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Molecular Differentiation between Two Varieties of *Rosmarinus officinalis* Grown in North East Region of Iraq

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Abstract. In an attempt to differentiate and study one of the most popular medicinal plants (*Rosmarinus officinalis* L.) grown in the North East region of Iraq, a number of experiments were carried out to differentiate between subspecies available in Kurdistan Province at the morphological and molecular levels. 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 East of Iraq. Subspecies A was characterized by a straight shoot growth, dark green leaves and whitish blue flowers while subspecies B has random growth, green leaves and white flowers. In order to investigate the genetic variation, the DNA was 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 for 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 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 is concluded that there are obvious genomic differences in the rDNA in 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 was present in subspecies B.

Keywords: molecular differentiation, *Rosmarinus officinalis* L., rDNA, chromosomal DNA, PCR

INTRODUCTION

Rosemary (*Rosmarinus officinalis* L.) is a member of Lamiaceae family. Rosemary extract had been widely used for topical applications for a wound-healing, anti-aging 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). 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).

Rosmarinus 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 regions of 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. The genetic classification system which reflects the actual route of evolution should be adopted. The many different systems proposed are not necessarily right (Anonymous, 2000).

Restriction Fragment Length Polymorphism (RFLP) analysis is a pioneer method for 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 (Mullis *et al.,* 1986).

In this work each one of 18S, 5.8S and 26S RNA genes with two Internal Transcribed Spacer (ITS) regions of ribosomal DNA (rDNA) sequence were considered for *R. officinalis* species identification in the North of Iraq using PCR based RFLP techniques. Due to the importance of this locally grown plant as a potential source of phytochemicals, this research work was conducted to characterize morphological differences among the two subspecies. Thus, it is possible to utilize the more producible subspecies from phytochemicals. In addition to study the genomic DNA of the two subspecies by using PCR based RFLP technique in order to differentiate between the subspecies at the molecular level as this would ease and speed up the process of differentiation between species and even varieties for better traits. In addition, the genomic DNA study of the two subspecies by using PCR based RFLP techniques were used in order to differentiate the subspecies at the molecular level.

MATERIALS AND METHODS

Plant Material

Rosemary (*R. officinalis*) plant samples were collected from different geographical sites located in Northern region of Iraq including: a) Erbil city which represents the plane land, moderate temperature with long sunny periods throughout the year. b) Sulaimania city, which represents the mountain land with low temperature and shorter sunny periods throughout the year. A morphological study was conducted on plant samples including: Leaves (shape and color), flowers (shape and color), and stem growth directions. Two subspecies were found with obvious different characteristics particularly in stem growth directions and were nominated as subspecies A and subspecies B. The two mentioned genotypes were collected and subjected to intensive molecular investigation.

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 for Biotechnology Information (NCBI). The following techniques were implemented in this study: DNA isolation, purification, gel electrophoresis and digestion by restriction enzymes (Sambrook *et al.,* 2003). DNA purification kit was used according to the manufacturer instructions (Fermentas, Germany) and gel electrophoresis was conducted according to 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 for Biotechnology Information), which consists 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 by selecting the first 23bp repeating sequence (Sense) of rDNA template from the 5` to 3` end as a first forward primer which was: 5`-GTT TCC GTA GGT GAA CCT GCG GA-3`, named as (R18SF) and representing 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 was:
(5`-TGA CCT GGG GTC GCG GTC GA-3`), and named as (R26SR) which was explained as: R: For Rosemary 26S: For the site in which primer sequence located in rDNA (26S RNA Gene). R: for reverse primer.
R18SF primer 23bp origin 5`end
1 gtttccgtag gtgaacctgc ggaaggatca ttgtcgaaac ctgcaaagca gaccgcgaac
61 acgtgtttaa cgccatcggg ggcacgacgt gggggcaacc cccatcgtgc caccggcccc
121 ccgcccggca tgttccctcg ggccatgtck tgcgggctaa cgaaccccgg cgcggaatgc
181 gccaaggaaa actaaacgaa gcgtcygcct cccgcacccc gttcgcggaa cgtgcgtggg
241 gatcggatgt ctgtcaaatg tcaaaacgac tctcggcaac ggatatctcg gctctcgcat
301 cgatgaagaa cgtagcgaaa tgcgatactt ggtgtgaatt gcagaatccc gtgaaccatc
361 gagtctttga acgcaagttg cgcccgaagc cattaggccg agggcacgtc tgcctgggcg
421 tcacgcatcg cgtcgccccc catcccagcg taaagcagcg gttgtggggc ggayattggc
481 ctcccgtgcg tctcgatgcg cggttggccc aaatgcgatc cctcggcgac tcgtgtcacg
541 ataagtggtg gttgaacaac tcaatctcgc gcgccgtcgt gccactgcgt cgtccgcatg
601 ggcatccatc aatgacccaa tggtatggtg ccttagggcg ctctaccttc gaccgcgacc
661 ccaggtca 3`end ag ctggcgctcc
Ggtccagt

