Journal of Plant Physiology and Breeding
2011, 1(2): 39-51 ISSN: 2008-5168

Patterns of Population Diversity in Lemon balm (*Melissa officinalis* L.) as Revealed by IRAP Markers

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Received: April 7, 2011 Accepted: October 22, 2011
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Abstract

Analysis of genetic diversity and population structure in medicinal plants is essential step in their conservation, utilization and breeding. In the present study, seven primers designed based on long terminal repeats (LTRs) of barley retrotransposons and their combinations were used to amplify DNA fragments from 12 Iranian ecotypes as well as two populations from Germany and Japan based on inter-retrotransposon amplified polymorphism (IRAP) technique. A set of eight most polymorphic primers out of 28 possible single and primer combinations yielded 456 reproducible informative data points, with 95% polymorphism.The mean of Nei's genetic diversity index and Shannon diversity index were 0.10 and 0.16, respectively. Polymorphism information content (PIC) ranged from 0.22 to 0.32 with an average of 0.27 and marker index (MI) varied in the range of 10.10-29.30 with an average of 14.39. Analysis of molecular variance revealed higher within population molecular variation (76.30%) compared with among populations variation (23.70%). The highest and lowest genetic heterogeneity were observed in Hamedan-2 and Qazvin-1 populations, respectively. The studied genotypes were assigned into five groups based on IRAP data using Neighbor-Joining algorithm and p-distance evolutionary distance coefficient. Populations relationships were resolved using Neighbor-Joining algorithm and Nei's genetic distance and the populations were grouped into three clusters. Our results suggest that retrotransposon based markers such as IRAP are efficient and reliable markers in determining level of genetic diversity and population structure in lemon balm.

Keywords: Genetic diversity, IRAP, Lemon balm, Population structure

Introduction

Based on World Health Organization report, approximately 80% of the world population trying to cure their health problems with herbal drugs and in the developed countries, 25% of the important medicines such as vimblastin, reserpine, aspirin, etc. were obtained from medicinal plants (Farnsworth 1990; Principe 1991). Lemon balm, (*Melissa officinalis* L.) member of the family *Lamiaceae*, is one of the important medicinal plant species mainly grown in natural flora and is native to southern Europe, Asia Minor and southern parts of North America (Simon *et al.* 1984). The centre of genetic diversity of lemon balm is in southern Europe and the eastern Mediterranean, and it was introduced to other

parts of Europe by monks. In Iran, this plant grows widely in Tehran, Golestan, Azarbayjan, Lorestan and Kermanshah provinces (Zargari 1991). There are three subspecies of *M. officinalis*: subsp. *officinalis*, subsp. *inodora* and subsp. *altissima*; however, only subsp. *officinalis* has commercial value (Craker and Simon 1992). Lemon balm is popular as an alternative to standard allopathic medicine for a variety of problems, including an over-active thyroid, heart palpitations, and depression as well as relaxing and reducing fevers (Sari 2002; Kennedy *et al.* 2004). Antiviral, antiulcerogenic, modulation of mood and cognitive performance as well as immune system stimulating (against anti HIV-1) effects of lemon balm were also reported (Yamasaki *et al.* 1998; Allahverdiyev *et al.* 2004).

There is general opinion on global decrease in diversity of medicinal plants due to exploitation of raw materials from native resources. In Iran, overexploitation of some native plant species for pharmaceutical and other processing industries has been the main reason that such species have become threatened and/or rare. Presently it is not possible to estimate general diversity loss of medicinal plants in Iran, because there is no reliable data available on the economy of the wild medicinal plants, which may become extinct within a few years (Koocheki *et al*. 2008). Referring to the general agreement on tariffs and trade (GATT), it is important to assess the value of these plants as important biological resources and to document the intellectual property rights. The first activity in this task is characterization of plant species and determination of their genetic diversity level and population structure.

Understanding the molecular basis of the essential biological phenomena in plants is crucial for the effective conservation, management and efficient utilization of plant genetic resources. In particular, an adequate knowledge of existing genetic diversity, where in plant population it is found and how to best utilize it, is of fundamental interest for basic science and applied aspects like the efficient management of genetic resources (Tang *et al.* 2007; Mondini *et al*. 2009). Molecular techniques, especially DNA-based markers were used for comprehensive analysis of genetic diversity and population structure in medicinal plants.

