated tRNA; P (peptidyl), which hold the tRNA with the nascent peptide chain; and E (exit), which holds the deacylated tRNA before it leaves the ribosome (Ramakrishnan, 2002).

2.13. Ribosomes Synthesis

The key steps in ribosome synthesis are: transcription of the pre-rRNA from the rDNA; covalent modification of the mature rRNA regions of the pre-rRNA; processing of the pre-rRNA to the mature rRNAs; and assembly of the rRNAs with the ribosomal proteins (Figure 4). In eukaryotes, additional steps include the import of r-proteins from the cytoplasm to the nucleus and the export of the ribosomal subunits from the nucleolus through the nucleoplasm and nuclear pore complexes to the cytoplasm (Denis *et al*., 2001).

After transcription of the pre-ribosomal RNAs, most steps in eukaryotic ribosome synthesis occur within the nucleolus. The pre-rRNAs are processed to yield the mature rRNA species, which also undergo extensive covalent modification. In bacteria, rRNA modifications are made by conventional enzymes, but in eukaryotes most modification involves methylation of the sugar 2′ hydroxyl group (2′-*O*-methylation) or pseudouridine formation, which occur at sites that are selected by base pairing with a host of Small Nucleolar Ribonucleoprotien (snoRNP) particles (Lafontaine and Tollervey, 1998).

Human cells contain over 100 species of snoRNP, and each pre-rRNA molecule must transiently associate with a member of each species. During pre-rRNA transcription and processing, many of the ribosomal proteins assemble onto the mature rRNA regions of the pre-RNA (Moy and Silver, 1999).

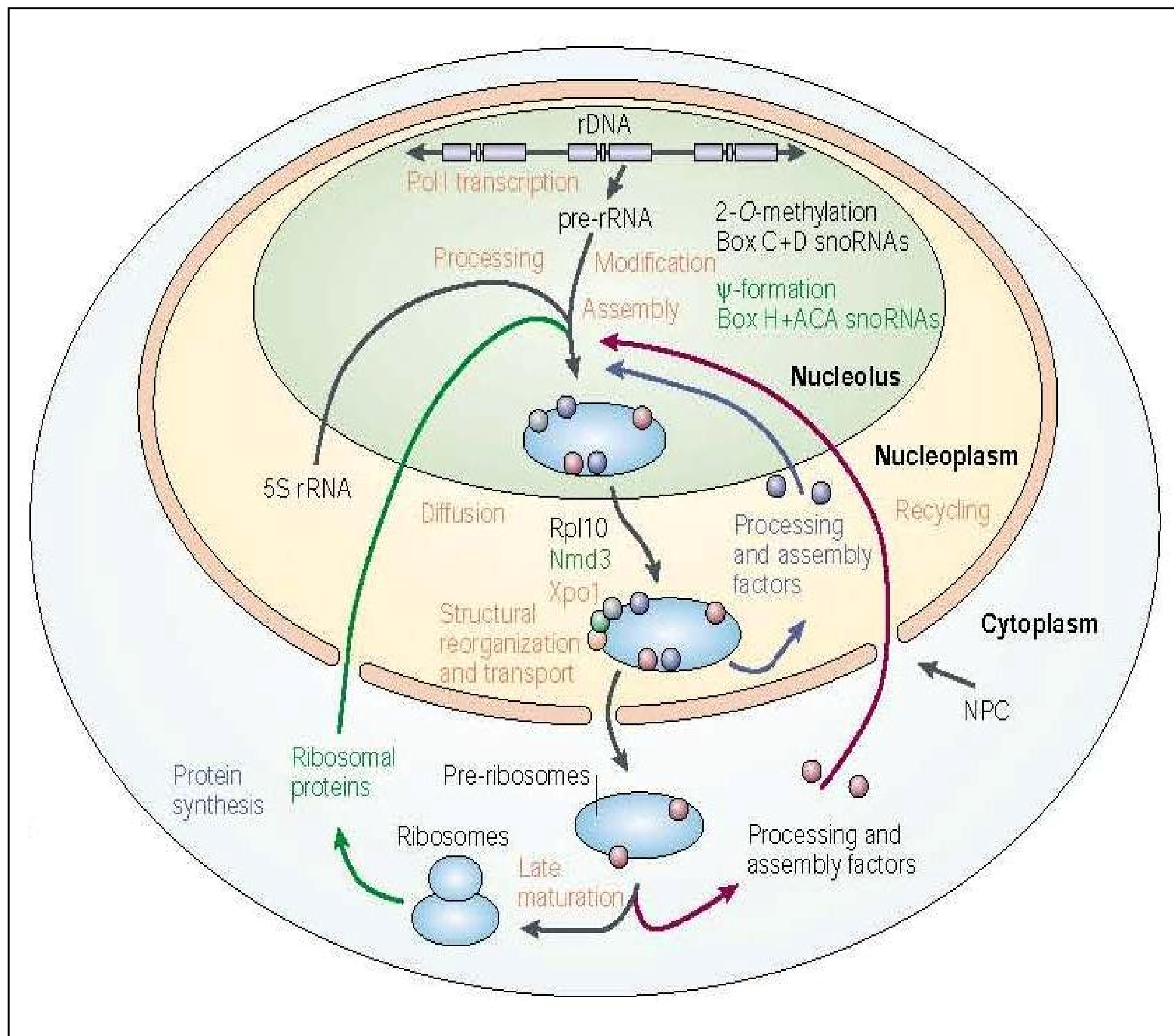

Figure (4): Ribosomes synthesis steps (Denis *et al*., 2001).

During maturation, the pre-ribosomal particles are released from association with nucleolar structures, and are believed to diffuse to the nuclear pore complex. In yeast, nuclear export of pre-60S particles is mediated at least

in part by the small GTPase Ran and the export factor, which binds to the ribosomal protein through an adaptor (Stage-Zimmermann *et al*., 2000).

Export of the pre-40S subunit also requires Ran (Moy and Silver, 1999), but no specific export factors have been identified. Passage through the nuclear pores is likely to be preceded by structural rearrangements and the release of pre-ribosome-associated proteins, including processing and assembly factors.

It seems likely that further ribosome synthesis factors will be released during late structural rearrangements in the cytoplasm that convert the pre ribosomal particles to the mature ribosomal subunits (Kressler *et al*., 1999 ; Venema and Tollervey, 1999).

2.14. Translation

In translation, the sequence of codons on mRNA directs the synthesis of a polypeptide chain. This process takes place on the ribosome, and the movement of tRNA and mRNA through the ribosome is a complicated process that combines high speed with high accuracy (Green and Noller, 1997).

2.14.1. Initiation

Initiation in bacteria involves the interaction of the 30S subunit with the Shine-Dalgarno sequence on mRNA that is complementary to the 3` end of 16S RNA. The process also involves three initiation factors, IF1, IF2, and IF3 (Gualerzi and Pon, 1990). IF3 is known to bind strongly to the 30S subunit and prevents its association with the 50S subunit. It also helps in the selection of initiator tRNA (fMet-tRNA) by destabilizing the binding of other tRNAs in the P site of the ribosome (Hartz *et al*., 1990). In a possibly related function, IF3 has been found to dissociate deacylated tRNA from the 30S subunit in the

last step of termination before it is recycled in a new round of protein synthesis (Karimi *et al*., 1999). IF2 is a GTPase that binds preferentially to fmet-tRNA, and its affinity for the ribosome is increased by IF1 (Zucker and Hershey, 1986).

Recent kinetic data indicate that the GTPase activity of IF2 is required neither for the proper placement of initiator tRNA in the P site nor for IF2 release (Tomsic *et al*., 2000).

2.14.2. Elongation

The end of the initiation process leaves an aminoacylated initiator tRNA in the P site of the ribosome and an empty A site, which serves to start the elongation cycle (Ramakrishnan, 2002).

Aminoacylated tRNA is brought into the A site as a ternary complex with EF-Tu and GTP. Correct codon-anticodon interactions result in conformational changes in the ribosome which stabilize tRNA binding and trigger GTP hydrolysis by EF-Tu. This leads to the release of the aminoacyl end of A-site tRNA by EF-Tu; the tRNA then swings into the peptidyl transferase site of the 50S subunit in a process called accommodation (Lafontaine and Tollervey, 1998)

Peptide bond formation, which involves the deacylation of P-site tRNA and the transfer of the peptide chain to A-site tRNA, is then essentially spontaneous. Following peptidyl transfer, the ribosome has a deacylated tRNA in the P site and peptidyl tRNA in the A site. Translocation of the tRNAs and mRNA is facilitated by EF-G, which is also a GTPase. The result is a ribosome ready for the next round of elongation, with deacylated tRNA in the E site, peptidyl tRNA in the P site, and an empty A site that is ready to receive the next cognate ternary complex (Ramakrishnan, 2002).