After the completion of primer design, the two primers were then ordered and synthesized from (Cybergene AB, Stockholm, Sweden), (www.cybegene.se). Finding specific primer (s) for differentiation is of a vital importance to build up genomic data bases.

Morphological Differentiation

It was found that there are two common subspecies with clear differences in morphology. For this reason they were transferred to a 20 cm in diameter black plastic pots and subjected to further investigation.

RESULTS AND DISCUSSION

Subspecies A was characterized by tall and upward stem direction, whitish blue flowers, less branches, dark green leaves and thin pin like leaf shape (Fig. 1). Subspecies B was characterized by random growth direction, white flowers, green leaves, more branches and wide pin like leaf shape.

Molecular Differentiation:

Clear DNA smear was appeared on gel for both subspecies A and B representing complete chromosomal DNA of the plant cell (Fig. 2). 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).

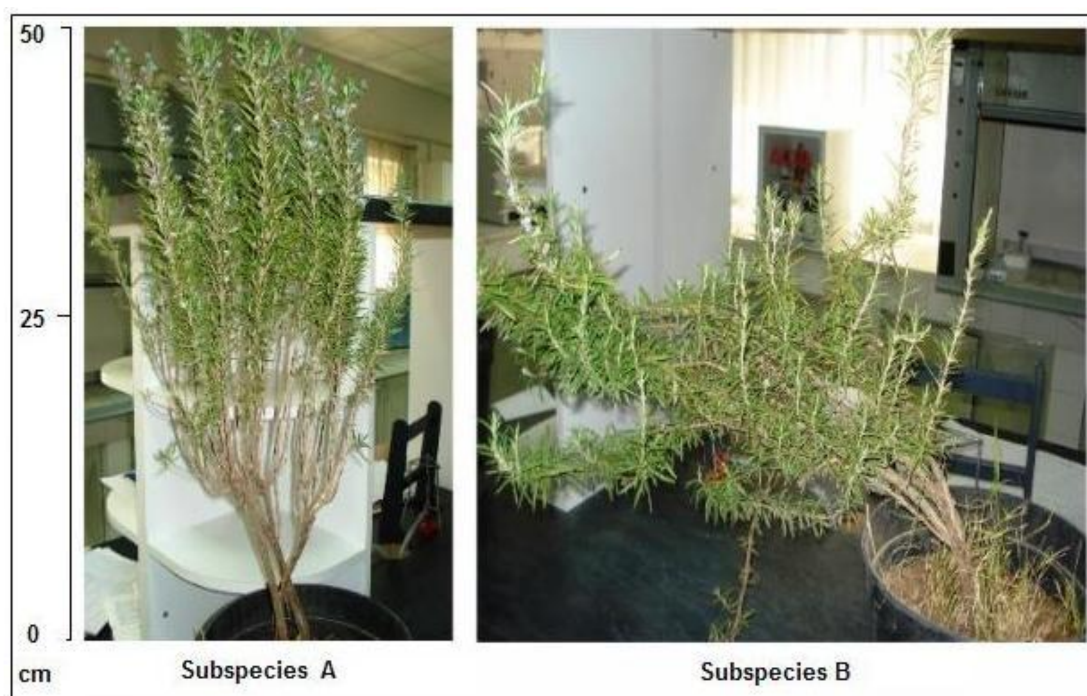

Fig. 1. Morphological differences between the two subspecies A and B of *R. officinalis*

