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Cytogenetic diversity of Iranian balm (*Melissa officinalis*) landraces and genetic relationship within and between them using ITS markers

Parviz HEIDARI[1], Ali Ashraf MEHRABI[2] and Ali Asghar NASROLAH NEZHAD GHOMI[3]

1. Ph.D Student in Plant Breeding, Sari Agricultural Sciences and Natural Resources University, Sari, Iran.
2. Department of Agronomy, University of Ilam, Ilam, Iran.
3. Department Plant Breeding and Biotechnology of Agricultural Sciences and Natural Resources University of Gorgan, Gorgan, Iran.
*Corresponding author, P. Heidari, Tel.: +98-9193803455, Email address: hidarip@yahoo.com

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Abstract. The lemon balm (*Melissa officinalis L.*) a perennial medicinal plant, of Labiateae family is grown in the most parts of Iran and it have shown the sedative, hypnotic, analgesic, antiviral and antimicrobial effects. In this study, 14 landraces of balm with two out group (Basil and Moldavian balm) were investigated by Cytogenetic and PCR-RFLP of rDNA ITS region (ITS1, ITS2, 5.8S). Cytogenetic results showed that all of the Balm landraces, Moldavian balm and Basil had chromosome number 2n=2x=32, 2n=2x=10 and 2n=6x=72 respectively. In order to study variation within and between landraces of balm ITS (internal transcribed spacer) region was used to design markers. Also four enzymes *Taq*1, *EcoR* v, *BssM*1 and *AspLE*1 were used for investigating polymorphism in ITS region. Enzyme *BssM*1 showed the polymorphism in number and length of digested fragments better than other enzymes. Diversity was observed just for five of landraces (3, 7, 8, 9 and 11). Results of cluster analysis separated landraces on eight groups and Basil genetic distances were less than Moldavian balm to the Balm.

Keywords: lemon balm, the number of chromosome, ITS, genetic diversity.

Introduction

The lemon balm (*Melissa officinalis L.*) a perennial medicinal plant, of Labiateae family is grown in the most parts of Iran and it have shown the sedative, hypnotic, analgesic, antiviral and antimicrobial effects (Bagdat & Cosge 2006). The karyotypic description, such as morphology and number of chromosomes, is of great value for the understanding of the evolutionary processes of a species (Mukherjee et al. 2005, Tarinejad & Mirshekari 2010). The chromosome number and morphology of the genus Melissa are little known.

rDNA polymorphisms have been used for identification in systematic and phylogenetic studies of plants, animals and fungi (Souframanien et al, 2003). Internal transcribed spacers are sequences located in eukaryotic rRNA genes between the 18S and 5.8S rRNA coding regions (ITS1) and between the 5.8S and 25S rRNA coding regions (ITS2) (Adams et al. 1998 and Adlard et al. 1993). Studies of restriction site variation in the ribosomal DNA (rDNA) in populations of animals and plants have shown that while coding regions are conserved, the spacer regions are variable (Gerbi, 1985). These spacer sequences have a high evolution rate and are present in all known nuclear rRNA genes of eukaryotes (Jorgensen et al. 1987). They are useful for phylogenetic analysis among related species and/or among populations within a species (Doyle & Beachy 1985).

The objective of the present study was to determine the ploidy levels, chromosome numbers and genetic relationship among the Melissa land races.

Material and method

Plant material

All plant materials used in the experiments is given in Table.1 and out group (*Deracocephalum moldavica* and basil), were collected from different regions of Iran.

Preparing karyotype

About 50 seeds of *Melissa officinalis* were randomly selected from the original pools of seeds and germinated in petri dish at room temperature (24±2°C) for 10 days, containing germ test paper moistened with distilled water. The seeds that had 0.5 to 1.0 cm long roots were submitted to four types of pretreatments: 0.0029 M 8-hydroxyquinoline (8-HQ) for 2 to 5 h, at room temperature. Next, roots were washed in distilled water for 5 min. The roots tips were then fixed in Carnoy solution (three parts ethyl alcohol: one part acetic acid) for 12 h at room temperature. The material was hydrolyzed in 1 N HC1 at 60°C for 9 min, and then stained with the Schiff reagent for 90 min. The material was then squashed on slides containing a drop of 1% acetic carmin. Melissa officinalis cells in mitotic metaphases were analyzed to establish the chromosome number.