2.14.3. Termination

The process of termination begins when a stop codon on mRNA is encountered in the A site. In bacteria, recognition of the stop codon involves two release factors, RF1 and RF2. Both factors recognize UAA; however, UAG is recognized by RF1 while UGA is recognized by RF2. In eukaryotes, a single factor, eRF1, recognizes all three stop codons (Kisselev and Buckingham, 2000).

2.15. The Use of rDNA for Species Identification

Many reports has conducted the use of rDNA or its genes with the ITS regions that separate them, as a standard gene for differentiation and identification between species using RFLP which is considered the easiest way for differentiation that is less time consuming and more accurate than traditional methods of differentiation.

rDNA and ITS regions has been used as a template DNA by Merhendi (2004) for species identification between *Candida albican* and *Candida dubliniensis* using PCR-RFLP technique.

Vaneechoutte *et al.* (1998) considered the use of 16S rDNA for species identification in Listera species isolates by PCR-RFLP, also Misbah *et al.* (2005) used 16S rDNA gene for Acinetobacter identification but by PCR and DNA sequencing only, while Esteve-Zarzoso *et al.* (1999) considered the use of 5.8S RNA gene with two ITS regions by RFLP for species identification of different yeast species.

Chapter Two

Materials and Methods

1. Materials

1.1. Apparatus and equipments

The following equipments and apparatus were used throughout the experimental work:

Apparatus	Company
Hood cabinet	Fisher Hamilton / USA
Vortex mixer	Eppendorf / Germany
Centrifuge	Eppendorf / Germany
Micropipette (Different Sizes)	Eppendorf / Germany
Sensitive balance	Ohaus / Switzerland
Freezer (- 70˚C)	Thermo Electron Corporation / USA
Freezer (- 20˚C)	General / USA
Tips (Different sizes)	Jippo / Japan
Micro centrifuge tube (1.5mL)	Jippo / Japan
Water bath	Fisher Scientific / USA
Mortar and pestle	Jippo / Japan
Microwave oven	General / USA
Gel electrophoresis system	Thermo / USA
PCR Thermal cycler	Thermo / USA
PCR Tubes	Eppendorf / Germany
Concentrator	Eppendorf / Germany
UV trans-illuminator	Spectroline / USA

Digital Camera	Canon / China
Ice maker	Scotsman / Italy
Incubator	Barnstead international / Switzerland
Autoclave	Thermo / USA

1.2. Chemicals

The following chemicals were used through out the experimental work:

Chemicals	Company
Ethyl alcohol	Fisher Scientific
MgCl2	Fermentas
Chloroform (Molecular Biology Grade)	Fisher Scientific
Tris Base	BDH
Boric acid	BDH
EDTA	BDH
Ethidium Bromide	BDH
Agarose	BDH
DNA Marker (1kb)	Fermentas
dNTP's	Fermentas

1.3. Enzymes

Restriction enzymes and Taq polymerase enzyme were ordered from Fermentas/Germany and used through out the experimental work:

Enzyme	Company
*Eco*RI	Fermentas / Germany
*Eco*RV	Fermentas / Germany
*Hin*dIII	Fermentas / Germany
*Bam*HI	Fermentas / Germany
*Sal*I	Fermentas / Germany
*Sma*I	Fermentas / Germany
DNA Taq Polymerase	Fermentas / Germany

1.4. Standard Buffers

Standard buffers were ordered and used for PCR amplification and DNA digestion:

Buffer	Company
PCR Buffer	Fermentas / Germany
Fast Digest Buffer	Fermentas / Germany
Gel Loading Buffer	Fermentas / Germany

1.5. Ready-to-use Kit

Ready to use kit was ordered for DNA Isolation and Purification:

Kit Name	Components	Company
Genomic DNA Purification Kit	Lysis Solution, 40ml of ready-to-use solution	Fermentas / Germany
	Precipitation Solution, 8ml of 10X concentrated solution.	
	NaCl Solution, 10ml of 1.2M Sodium Chloride.	

2. Methods

This study was carried out in Kurdistan Medical Research Center/Ministry of higher Education and Scientific Research/Kurdistan Province/Iraq, during the period from 1/11/2006 to 1/10/2008.

2.1. Morphological Differentiation

2.2. Plant Material

Rosemary Plant, (*R. officinalis*) was collected from different geographical places in Northern region of Iraq (Figure 5), including:

a) Erbil city which represents the extending land, moderated temperatures and long sunny periods through out the year.
b) Sulaymania city, which represents the mountain land, lower temperatures and shorter sunny periods through out the year (Figure 5).

A morphological study was conducted on plant samples including:

- Leaves (shape and color).

- Flowers (shape and color).
- Stem growth directions.

Two subspecies were found with obvious different characteristics particularly in stem growth directions. Those were nominated as subspecies A and subspecies B. The two were collected and subjected to intensive molecular investigation.

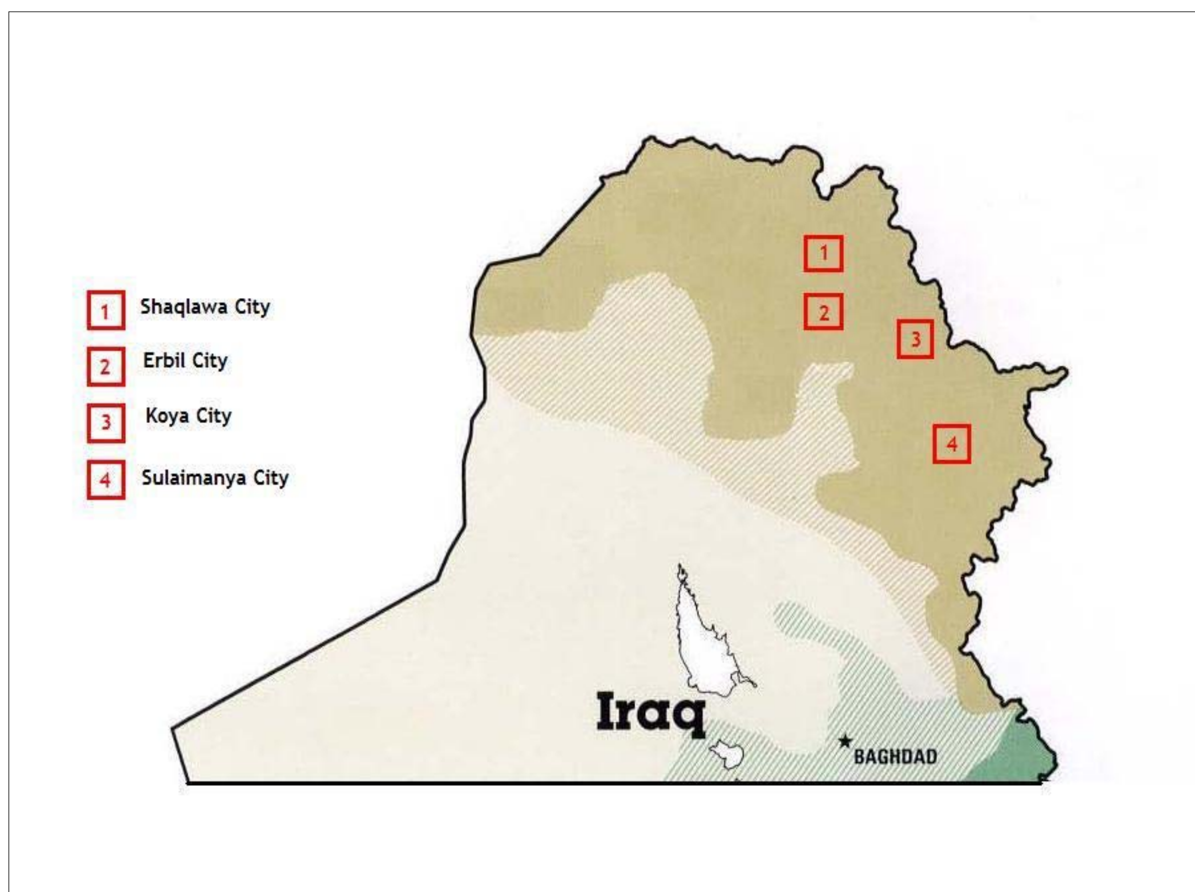

Figure (5): Northern region of Iraq map shows the surveyed zones for *R. officinalis*.

2.3. Molecular Differentiation

Ribosomal genomic DNA was used as a standard, and the template DNA of *R. officinalis* was obtained from the genomic database of the National Center of Biotechnology Information (NCBI).