Several DNA markers including RAPD, AFLP, ISSR and ribosomal-DNA were commonly used for analysis of genetic diversity and population structure in medicinal plants (Joshi *et al.* 2004; Canter *et al.* 2005; Xia *et al.* 2007; Rahimmalek *et al.* 2009; Tatikonda *et al.* 2009). Efforts have also been made for the development of PCR-based high throughput markers (e.g. SSR, SNP) in some medicinal plants, but they need to be developed in all medicinal plants (Kumar and Gupta 2008). Retrotransposon-based markers have been recently examined in various plant species for diverse applications. These methods rely on PCR amplification between a conserved retrotransposon feature, most often the long terminal repeat (LTR), and another dispersed and conserved feature in the genome (Schulman *et al.* 2004). The inter-retrotransposon amplified polymorphism (IRAP) method displays insertional polymorphisms by amplifying the segments of DNA between two retrotransposons. It has been used in some studies for analysis of genetic

diversity (e.g. Antonius-Klemola *et al*. 2006; Kalendar and Schulman 2006; Smýkal *et al.* 2008; Vukich *et al.* 2009). Here, we report the first application of barley retrotransposon LTR primers to develop IRAP markers in *M. officinalis* for the analysis of genetic diversity and population structure.

Materials and Methods

Plant materials and DNA extraction

Twelve populations collected from natural habitats of *M. officinals* in Iran, including Ardabil, Esfahan, Fars, Kurdestan, Tehran, Hamedan (two), Karaj (two) and Qazvin (three]) were obtained from Research Institute of Forest and Rangelands, Iran. In addition, two populations from Germany and Japan were used. For each population, fresh leaves from 5-10 field grown plants were sampled and used for DNA isolation. Total genomic DNA was extracted using genomic DNA purification kit (Fermentase). The quantity and quality of DNA were evaluated by 0.8% agarose gel electrophoresis and a spectrophotometer.

IRAP analysis

The amplification reaction was performed according to the protocol described by Kalendar *et al.* (1999). Seven LTR primers from barley *Nikita*, *Sukkula* and *BARE-1* retrotransposons were used (Table 1). The combination of seven oligos as single and paired, referred to as Nikita, Sukkula, LTR6149, 3′LTR, LTR6150, 5′LTR1 and 5′LTR2, enabled the use of 28 distinct primer combinations. IRAP amplifications were performed in a final volume of 10 μL containing 40 ng DNA, 1× PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl, 4 mM MgCl2), 200 nM dNTPs, 1U *Taq* DNA polymerase (Cinagen, Iran), 1.5 mM $MgCl_2$, 5 mM BSA and 12 pmol of each LTR primer. Amplification was carried out in a Bio-Rad thermocycler in 0.2-mL microtubes. Amplification program consisted of 94°C for 4 min, followed by 40 cycles composed of 94°C for 1 min, 55-70°C (Table 2) for 2 min and 74°C for 2 min for denaturation, annealing and extension, respectively. After amplification, a final extension step was performed at 74°C for 9 min. The PCR product was separated in 4% (0.2 mm) non-denaturing polyacrylamide gels, by ethidium bromide staining, using a Gel-Scan 3000 electrophoresis system (Corbett Robotics, Australia).

Table 1. Primer name, retrotransposon type, position and sequences used to measure within and among populations variation in 14 populations of lemon balm (*Melissa officinalis*)

Name and orientation	Element origin in barley	Position	Sequence
Nikita →	Nikita	1-22	CGCATTTGTTCAAGCCTAAACC
Sukkula →	Sukkula	4301-4326	GATAGGGTCGCATCTTGGGCGTGAC
LT R6149 →	*BARE-1*	1993-2012	CTCGCTCGCCCACTACATCAACCGCGTTTATT
3′LT R →	*BARE-1*	2112-2138	TGTTTCCCATGCGACGTTCCCCAACA
LT R6150 ←	*BARE-1*	418-439	CTGGTTCGGCCCATGTCTATGTATCCACACATGTA
5′LT R1 ←	*BARE-1*	1-26	TTGCCTCTAGGGCATATTTCCAACA
5′LT R2 ←	*BARE-1*	314-338	ATCATTCCCTCTAGGGCATAATTC