PCR-RFLP

Genomic DNA was isolated from young leaves using the method of Dellaporta et al (1985). To study within variation of landraces, two plants were selected from each landrace. The primer sequences were as follows: ITS1F (AAGTCGTAACAAGGTTTCCGTAG) and ITS1R (AAAGACTCGATGGTTCACG) primers were designed based on the ITS region of balm (DQ667291). Amplifications were done in 30 ml reaction volume containing 1X Taq polymerase assay buffer (10 mM Tris-HCl pH 8.0, 50 mM KCl, 2.5 mM MgCl2 and 0.01% gelatin), 0.1 mM of each dNTP, 0.5 mM of both forward and reverse primer, 0.5 units of Taq polymerase (Takapouzist, Iran) and 100 ng of DNA. The PCR thermocycle was: 95°C for 3 min, then 45 cycles of 95°C for 40 sec, 47°C for 70 sec, and 72oC for 3 min, followed by a final extension at 72°C for 10 min. Amplified products were resolved in 2% agarose gel electrophoretically at 75 V. The restriction enzyme digestion analyses were performed using of the amplified PCR product using 10 units of enzyme overnight. The following enzymes were used: Taq1, EcoR v, BssM1 and AspLE1 (Table 2), as per the specifications of the manufacturers (Takapouzist, Iran). The restriction fragments were size separated by electrophoresis on 2% agarose gel at 50 V for 3 h and 10% polyacrylamide gel. The results were analyzed using the NTsys software (version 2.0).

Results & Discussion

Karyotype results showed that all populations of *Melissa* is diploid and have 32 chromosomes (Fig. 1). These results indicate that no differences between all landraces collected in lemon balm on chromosome number. The results of this study showed that basil contains 2n=6x=72 chromosomes (Fig. 2a). Cytogenetic studies were performed *Deracocepha-*

Table 1. Materials used in the experiment.

Landrace number	Collection location	Landrace number	Collection location
1	Zanjan	8	shiraz
2	Urmia	9	Qazvin
3	Ilam	10	Karaj
4	Kermanshah	11	Isfahan
5	Kurdistan	12	North Khorasan
6	Tehran	13	Hamadan
7	Tabriz	14	Ardabil

Table 2. Restriction enzymes used in the experiment.

Restriction enzyme	Activity temperature	Restriction sites
Taq 1	65	5´……TCGA…..3´
		3´……AGCT…..5´
EcoR v	37	5´…GATATC…..3´
		3´…CTATAG…..5´
AspLE 1	37	5´……GCGC…..3´
		3´……CGCG…..5´
BssM 1	37	5´……GATC…..3´
		3´……CTAG…..5´

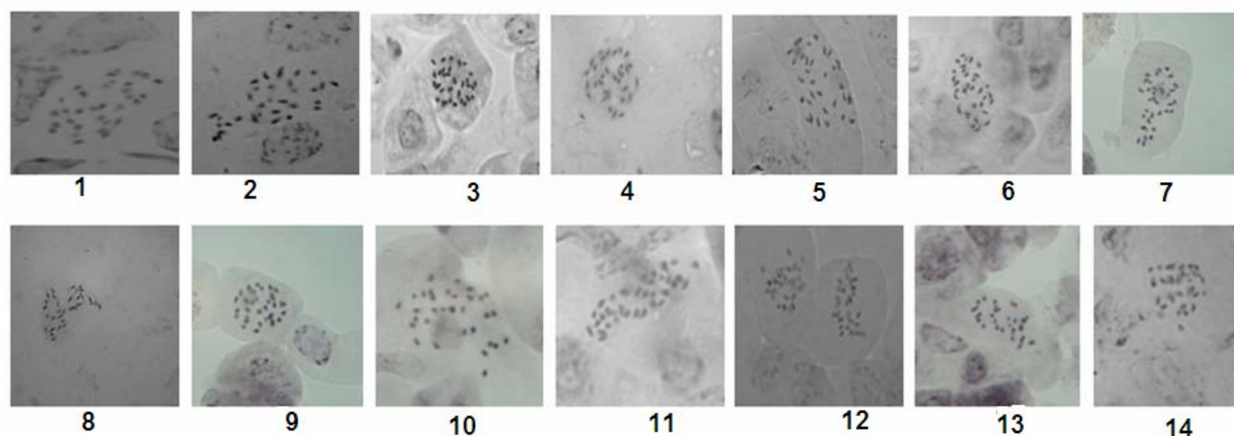

Figure 1. Melissa metaphase chromosomes range (populations of 1 to 14).