The following techniques were used in this study:

- DNA isolation and Purification.
- Gel Electrophoresis.
- Restriction Enzymes Digestions.
- PCR Amplifications for digested DNA.

2.4. DNA Isolation and Purification

An amount of 100mg of rosemary leaves of each subspecies was macerated in cold ethanol using mortar and pestle. The powder of the plant leaves was placed in a 1.5ml microcentrifuge tube and resuspended in 200 µl of DDH2O, samples stored at -70°C until use.

A DNA purification kit was used according to the manufacturer instructions (Fermentas/Germany):

- A liquot of 200µl was mixed with 400µl of lysis solution, incubated at 65°C for 20 min.
- Aliquot of 600µl chloroform was added immediately, gently emulsified by inversion (3-5 times) and centrifuged at 10,000rpm (6708 g) for 2min.
- Precipitation solution was prepared by mixing 720µl of sterile deionized water with 80µl of supplied 10x concentrated solution.
- The upper aqueous phase containing DNA was transferred to a new tube and added to 800µl of the freshly prepared precipitation solution, mixed gently by several inversions at room temperature for 1-2min and centrifuged at 10000 rpm (6708 g) for 2min.
- The supernatant was completely removed and the DNA pellet was dissolved in 100µl of 1.2M NaCl solution by gentle vortexing.

- Aliquot of 300µl of cold ethanol was added, DNA was left to precipitate (for 10 min at -20ºC) and centrifuged at (10000rpm (6708 g), 3-4 min), and the ethanol was decanted, then pellets washed once with 70% cold ethanol. DNA was then dissolved in 100µl of sterile deionized water with gentle vortexing.
- DNA concentration was measured visually under UV illuminator after electrophoresis in agarose gel and photographed by digital camera.

Isolation of genomic DNA was carried out by macerating the plant leaves with mortar and pestle in cold ethanol, incubated overnight at -70°C then maceration repeated. This procedure can be used instead of maceration with liquid nitrogen.

During the lyses process, a volume of 400 µl of lysis solution was added to 200µl of plant sample and incubated at 65°C for 20 minutes instead of 5 minutes, since the plant cells are coated by a rigid cellulosic cell wall which requires more time to be lysed.

2.5. Gel Electrophoresis (Sambrook *et al*., 2003)

The following procedure was used for gel electrophoresis establishment:

- The edges of a clean, dry glass plate were sealed with tape to form a mold. It was then placed on a horizontal section of the bench.
- Sufficient quantity of electrophoresis buffer (0.5X TBE) was prepared to fill the electrophoresis tank and to cast the gel.
- A solution of agarose was prepared in electrophoresis buffer at a concentration appropriate for separating the particular size fragments expected in the DNA samples. The correct amount of powdered agarose was added to a known quantity of electrophoresis buffer in a flask or a glass bottle.

- The neck of the flask was loosely plugged with Kim wipes. The slurry was heated in a microwave oven until the agarose was dissolved.
- Insulated gloves or tongs were used to transfer the flask/bottle into a water bath at 55ºC. When the molten gel had cooled, ethidium bromide was added to a final concentration of 0.5 g/ml, the gel solution mixed thoroughly by gentle swirling.
- While the agarose solution was cooling, an appropriate comb was chosen for forming the sample slots in the gel. The comb was positioned 0.5-1.0 mm above the plate so that a complete well was formed when the agarose was added to the mold.
- The warm agarose solution was poured into the mold.
- The gel was allowed to set completely (30-45 minutes at room temperature), small amount of electrophoresis buffer was poured on the top of the gel, and the comb was carefully removed. The electrophoresis buffer was decanted and the tape was carefully removed. The gel was mounted in the electrophoresis tank.
- Enough electrophoresis buffer was added to cover the gel to a depth of 1mm.
- The samples of DNA were mixed with 0.20 volumes of the desired 6x gel-loading buffer.
- The sample mixture was loaded slowly into the slots of the submerged gel using an automatic micropipette. Loading size standards into slots on both the right and left sides of the gel.
- The lid of the gel tank was closed and the electrical leads were attached so that the DNA would migrate toward the positively charged anode (red lead). A voltage of 1-5 V/cm was applied (measured as the distance between the positive and negative electrodes). If the leads were

attached correctly, bubbles should be generated at the anode and cathode (due to electrolysis), and within a few minutes, the bromophenol blue should migrate from the wells into the body of the gel. The gel was run until the bromophenol blue and xylene cyanol (that are already included in the loading buffer) had migrated an appropriate distance through the gel.

- When the DNA samples or dyes were migrated a sufficient distance through the gel, turned off the electric current and removed the leads and lid from the gel tank. If ethidiun bromide was presented in the gel and electrophoresis buffer, the gel should be examined by UV illuminator and photographed.

2.6. Digestion by Restriction Enzymes

- The Restriction mixture was prepared for all types of the used enzymes. This procedure was performed six times for each one of the six restriction enzymes. The mixture was composed of:
  - 15µl of DDH2O.
  - 2µl of 10X digestion buffer
  - 2µl of DNA sample
  - 1µl of the desired restriction enzyme.

The total volume was 20µl, and the volume of the 10X buffer was equal to the 0.1 of the total volume.

- The concentrations of EcoRI, BamHI, HindIII and SalI was 10,000 Unit/µl and for EcoRV was 4000 Unit/µl and for SalI was 2000Unit/µl.
- Gently mixed by vortex and centrifuged.
- Incubated at 37ºC in a water bath for 20 minutes.

2.7. Primers Design (Sambrook *et al*., 2003)

The following procedure was followed for primers design:

- Template ribosomal DNA (rDNA) of *R. officinalis* was obtained from the NCBI genomic data base (National Center of Biotechnology Information), which consist of three repeating RNA genes transcribed as a single united code for the 18S, 5.8S and 26S RNA genes that were highly conserved, and separated by two ITS regions.
- Two primers were designed (Figure 6) by selecting the first 23bp repeating sequence (Sense) of rDNA template from the 5` to 3` end as a first forward primer which is:

(5`-GTT TCC GTA GGT GAA CCT GCG GA-3`), and named as (R18SF) which represents:

R: For Rosemary

18S: For the site in which primer sequence locates in rDNA (18S RNA gene).

F: For Forward Primer

The last 20 bp reverse (Anti-sense) sequence from the 3` to 5` end of the rDNA template as the second reverse primer, which is:

(5`-TGA CCT GGG GTC GCG GTC GA-3`), and named as (R26SR) which is explained as:

R: For Rosemary

26S: For the site in which primer sequence locate in rDNA (26S RNA Gene).