Table 2. Annealing temperature and total number of bands (N), number of polymorphic bands (Np), polymorphic information content (PIC) and marker index (MI) of each primer/primer combination, considering 14 evaluated lemon balm (*Melissa officinalis*) populations

Primer/primer combinations	Annealing temperature (°C)	N	Np	PIC	MI
3'LTR	70	36	35	0.25	10.06
5'LTR1	65	47	44	0.29	11.85
5'LTR2	60	31	30	0.27	7.83
Sukkula	60	62	54	0.26	12.34
Nikkita	55	114	111	0.27	29.33
5'LTR1/5'LTR2	65	39	37	0.32	11.22
5'LTR2/Nikkita	65	76	75	0.29	21.28
5'LTR2/ LTR6150	65	51	51	0.22	11.15

Data analyses

Each IRAP band was treated as a single locus. The presence or absence of a fragment of a given length was recorded in binary code using 1Dscan EX 3.1 software (Scanalytics Inc., V3.1.0). Polymorphic information content (PIC) for each IRAP marker was calculated as $PIC_i=1-f_i(1-f_i)$, where PIC_i is the polymorphic information content of marker *i*, f_i is the frequency of the i^{th} marker fragment when present and $1-f_i$ is the frequency of the i^{th} marker fragment when absent (Roldan-Ruiz *et al.* 2000). The marker index (MI) was also calculated as MI=PIC x EMR, where EMR is "the effective multiplex ratio (*E*) defined as the product of the total number of loci/fragments per primer (*n*) and the fraction of polymorphic loci/fragments (β) ($E=n.\beta$)" (Tatikonda *et al*. 2009). Nei's (1978) unbiased genetic distance coefficient was used to estimate the genetic relationships between populations. Analysis of molecular variance (AMOVA) was performed to partition the total molecular genetics variance into components attributable to the variance among and within populations (Excoffier *et al.* 1992). All genetic diversity and population structure parameters were estimated by GenAlex6.4 software (Peakall and Smouse 2010). The relationships among genotypes and populations were analyzed using Neighbor-Joining algorithm and p-distance evolutionary distance coefficient and pairwise unbiased Nei's genetic distance, respectively, using MEGA V4.0 (Kumar *et al.* 2004). Principal coordinate analysis (PCoA) was also performed using GenAlEex v6.2 software (Peakall and Smouse, 2006) to ascertain genetic relationships.

Results and Discussion

IRAP polymorphism and efficiency

In the absence of characterized native retrotransposons, primers matching retrotransposons from barley were tested for use in *M. officinalis*. The approach seemed reasonable because active retrotransposon families appear to cross generic boundaries in the plants (Vicient *et al.* 1999; AlaviKia *et al.*, 2008). Several LTR primers designed for barley were tested in single- and paired-primer amplifications. Only those producing numerous, strong bands and as well as polymorphism, were selected for IRAP analysis. Because the annealing condition was stringent, reproducible band patterns were expected.

Reproducibility was tested and polymorphism confirmed by multiple reactions and electrophoretic separations.