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 the 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 includes 18S, 5.8S and 26S RNA genes separated by two ITS sequences representing the non-coding region of interest for species identification (Fig. 3). Nevertheless, rDNA genes were considered and separated by two spacer regions in which the genetic variations were reflected. During primers design, the template rDNA was compared and studied with some other plants related to *R. officinalis* in a preliminary study (data not shown) 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. This study showed that ITS1 region in rDNA gene always begins with the TCG sequence and 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. 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).

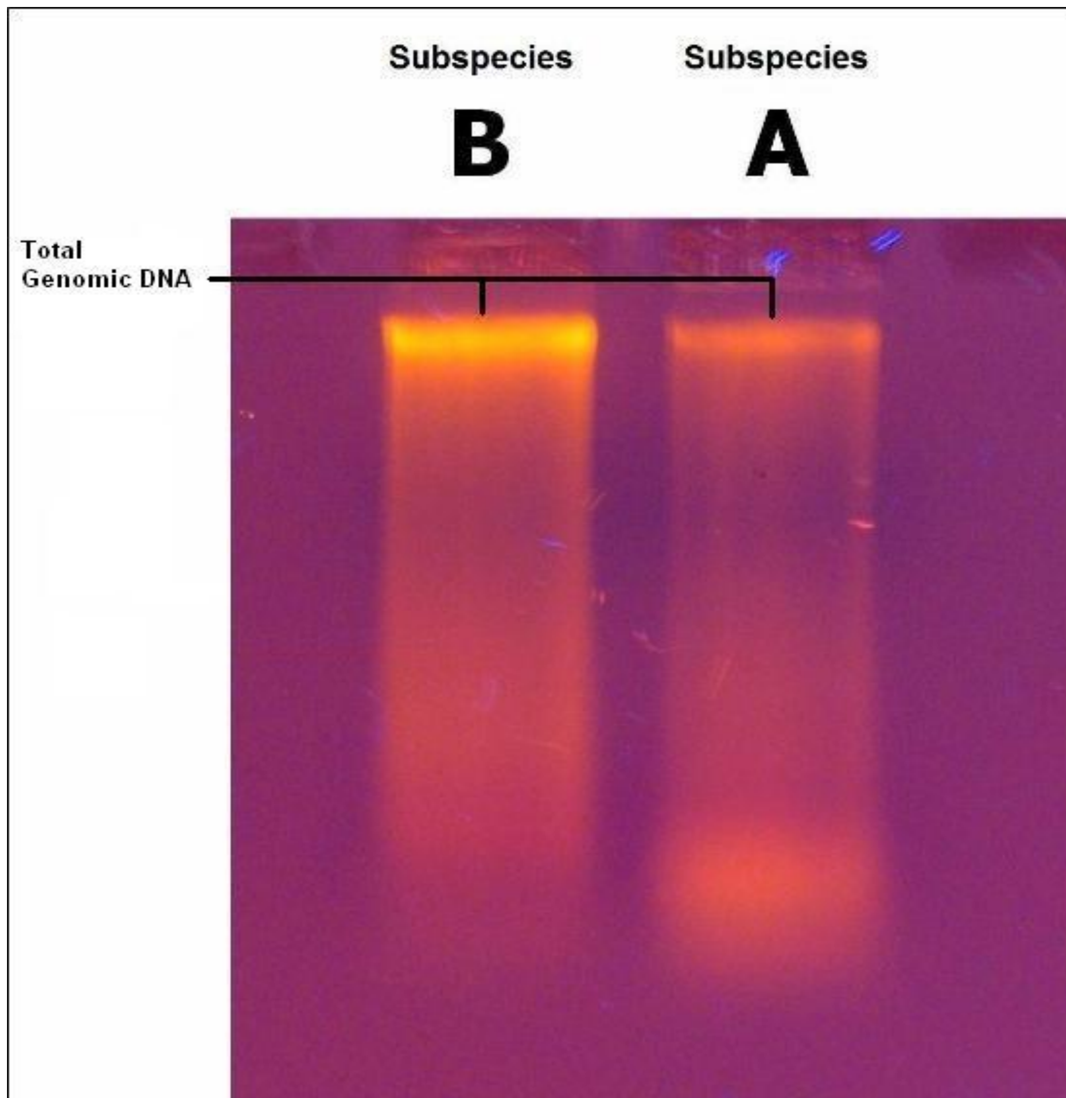

Fig. 2. Total Genomic DNA for both subspecies A and B of *R. officinalis* after electrophoresis on agarose gel

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 for both subspecies A and B, this would reflect the genetic stability of the two subspecies, and which one is more subjected to genetic variations. DNA Marker (1kb) was used to estimate the size of the amplified region (rDNA) which was 668 bp long. Clear bands appeared on agarose 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.