R: For Reverse Primer

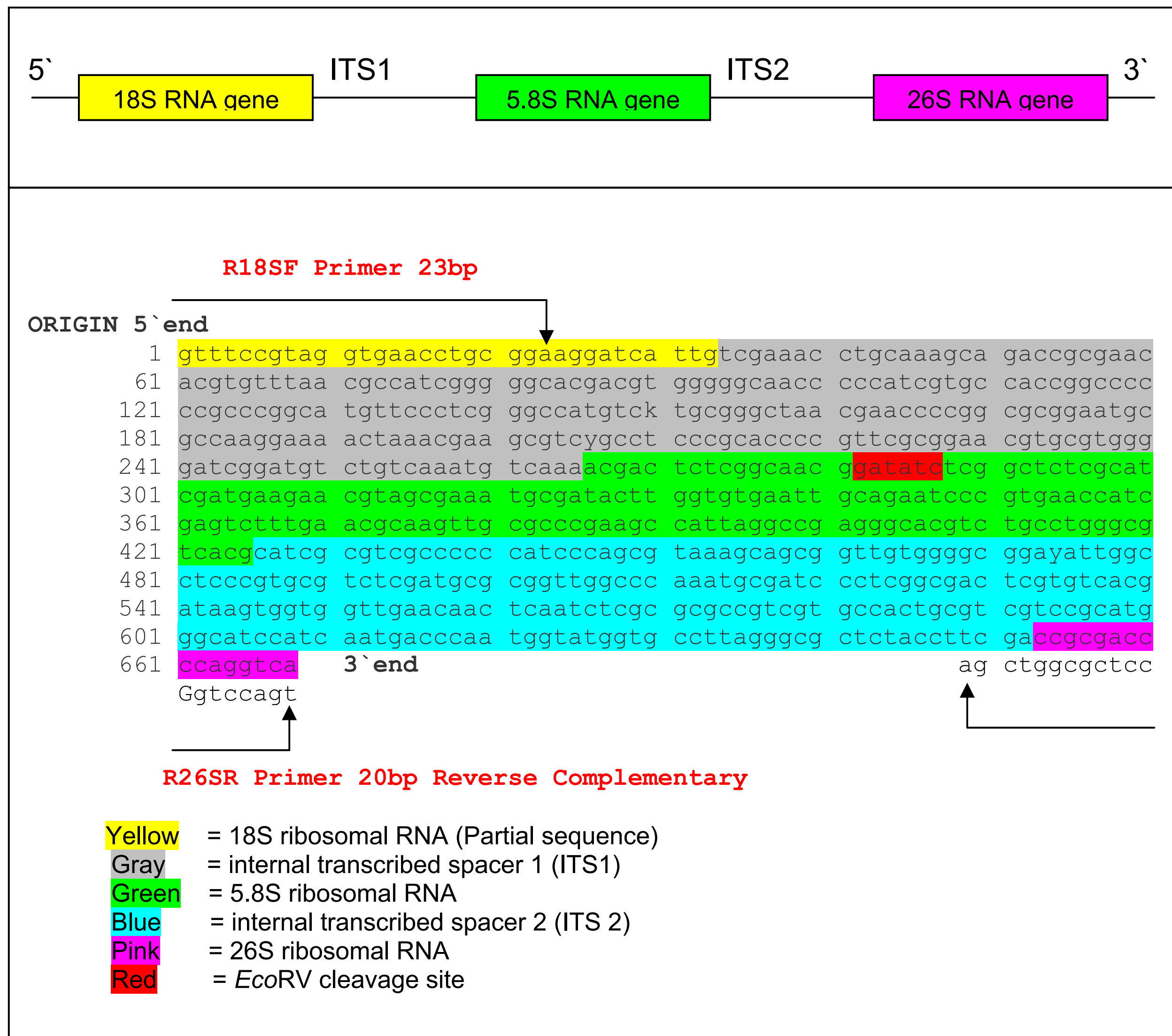

Figure (6): Alignment of rDNA for *R. officinalis* including the coding genes of 18S, 5.8S and 26S RNA separated by two non-coding ITS regions (NCBI, 2008).

- Some factors were considered in primer selection and design which were:

- GC and AT contents in the primer sequence that are strongly affect the annealing temperature T(a) and melting temperature T(m) of the primer with the template DNA during PCR thermal cycles.

- It is important to set the same T(a) of both forward and reverse primers and that is done by increasing or decreasing one or more GC and AT base

pairs from the primer sequence. This was achieved by increasing or decreasing the three binding bonds between GC bp and two binding bonds between AT bp that require more heat to anneal.

- T(a) and T(m) of the primers were estimated using the following equation:

$T(m) = (\text{No. of GC bp} \times 4) + (\text{No. of AT bp} \times 2)$

$T(a) = T(m) - 20$

So T(a) for the R18SF primer is:

$T(m) = 13\times4 + 10\times2 = 52 + 20 = 72\ °C$

$T(a) = 72 – 20 = 52\ °C$

While for R26SR primer is:

$T(m) = 14\times4 + 6\times2 = 56 + 12 = 68\ °C$

$T(a) = 68 – 20 = 48\ °C$

- After the completion of primer design, the two primers were then ordered and synthesized from (Cybergene AB/Stockholm/Sweden/ (www.cybegene.se).
- The primer set was delivered with the specifications shown in Table(1), and diluted to get 10x stock for PCR work. Primer dilution is done by getting N µl of the primer and completing the volume to 100µl with DDH2O. The value of N depends on the concentration of primers shown on the primer label and using the following equation:

$N = 10 / \text{primer concentration} \times 100$

So the value of N for (R18SF) primer with 105.55pmol/µl concentration is:

$N = 10 / 105.55 \times 100 = 9.47$ µl of primer added and completed the volume to 100µl to get 10X solution.

While the value of N for (R26SR) Primer with 116.99 pmol/µl concentration is:

N = 10 / 116.99 × 100 = 8.54µl

Table (1): Primer set specification for rDNA amplification of rosemary

Name	Sequence	M/W	Concentration (pmol/µl)	OD	Delivery Form
R18SF	5`-GTTTCCGTAGGTGA ACCTGCGGA-3`	7096.67	105.55	10.21	Diss. in water
R26SR	5`-TGACCTGGGGTCGC GGTCGA-3`	6191.06	116.99	9,87	Diss. in water

2.8. PCR amplification (Sambrook *et al*., 2003)

- Using a sterile 0.5 ml microcentrifuge tube, (PCR amplification tube), The following were mixed:

DDH2O	24 µ1
l0x amplification buffer	5 µl
20 mM solution of four dNTPs	8 µl
20 µM forward primer	4 µl
20 µM reverse primer	4 µl
Template DNA	5 µl

- Total volume of the mixture was set at 50 µl and then DNA Taq Polymerase (0.3 µl) was added.
- dNTP solution was prepared by mixing 10µl of each dATP, dTTP, dCTP and dGTP, then volume completed to 100µl with DDH2O.

- DNA was amplified using a programable PCR thermal cycler with the denaturation, annealing, and polymerization times and temperatures listed below:

Cycle Number	Denaturation	Annealing	Extension
30 Cycles	1 min at 94ºC	1 min at 52ºC	1 min at 72ºC

- A sample (5-10 µ1) was withdrawn from the test reaction mixture and and analyzed by electrophoresis through an agarose gel. DNA marker of an appropriate size (1kb) was included with DNA. The gel was stained with ethidium bromide to visualize the DNA on UV illuminator and photographed for documentation.

Chapter Three

Results and Discussion

1. Morphological Differentiation

In this study was found that there are two common subspecies with clear differences in morphology, they were transferred to a 20cm in diameter pots and subjected for further investigation (Figure 7: A, B).

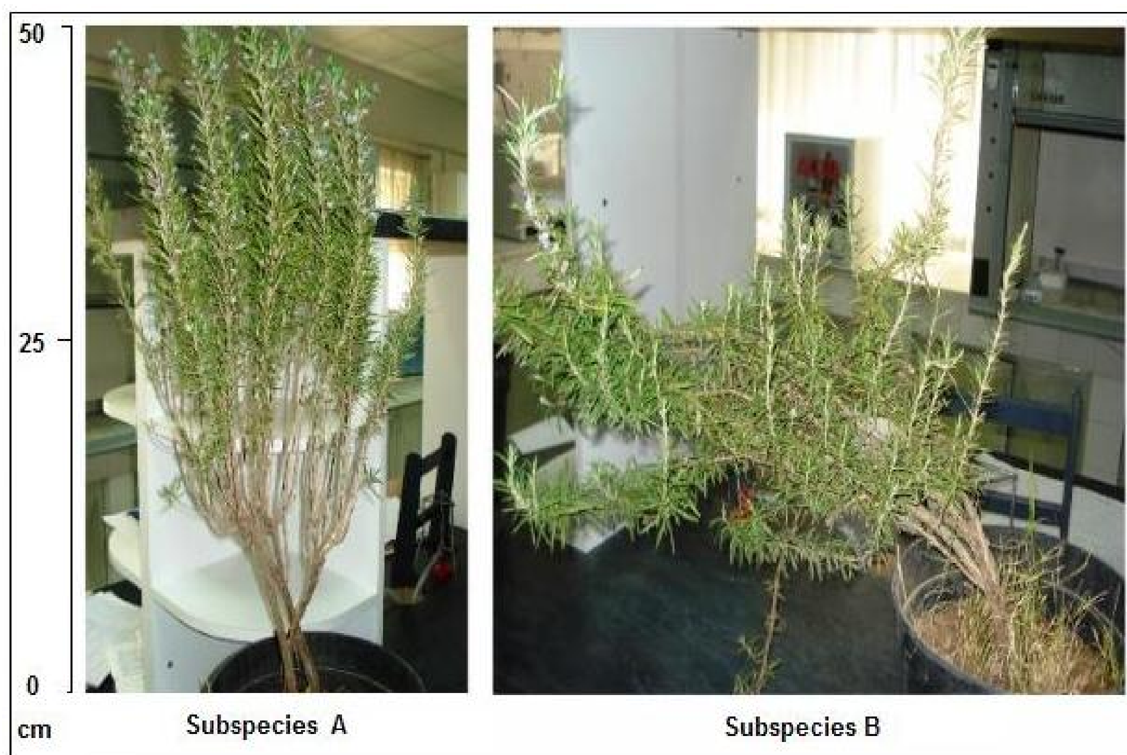

Figure (7): *Rosmarinus officinalis* plant
A: The collected subspecies A with upward growth
B: The collected subspecies B with random growth

1.2. Samples Collection

A local survey in the northern region of Iraq was performed for *R. officinalis* subspecies available in different geographical sites. These sites differ in their ecological conditions that can affect the morphology of *R. officinalis* subspecies.