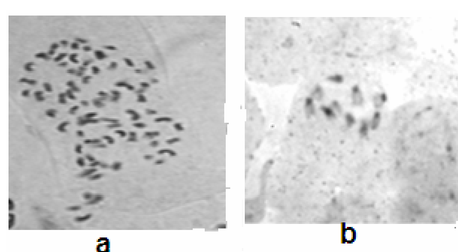

Figure 2. Basil metaphase chromosomes range (a) and Moldavian balm (b).

lum moldavica L. diploid chromosomes and three pairs of chromosomes are metacentric and two submetacentric chromosome pairs (Fig. 2b). Some examples of this plant cells was observed with 8 chromosomes. Aneuploidy was presented in plant changes according to research conducted in *Deracocephalum moldavica L.* (Irving, 1976) labiates family of plant genetic changes than other families there. Primers ITS1F and ITS2R were used to amplify the entire ITS region and was approximately 400 bp (Fig. 3). Primers ITSF and ITSR were used to amplify the entire ITS region and was approximately 700 bp. There was no size variation in the PCR product from the fourteen landraces of *Melissa oficinalis* and two out groups studied. The amplified product from all the samples had very similar molecular size. This would mean that during evolution and selection there has been no selection for or against ITS length variation. The results of the amplified fragment length matched the result reported by Souframanien et al. (2003) also. The digestion results showed polymorphism of the amplified fragment (Figs 3 and 4). Enzyme BssM1 showed the polymorphism in number and length of digested fragments better than other enzymes (Fig. 3c). Based on restriction fragment size and pattern analyses, the genetic similarity matrix was obtained (data not shown) and a dendrogram constructed. Similarity among the 14 landraces ranged from a high of 100% to a low of 60% by the Neighbor-Joining method. Cluster analysis separated the 14 landraces into six major groups on the basis of a 93% similarity cut off (Fig. 5). Two out group landraces were found to be distantly related to other lemon balm landraces. Populations 3, 8, 7, and 11 had more variation than the other landraces. Therefore, it is necessary to collect germplasm Melissa's attention to geographical areas. Absolute maximum landrace variation within populations was 0.077 in Ilam and Shiraz landraces. The present study has shown that the digestion of