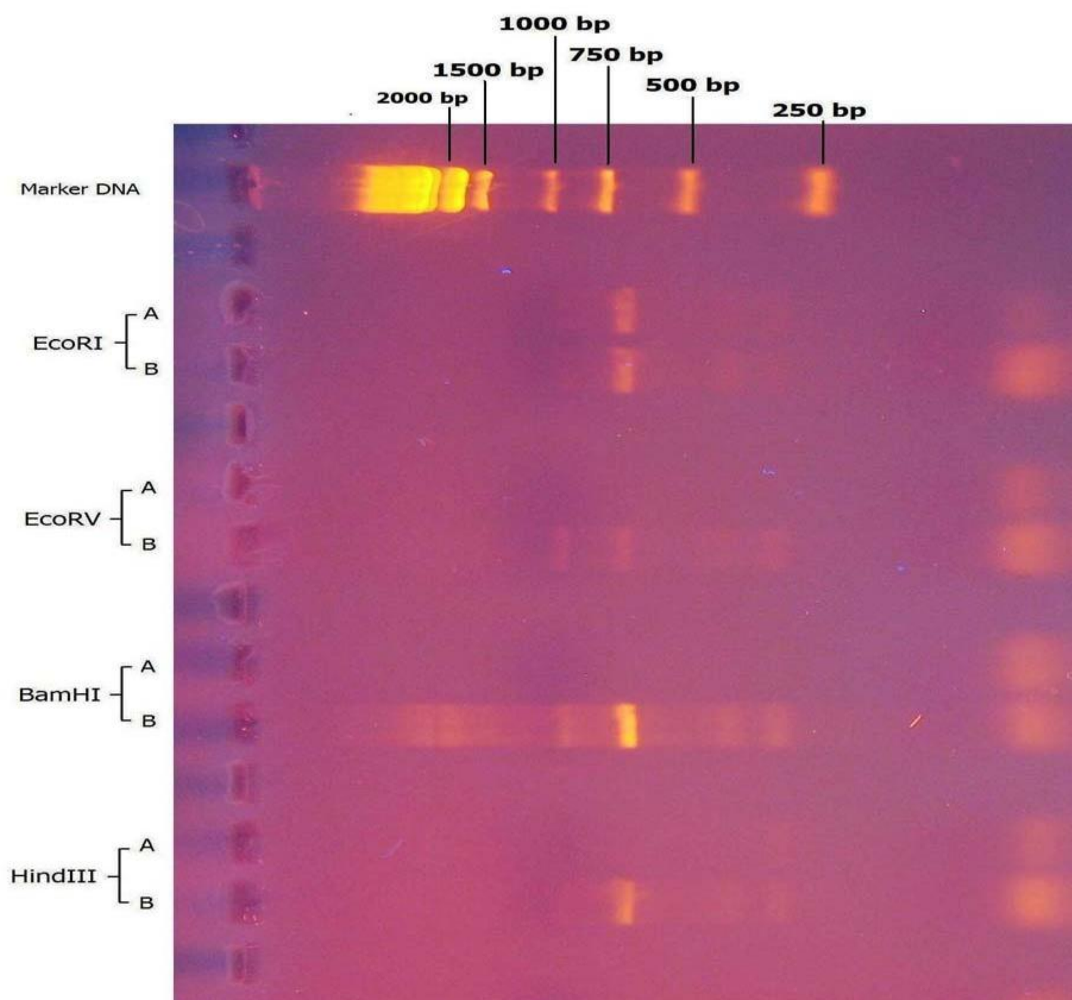

Fig. 3. PCR based RFLP pattern for the two subspecies A and B of *Rosmarinus officinalis*

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 and thus 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, reflecting the presence of 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 (Nei and Li, 1979). The *Eco*RV recognition site disappeared from B subspecies. A clear band was appeared, which means that there was 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 causes disappearance of the *Eco*RV cleavage site (Misbah *et al.,* 2005). 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.

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 due to 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) retarded the migration of DNA and distorted the electrophoresis of DNA in the adjacent wells (Merhendi *et al*., 2004). 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 the disappearance of bands in RFLP pattern.

CONCLUSIONS

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 and 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, there was a non-conserved gene (5.8S RNA Gene) in the rDNA, then this plant (Subspecies B) 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 throughout generations as morphological differences.

REFERENCES

1. Al-Sereitia, M. R., K. M. Abu-Amerb and P. Sena (1999). Pharmacology of rosemary (*Rosmarinus officinalis* Linn.) and its therapeutic potentials, Ind. J. of Exper. Biol. 37:124-131.