Table (2) shows the main morphological differences between subspecies A and B according to a naked eye examination.

Subspecies A was characterized by, tall and upward stem direction, whitish blue flower color, less branches, dark green leaves and thin pin like leaf shape.

Subspecies B was characterized by, random growth direction, white flower color, green leaf, more branches, and wide pin like leaf shape, the two subspecies were subjected to investigation at the molecular level.

Table (2): Morphological difference between subspecies A and B.

Character	Subspecies A	Subspecies B
1. Growth Direction	unique, elongated and straight directions	Random directions
2. Flower Color	Whitish Blue	white
3. Branching	Less branches	More Branches
4. Leaf Color	Dark Green	Green
5. Leaf Shape	Pin like shape / thin	Pin like shape / wide

2. Molecular Differentiation

2.1. DNA Isolation and Purification

Genomic DNA of both subspecies was isolated, purified, and electrophorized on agaros gel to examine the purity after staining with Ethidium Bromide (Figure 8).

Clear DNA smear was appeared on gel for both subspecies A and B that represents complete chromosomal DNA of the plant cell. This smear ensures that DNA was correctly isolated and purified, if this smear appears as

separated bands, it indicates that the DNA is digested with restriction enzymes or there is an amplification of specific gene (Sambrook *et al*., 2003).

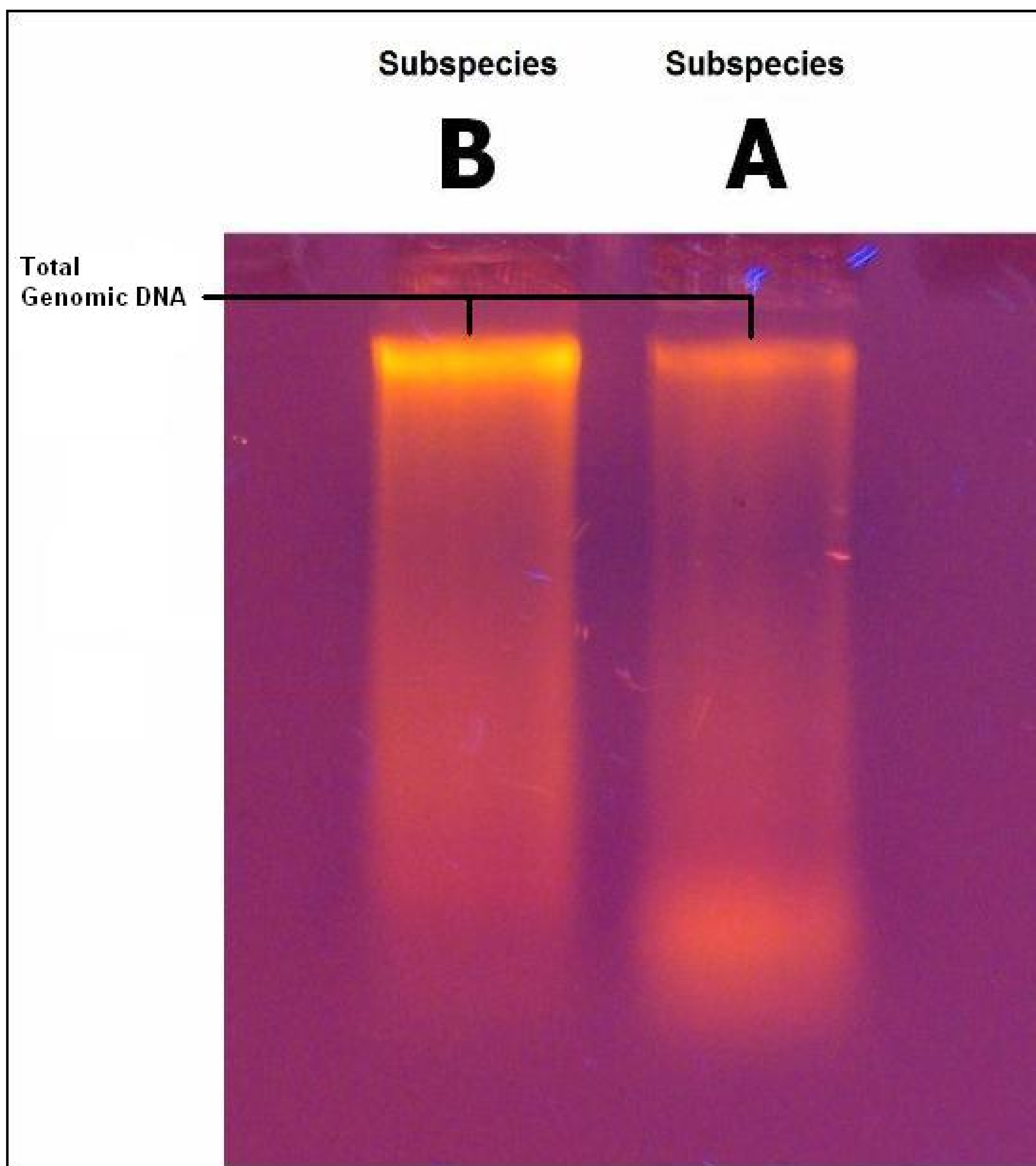

Figure (8): Total Genomic DNA of both subspecies A and B of *R. officinalis* after electrophoresis on agarose gel.

2.2. Ribosomal DNA Template Selection

Since the entire genome sequencing of rosemary was not achieved, therefore it was difficult to find out specific genes that might reflect the molecular differences between subspecies A and B.

The rDNA of rosemary was selected from NCBI Data Base, to be considered as the template DNA of 668 base pairs length used for species identification, which include 18S, 5.8S and 26S RNA genes, separated by two ITS sequences that represent the non-coding region of interest for species identification (Figure 8). Nevertheless, rDNA genes were considered, which were separated by two spacer regions in which the genetic variations should be reflected.

2.3. Primers Design and Synthesis

Two (Forward and Reverse) primers were selected, designed and synthesized by Cybergene AB/Sweden. Primers were delivered with the specifications shown in.

During primers design, the template rDNA was compared and studied with some other plants related to *R. officinalis* which belong to the family Lamiaceae. It was found that the ITS1 and ITS2 regions of each rDNA begins and ends with the same (3-6 bp) nucleotides sequence in most species of Lamiaceae family (Appendix 1).

This study showed that ITS1 region in rDNA gene always begins with the TCG sequence.

It was found that the ITS2 region always starts with CATCG sequence and ends with TCGA sequence. In fact this is the idea of DNA finger print when similarities and dissimilarities between species are required in the non-coding regions (not conserved sequences).

According to this study it was possible to choose four base pairs from ITS2 region during R26SR primer selection as they are the same base pairs for the subspecies under investigation.

2.4 Digestion by Restriction Enzymes

Two samples of DNA (Subspecies A and B) were digested with six different restriction enzymes which were (*Eco*RI, *Eco*RV, *Bam*HI, *Hin*dIII, *Sal*I and *Sma*I). The selected template rDNA showed the availability of *Eco*RV cleavage site, while none of the other five restriction enzymes cleavage sites appeared on the restriction map of the sequence that was obtained by using BioEdit computer based software.

The *Eco*RV enzyme was chosen to test the availability of its cleavage site between rDNA gene of both subspecies A and B, this would reflect the genetic stability of the two subspecies, and which one is more subjected to genetic variations.

2.5. PCR Based RFLP Pattern

Digested DNA for both subspecies with six restriction enzymes was amplified for the rDNA using primers specially developed for this region (Figure 9).

DNA Marker (1kb) was used to estimate the size of the amplified region (rDNA) which was 668 bp long. There were clear bands appeared on agaros gel for samples that lie in a region between 500bp and 750bp bands of the DNA marker at the 668bp region. These bands represent the amplified rDNA by PCR for both subspecies A and B.

According to the PCR based RFLP results, there was a clear genetic variation between the subspecies in the rDNA sequence. For *Eco*RI restriction enzyme digestion and amplification (for both A and B), it was found that the

amplified region appeared as clear bands, which means that there was no recognition site for *Eco*RI (5`-G^AATTC-3`) at the ITS1 and ITS2 regions of the rDNA. It is assumed that if there was a cutting site then the band will not appear as there will be no amplification for the whole rDNA.

DNA amplification after digestion with *Eco*RV, showed no DNA band between the 500-750bp bands of DNA marker for subspecies A. That means that there is a cutting site for *Eco*RV (5`-GAT^ATC-3`) in the rDNA sequence of A. In fact this result was expected as the recognition site of *Eco*RV was already exist in the template DNA in a conserved region of 5.8S gene and not in the ITS region.