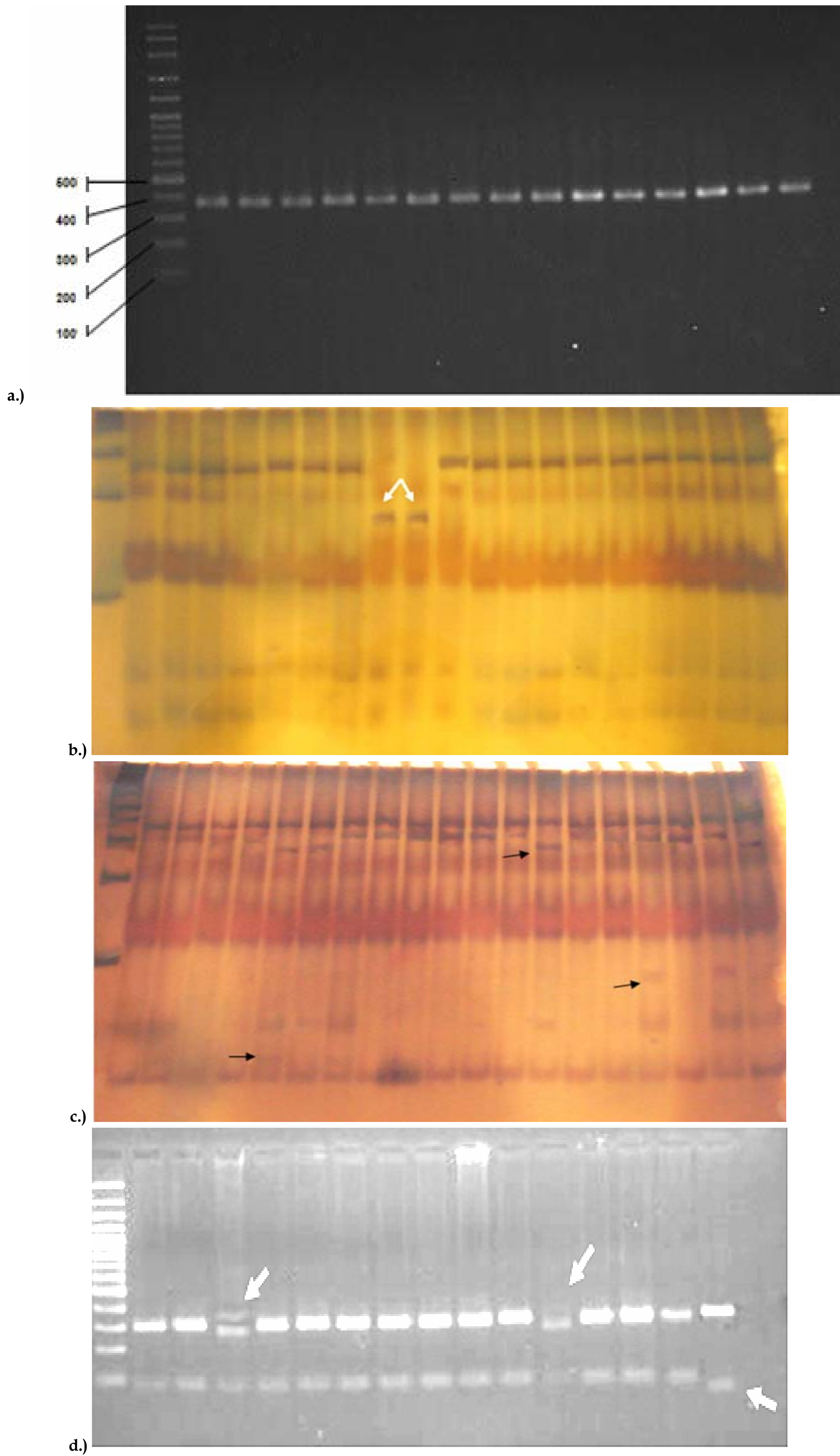

Figure 3. PCR amplified rDNA ITS region with ITS1F and ITS2R primers. M, marker 100 bp ladder (a) Restriction pattern of ITS1 amplified product with *Taq1* (b), BssM **1** (c) and EcoR V (c).

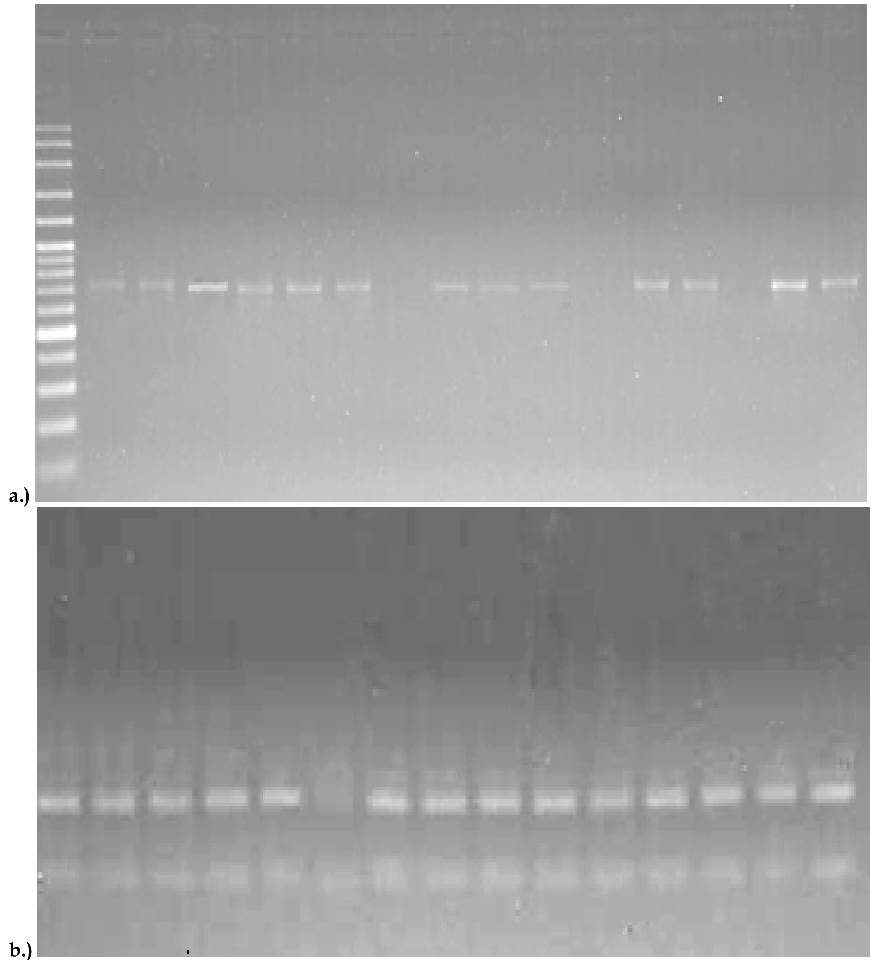

Figure 4. PCR amplified rDNA ITS region with ITS F and ITS R primers. M, marker 100 bp ladder (a), Restriction pattern of ITS amplified product with *Taq1* (b).