2. Anonymous, (2000). Kew Royal Botanic Gardens. Studying plant diversity, plant classification, Kew information sheet B1, London.

3. Kowalchik, C. and W. H. Hylton (1987). Rodales Illustrated Encyclopedia of Herbs. Rodale Press. Emmaus, Pennsylvania, USA.

4. Leithy, S., T. A. El-Meseiry and E. F. Abdallah (2006). Effect of biofertilizer, cell stabilizer and irrigation regime on rosemary herbage oil yield and quality. J. of Appl. Sci. Res. 2(10):773-779.

5. Merhendi, H., K. Makimora, K. Zomorodian, N. Maeda, T. Ohshima and H. Yamaguchi (2004). Differentiation of *Candida albicans* and *Candida dubliniensis* using single enzyme PCR-RFLP method. Jap. J. Infect. Dis. 58:235-237.

6. Misbah, S., H. Hassan, M. Y. Yusof, Y. A. Hanifah, S. AbuBakar (2005). Genomic species identification of Acinetobacter of clinical isolates by 16S rDNA sequencing. Singapore Med. J. 46(9):461-462.

7. Mullis, K., F. Faloona, S. Scharf, R. Saiki, G. Horn, and H. Erlich (1986). Specific enzymatic amplification of DNA *in vitro*: The polymerase chain reaction. Cold Spring Harbor Symp. Quant. Biol. 51:263-273.

8. NCBI, National Center for Biotechnology Information /www.ncbi.nlm.mih.gov/rDNA sequence of *Rosemarinus officinalis*.

9. Nei, M. and W. H. Li (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. 76:5269-5273.

10. Sambrook, J., E. F. Fitsch and T. Maniates (2003). Molecular Cloning: a Laboratory Manual. 2nd ed. Cold Spring Harbor Lab. Press. New York.

11. Tyler, V. E., L. R. Brady and J. E. Robbers (1976). Pharmacognosy. Lea and Febiger Press, Philadelphia.

12. www.cybegene.se.