The *Eco*RV recognition site disappeared from B subspecies. A clear band was appeared, which means that there is no *Eco*RV digestion occurred since the site in the template DNA occurs at a conserved region (5.8S gene). There may be a type of modification or mutation occurred in one base pair in this site without affecting the function of the gene for 5.8S RNA synthesis which may lead *Eco*RV not to recognize and cut rDNA sequence in subspecies B.

Possible explanation that there may be a shifting in one nucleotide base pair of the gene led to the synthesis of other amino acids included in product synthesis. This shifting lead to the disappearance of the *Eco*RV cleavage site.

This result gives an idea about the 5.8S RNA gene that is not conserved and subjected to mutation in subspecies B. However, subspecies A seemed to conserve the gene.

*Bam*HI RFLP pattern showed an amplification band in subspecies B while no band in subspecies A, that means that there was a recognition sequence for *Bam*HI (5`-G^GATCC- 3`) in subspecies A while not in B.

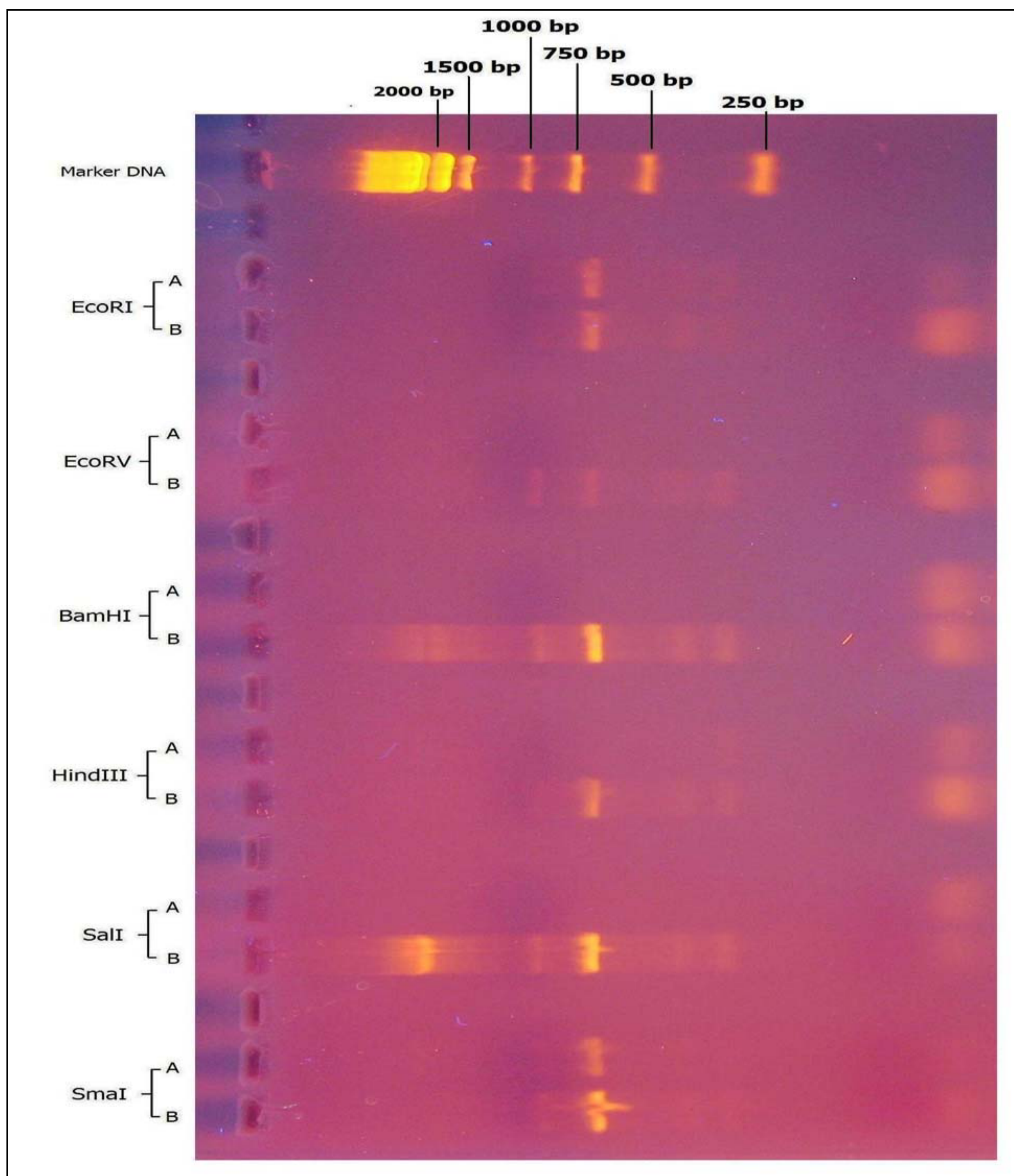

Figure (9): PCR based RFLP pattern of two subspecies A and B of *R. officinalis*.

Additionally for the recognition site of *Hin*dIII (5`-A^AGCTT-3`), a band appeared in the pattern of subspecies B while not in A. This may mean

that the digestion of double stranded DNA occurred in the rDNA of subspecies A while not in B.

The high concentrations of salt in certain restriction enzyme buffers (e.g., *Bam*HI and *Eco*RI) retard the migration of DNA and distort the electrophoresis of DNA in the adjacent wells.

The recognition site of *Sal*I (5`-G^TCGAC-3`) is found in rDNA of subspecies A but not in B, while both subspecies did not contain any recognition site for *Sma*I (5`-CCC^GGG-3`) restriction enzyme in both subspecies, this may due to disappearance of bands in both of them in RFLP pattern.

According to the above molecular differentiation results, it was found that there are many cleavage sites for restriction enzymes in subspecies A, namely, *Eco*RI, *Bam*HI, *Hin*dIII, and *Sma*I, none of the six restriction enzymes used contain any recognition sites in the subspecies B.

Also it could be concluded that, because of the absence of six restriction enzymes cleavage site from subspecies B (Table 3) and there is a non-conserved gene (5.8S RNA Gene) in the rDNA, then this plant is more subjected to mutations and modifications naturally, and this may led to the random growth and differences in shape from subspecies A, that appeared through out generations as a morphological differences.

Table (3): Genetic Variation between subspecies A and B according to PCR based RFLP pattern of rDNA.

Restriction Enzyme	Recognition Site	Subspecies A	Subspecies B
*Eco*RI	5`-G^AATTC-3`	Absent	Absent
*Eco*RV	5`-GAT^ATC-3`	Present	Absent
*Bam*HI	5`-G^GATCC- 3`	Present	Absent
*Hin*dIII	5`-A^AGCTT-3`	Present	Absent
*Sal*I	5`-G^TCGAC-3`	Present	Absent
*Sma*I	5`-CCC^GGG-3`	Absent	Absent

Conclusions

1) Establishment and application of PCR based RFLP technique (DNA Finger Print) for species identification of *R. officinalis* L. grown in the northern region Iraq.

2) According to our survey in east north region of iraq, we found that there are two subspecies differ morphologically, classified as subspecies A and B.

3) DNA successfully isolated and purified from plant tissues using maceration with cold ethanol under very low temperatures instead of the use of liquid nitrogen.

4) ITS1 non-coding regions of the rDNA begin with the same (3 bp) nucleotides (TCG sequence) in Lamiaceae family and ITS2 non-coding regions in the rDNA always start with CATCG sequence and end with TCGA sequence of the rDNA in Lamiaceae family.

5) *Eco*RV, *Bam*HI, *Hin*dIII and *Sal*I recognition sites was present in subspecies A but not in B, while both subspecies A and B did not contain a recognition sites for *Eco*RI and *Sma*I restriction enzyme in the ribosomal DNA.

6) Subspecies B could be subjected to mutations more than subspecies A. This may led to the variation in morphology.

Recommendations

1) Complete genomic DNA sequencing is required for *R. officinalis*.

2) Investigation of the molecular make up of other medicinal plants in Iraq.

3) Genetic manipulation of some genes of rosemary that could results in increasing the production of some interesting secondary metabolites.

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Appendix

Appendix 1: Alignment of rDNA gene for ten different genera of Lamiaceae family (NCBI, 2008).