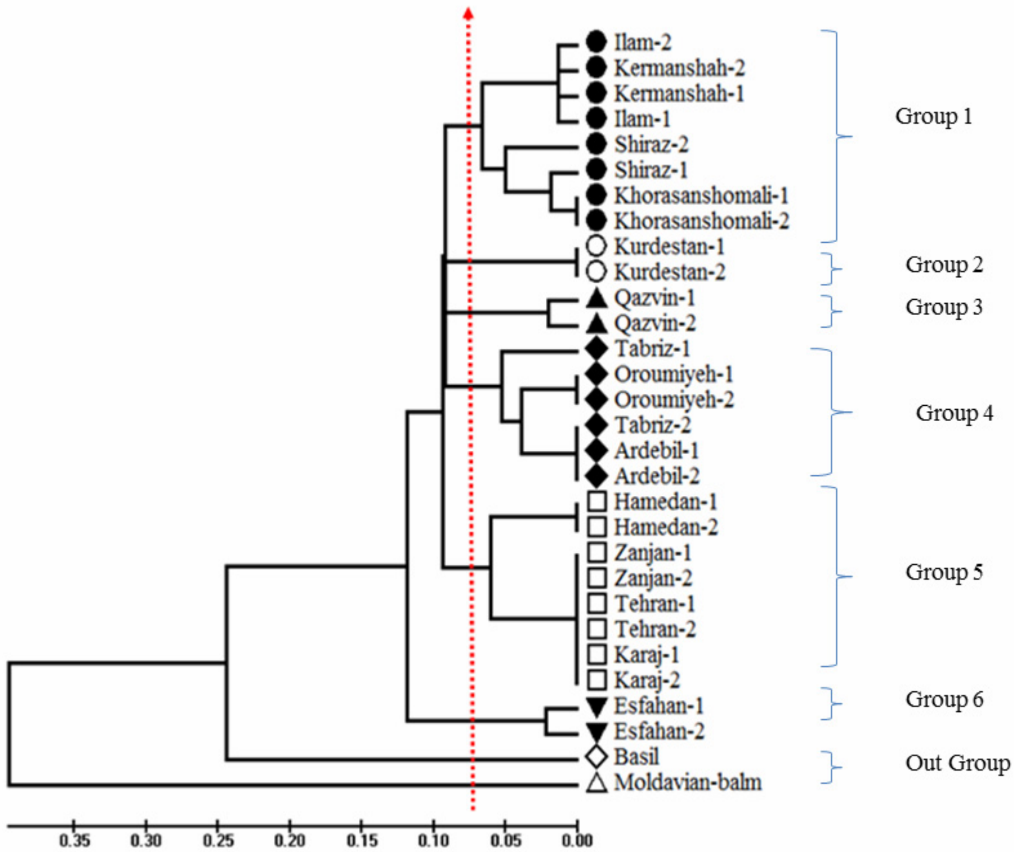

Figure 5. Similarity based on cluster analysis with data obtained from ITS-RFLP of PCR product among 14 landraces of *Melissa officinalic* with two out groups by using Neighbor-Joining algorithm.

PCR amplified rDNA ITS region with restriction endonucleases *Taq1, EcoR v, BssM1 and AspLE1* detect variation among the balm landraces. In general all cultivars developed and released for different geographical locations did not show any variation in the ITS region (Doyle & Beachy 1985). Conserved sequences in internal transcribed spacers have been observed in *Glycine* (Kollipara et al. 1997) and in other plant nuclear rRNA genes (Liu & Schardl 1994). However, variation was found to exist among *V. mungo* var *silvestris*, *V. trilobata* and *V. glabrescens* (Souframanien et al, 2003). Different regions of the rDNA evolve at different rates and provide different levels of resolution for addressing specific questions at appropriate taxonomic levels (Doyle & Beachy 1985). Repeated gene families like rDNA genes including spacer regions are subject to concerted evolution (Souframanien et al. 2003), which means variation in the rDNA family is homogenized among the repeating units within an individual and among members of a population. This phenomenon was postulated to occur through mechanisms like unequal crossing over and biased gene conversion. The study showed that the ability to discriminate ITS subtypes and genotypes within populations are from each other.

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