Melissa officinalis:

```
  1 gtttccgtag gtgaacctgc ggaaggatca ttgtcgaaac ctgcaaagca gaccgcgaac
 61 acgtgtttaa caccacgggg cacgacgtgg gggcatgctc cccgtcgtgt ctccgttcct
121 gccggcgtgc tccctcgggt cacgtcgtgc ggactaacga accccggcgc ggaatgcgcc
181 aaggaaaact aaacgaagcg accgcctcct gcatcccgtt cgcggagtgt gtgggaggat
241 tgggcgtcta tcaaatgtca taacgactct cggcaacgga tatctcggct ctcgcatcga
301 tgaagaacgt agcgaaatgc gatacttggt gtgaattgca gaatcccgtg aaccatcgag
361 tctttgaacg caagttgcgc ccgaagccac taggccgagg gcacgtctgc ctgggcgtca
421 cgcatcgcgt cgcccccctt ccccgcgcac cgcgctggtt atgggggcgg atattggcct
481 cccgtgtgct tcggcacgcg gctggcccaa atgcgatccc tcggcgactc atgtcacgac
541 aagtggtggt tgaacactca atctcgcgcg tcgtcgtgct actgagtcgt cagtatgggc
601 atccatgaac gaccctatgg tgtcggtgcc tcacagcacc tcactttcga ccgcgacccc
661 aggtca
```

Mentha arvensis

```
  1 atcatttaga ggaaggagaa gtcgtaacaa ggtttccgta ggtgaacctg cggaaggatc
 61 attgtcgaaa cctgcaaagc agaccgcgaa ctcgtaacta acgccgcggg gcacggcacg
121 ggggagaccc cctgccgcat cccgtctcct gccggcttgc tccctcgggg gcacgccgtg
181 cgggctaacg aaccccggcg cggaacgcgc caaggaaaac caaacgaagc gtccgccccc
241 ggcatcccgt tcgcggggcg tgccgtggga tcgggcgtct atcaaatgtc aaaacgactc
301 tcggcaacgg atatctcggc tctcgcatcg atgaagaacg tagcgaaatg cgatacttgg
361 tgtgaattgc agaatcccgt gaaccatcga gtctttgaac gcaagttgcg cccgaagcca
421 ttaggccgag ggcacgtctg cctgggcgtc acgcatcgcg tcgcccccca cccccgcgcg
481 catcgccggg cagttggggg cggacactgg cctcccgtgc gcctcggcgt gcggccggcc
541 caaatgagat ccccgggcga ctggcgtcgc gacaagtggt ggttgaacat ctcaatctct
601 ctcgtggtcg tgccgccgtg tcgtcccgta cgggaatcga aaacgaccca acggtgctag
661 gcgcgaacag cgtctcacct tcgaccgcga ccccaggtca ggcgggatta cccgctgagt
721 ttaagc
```

Thymus pubescens

```
  1 ggtgaacctg cggaaggatc attgtcgaac ctttaaaaac agaccgcgaa cacgtgttta
 61 acaaagttgg ggacggtgcg gggggtaacc ctctgccgtg tcccatctcc tgccggcgtg
121 tatcttcggg tcatgtcgtg cgggctaacg aaccccggcg cggaatgcgc caaggaaaac
181 aaaacgaagc gtttcccccct ggcatcccgt tcgcggagtg tgctggggga gcagacgtct
241 atcaaatgtc aaaacgactc tcggcaacgg atatctcggc tctcgcatcg atgaagaacg
301 tagcgaaatg cgatacttgg tgtgaattgc agaatcccgt gaaccatcga gtctttgaac
361 gcaagttgcg cccgaagcca ttaggccgag ggcacgtctg cctgggcgtc acgcatcgcg
421 tcgcccccct tccccgcgct gaatgccggg cggtcggggg cggacattgg cctcccgtgc
481 acctccgtgc gcggctggcc caaatgcgat ccccgggcga ctggcgtcac gacaagtggt
541 ggttgaacat ctcaatctct ctcgtcgtcg tgccgtcctg tcgtcattac gggaatagtc
601 ataaacgacc caacggtgcc ggtgcttaac tgcacctcac cttcgaccgc gaccccaggt
661 caggcgggat tacccgctga gtttaagcat atcata
```

Thymus serpyllum

```
  1 gtttccgtag gtgaacctgc ggaaggatca ttgtcgaacc tttaaaaaya gaccgcgaac
 61 acgtgtttaa caccgttggg gacggtgcgg ggggtaaccc tctgccgtgt cccatctcct
121 gccggcgtgt atcttcgggt cacgtcgtgc gggctaacga accccggcgc ggaatgcgcc
181 aaggaaaaca aaaygaagcg tttcccccctg gcatcccgtt cgcggagtgt gctgggggag
241 cagacgtcta tcaaatgtca aaacgactct cggcaacgga tatctcggct ctcgcatcga
301 tgaagaacgt agcgaaatgc gatacttggt gtgaattgca gaatcccgtg aaccatcgag
361 tctttgaacg caagttgcgc ccgaagccat taggccgagg gcacgtctgc ctgggcgtca
421 cgcatcgcgt cgcccccctt ccccgtgctg aatgccgggc ggtcgggggc ggacattggc
481 ctcccgtgca cctccgtgcg cggctggccc aaatgcgatc cccgggcgac tggcgtcacg
541 acaagtggtg gttgaacatc tcaatctctc tcgtcgtcgt gccgtcctgt cgtcattacg
601 ggaatagtca taaacgaccc aacggtgccg gtgcttaact gyacctcacc ttcgaccgcg
661 accccaggtc a
```

Zhumeria majdae

```
  1 tagaggaagg agaagtcgta acaaggtttc cgtaggtgaa cctgcggaag gatcattgtc
 61 gaaacctgca aagcagaccg cgaacacgtg tttaacaccg acggtggcac ggcgaggggt
121 gacccccgtc gggccatcgt cccccnccgg cgtgttccct cgggtcacgt cgtgcgggct
181 aacgaacccc ggcgcggaat gcgccaagga aaactaaacr aagcgtccgc cccccgagcc
241 ccgttcrcgg tgcgcgcggg gggaccggat gtctgtcaaa tgtcaaaacg actctcggca
301 acggatatct cggctctcgc atcgatgaag aacgtagcga aatgcgatac ttggtgtgaa
361 ttgcagaatc ccgtgaacca tcgagtcttt gaacgcaagt tgcgcccgaa gccgtcaggc
421 cgagggcacg tctgcctggg cgtcacgcat cgcgtcgccc ccttccccgc gcartrcgcc
481 ggttacgggg gtggatattg gcctcccgtg cgctccggcg tgyggctggc ccaaatgcga
541 tacctcggcg actcgtgtcg cgacaagtgg tggttgaaca ctcaatctcg cgcgccgtcg
601 tgacaccgtg tcgtctgtac ggggatccat caatgaccca acggtgkagg tgcctcaggg
661 cgycccacct tcgaccgcga ccccaggtna ggcgggatta cccgctgagt tt
```

Salvia whitehousei

```
  1 tatcatttag aggaaggaga agtcgtaaca aggtttccgt aggtgaacct gcggaaggat
 61 cattgtcgaa acctgcaaag cagaccgcga acacgtgttt aacaccaatc ggcggtgcat
121 ggcgtggggg caaccccccg tcgtgtcgct gtcaaccccc gcctgcgtgc tccctcgggt
181 cacgtcgtgc gggctaacga accccggcgc ggaatgcgcc aaggaaaact aaacgaagca
241 tccacctcca gcaccccgtt cgcggagtgt gcaggggtat cgggtgtctt acaaatgtca
301 aaacgactct cggcaacgga tatctcggct ctcgcatcga tgaagaacgt agcgaaatgc
361 gatacttggt gtgaattgca gaatcccgtg aaccatcgag tctttgaacg caagttgcgc
421 ccgaagccat taggccgagg gcacgtctgc ctgggcgtca cgcatcgcgt cgccccccat
481 ccatgcgcac cgcgctggtt gcgggggcgg atattggcct cccgtgcgcc ttggcgtgcg
541 gctggcccaa atgcgatccc tcggcgacac atgtcacgac aagtggtggt tgaattctca
601 atctcgcgcg ccgtcgtgcc attgcgtcgt ccgtatgggc atccgtaaaa gacccaatgg
661 tgttggtgcc tcatggtgcc cccacctcga ccgcgacccc aggtcaggcg ggattacccg
721 ctgagtttaa g
```

Pycnanthemum virginianum

```
  1 taggtgaacc tgcggaagga tcattgtcga gacctgcaaa gcagaccgcg aacacgtaac
 61 taacaccgcg ggcgcggcgc gggggcgacc ccccgccgtg tcccgtctcc cgccggcgtg
121 ctccctcggg tcacgccgcg cgggctaacg aaacccggcg cggaatgcgc caaggaaaac
181 cgaacgaagc gtccgccccc ggcatcccgt tcgcggagcg tgccgcggga tcgggcgtct
241 atcaaaagtc aaaacgactc tcggcaacgg atatctcggc tctcgcatcg atgaagaacg
301 tagcgaaatg cgatacttgg tgtgaattgc agaatcccgt gaaccatcga gtctttgaac
361 gcaagttgcg cccgaagcca ttaggccgag ggcacgtctg cctgggcgtc acgcatcgcg
421 tcgccccccc caccccgcgc gcgtcgccgg gcggttgggg gcggaaactg gcccccccgtg
481 cgcctcggcg tgcggccggc ccaaatgaga tccccgggcg gctggcgtca cgacaagtgg
541 tggttgaaca tctcaatctc tctcgcggtc gtgccgccgt gtcgtcccgt gcgggaatcg
601 caaacgaccc aacggtgcac ggcgcgaaca gcgcctcacc ttcgaccgcg accccaggtc
661 aggcgggatt acccgctgag tttaagc
```

Clinopodium vulgare

```
  1 cttatcattt agaggaagga gaagtcgtaa caaggtttcc gtaggtgaac ctgcggaagg
 61 atcattgtcg agacctgcaa agcagaccgc gaacacgtaa ctaacgccgc nnggcgcggc
121 gggcgggcta cccccgccg agccccgttc cccgccggcg cgtccccccc cgggggccgc
181 gccgcgcggg ctaacgaacc ccggcgcgga acgcgccaag gaaaacgaaa cgaagcgccc
241 gcccccccc ggcatcccgt ccgcgggtcc tgccggggga ccgggcgtct gttaaatgtc
301 aaaacgactc tcggcaacgg atatctcggc tctcgcatcg atgaagaacg tagcgaaatg
361 cgatacttgg tgtgaattgc agaatcccgt gaaccatcga gtctttgaac gcaagttgcg
421 cccgaagcct ttaggccgag ggcacgtctg cctgggcgtc acgcatcgcg tcgcccccaa
481 tccccgcgcg catcgccggg cggtcggggg cggagattgg cctcccgtgc gcctcggcgc
541 gcggccggcc caaatgggat ccccgggcgg ctggcgtcgc gacaagtggt ggttgaacat
601 ctcaatctct ctcgcggtcg cgccgccgtg ccgtcccgca cgggcatcga taaacgaccc
661 aacggcgggc gggcgcgttc gttcgcgca
```

Monarda fistulosa

```
  1 tcatttagag gaaggagaag tcgtaacaag gtttccgtag gtgaacctgc ggaaggatca
 61 ttgtcgatac ctgcaaagca gaccgtgaac acgtaactaa caccgcggga gcggcgaggg
121 gcgacccccg ccgcgtcccg tctcccgacg gcgtgctccc tcgggtcacg ccgctcgggc
181 taacgaaccc cggcgcggaa tgcgccaagg aaaaccaact gaagcgttcg ccccccggca
241 tcccgttcgc ggagcgtgcc gcgggatcgg gcgtctatca aaagtcaaaa cgactctcgg
301 caacggatat ctcggctctc gcatcgatga agaacgtagc gaaatgcgat acttggtgtg
361 aattgcagaa tcccgtgaac catcgagtct ttgaacgcaa gttgcgcccg aagccattag
421 gccgagggca cgtctgcctg ggcgtcacgc atcgcgtcgc cccccacgcc ccgcgcgagt
481 cgccgggcgg ttgggggcgg acactggcct cccgtgtgcc tcggcgtgcg gccggcccaa
541 atgagatccc cgggcggctg gcgtcacgac aagtggtggt tgaacatctc aatctctctc
601 gcagtcgtgc cgccgtgtcg tcccgtgcgg gaatccaaaa acgacccaac ggtgcacggc
661 gcgaatagcg ccccaccttc gaccgcgacc ccaggtcagg cgggattacc cgctgagttt
721 a
```

Salvia austriaca

```
  1 gtaacaaggt ttccgtaggt gaacctgcgg aaggatcatt gtcgaaacct gcaaagcaga
 61 ccgcgaacac gtgtttaaca ccgaccgacg gcgcacggcg cgggggcgac ccccgtcgtg
121 ccgccgtcac ccccgcccgc gcgttccctc gggtcgcgcg gcgcggcta acgaaccccg
181 gcgcggaatg cgccaaggaa aactaaacga agcgtcctcc cccccgcgcc ccgttcgcgg
241 agtgcgcggg ggtgtcggac gtctatcaaa tgtcataacg actctcggca acggatatct
301 cggctctcgc atcgatgaag aacgtagcga aatgcgatac ttggtgtgaa ttgcagaatc
361 ccgtgaacca tcgagtcttt gaacgcaagt tgcgcccgaa gccattaggc cgagggcacg
421 tctgcctggg cgtcacgcat cgcgtcgccc ccccaccatg tgcgggggcg gatactggcc
481 tcctgtgcgc cccggcgcgc ggctggccca aatgcgatcc ctcggcgact catgtcacga
541 caagtggtgg ttgaaatctc aatctcttgc gccgtcgtgc cactgcgtcg tccgtacggg
601 catccatcaa cgacccaacg gtgggggagc ctcgcggcgc cccgaccttc gaccgcgac
```

Red = 18S RNA Gene (Partial Sequence)
Yellow = ITS1 Region (Complete Sequence)
Green = 5.8S RNA Gene (Complete Sequence)
Grey = ITS2 Region (Complete Sequence)
Pink = 26S or 28S RNA Gene (Partial Sequence)

بسْم أللهِ ألرَحمنْ ألرَحيمْ

نَرفَعُ دَرَجاتٍ منْ نَشاءُ وَفَوقَ كُلِ ذي عِلمٍ عَليمْ

صَدَقَ أللهِ ألعَظيمْ

سورة يوسف الاية 76

الملخص:

في محاولة لتمييز ودراسة واحد من اكثر النباتات الطبية شيوعاً (نبات الروزماري *Rosmarinus officinalis* L.) في شمال العراق، تم تنفيذ عدد من التجارب لهذا الغرض للتحري عن الاصناف المتوفرة في اقليم كردستان على المستويين المظهري والجزيئي.

اجري مسح ودراسة مظهرية لهذه الاصناف من الروزماري في شمال العراق وتحديدا مدينتي اربيل والسليمانية. وجد ان هناك اختلافات مظهرية في الاوراق، الازهار، التفرعات وشكل الاوراق، وطبقا لذلك فقد تم تسمية تحت صنفين (A) و (B) من نبات الروزماري في شمال العراق.

تحت الصنف (A) تميز بافرع طولية ومنتظمة، اوراق خضراء غامقة وازهار بلون الازرق المبيض، بينما تميز تحت الصنف (B) تميز بنموه العشوائي، غير منتظم، اوراق خضراء اللون وازهار بيضاء.

ومن اجل الكشف عن التغايرات على المستوى الجزيئي، فقد تم عزل وتنقية (DNA) من كلا الصنفين المعزولين، ومن ثم تم ترحيله على (Agarose Gel)، حيث وجد ان الـDNA قد ظهر على شكل مسحة واضحه لكلا الصنفين.

تم بعد ذلك تقطيع الـDNA المنقى وذلك باستخدام ستة انواع من الانزيمات القاطعة ولكل من الصنفين، والتي هي: *Eco*RI, *Eco*RV, *Bam*HI, *Hin*dIII, *Sal*I, *Sma*I

وتم بعد ذلك تضخيم الـDNA المقطع الناتج بتطبيق تقانة الـPCR للـDNA الرايبوسومي كسلسلة مستهدفة للتمييز بين الصنفين والمكونه من 668 زوج قاعدي والتي تحتوي على جينات التشفير للـ 18S، 5.8Sو ,26 للـRNA والمفصولة فيما بينها بواسطة منطقتين من ITS والتي تمثل المنطقة غير المشفرة المهمة في التمييز بين الاصناف.

وجد انه هناك اختلافات جينية واضحة بين تحت الصنفين وفي منطقة الـ DNA الرايبوسومي، وطبقا لتواجد شفرات التمييز والقطع للانزيمات القاطعة في الـ DNA المستهدف، فقد وجدت مناطق تمييز وقطع ولكل من الانزيمات EcoRV, BamHI, HindIII, و SalI في تحت الصنف (A)، بينما لا يوجد اي نوع من الانزيمات القاطعة الستة المستخدمة في تحت الصنف (B).

جمهورية العراق
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التمييز الجزيئي بين صنفين من نبات الروزماري النامية في المنطقة الشمالية الشرقية للعراق

رسالة

مقدمة الى كلية العلوم – جامعة النهرين وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانة الاحيائية

من قبل

طيف نزار محمود الشاكر

بكالوريوس تقانة احيائية جامعة النهرين 2004

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