The eight single primer or in combinations out of 28 tested produced a total of 456 IRAP markers, in the range of 100 to 3000 bp, and 437 were polymorphic (95%), with an average of six polymorphic bands per primer combination. The average Nei's genetic diversity index and Shannon's diversity index were 0.101 and 0.159, respectively. Boronnikova and Kalendar (2010) in analysis of six *Adonis vernalis* L. ecotypes' genetic diversity using five retrotransposon based primers amplified 127 markers with 93% polymorphism. Five out of eight IRAP band patterns were produced by application of single prime. The IRAP with one primer requires that elements of the same family be in a head-to-head or tail-to-tail orientation sufficiently close to produce amplification products. Therefore, the data suggested either that the copy number of these elements is high, or that they are clustered in the genome, or both (Kalendar and Schulman 2007). The number of polymorphic amplified fragments per primer/primer combination varied from 30 (5´LTR2) to 111 (Nikkita) with an average of 54.62 (Table 2). Figure 1 shows banding pattern of IRAP markers resulted from 5LTR2/Nikkita combination. In our study, successful amplification of genomic fragments using primers designed based on barley Nikkita, Sukkula and BARE-1 retrotransposon family indicates the presence of similar retrotransposon families in lemon balm genome. The abundance of marker bands indicates that retroelements closely related to many of those in barley are abundant in lemon balm and display a similar genome organization, similar to the pattern identified in barley (Kalendar *et al.* 2000). Among the studied primers, LTR6149 as a single primer or in combination with others could not amplify any genomic fragment from lemon balm. To increase the probability of finding bands, one can combine primers from both 5' and 3'LTR ends to amplify intervening genomic DNA (Branco *et al.* 2007). The IRAP markers are generated by the proximity of two LTRs using outward-facing primers annealing to LTR target sequences (Kalendar *et al*. 1999). Amplification of high number genomic fragments in our study compared with the studies using barley LTR primers could be due to the presence of large number of retrotransposon elements in lemon balm genome as well as diverse genetic materials and high resolution detection system used in this study. The efficiency of a molecular marker technique depends upon the amount of polymorphism it can detect among the genotypes under investigation (Tatikonda *et al*. 2009). A high level of IRAP polymorphism in the lemon balm populations indicates the accuracy of genetic diversity analysis and genetic differentiation. The efficiency of IRAP system in the determination of genetic variation and level of polymorphism was reported in *Hordeum* (Kalendar *et al*. 1999), *Oryza* (Branco *et al*. 2007), *Pisum* (Pearce *et al.* 2000), *Musa,* (Teo *et al*. 2002), *Spartina* (Baumel *et al*. 2002), *Diospyros* (Guo *et al*. 2006), *Crocus* (Alavi-Kia *et al*. 2008) and flax (Smýkal *et al.* 2011).

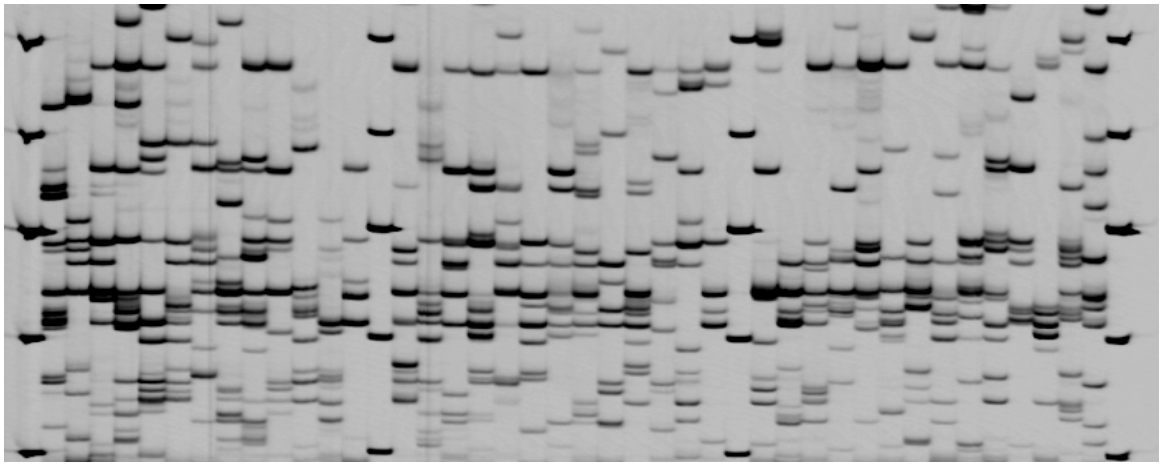

Figure 1. Electrophoretic pattern of lemon balm (*Melissa officinalis* L.) genotypes generated by IRAP, using primers 5'LTR2 and Nikkita.

The average polymorphic information content (PIC) was 0.28, ranging from 0.22 to 0.32. The lowest and the highest PIC values were recorded for primer 5´LTR2/LTR6150 and 5´LTR1/5´LTR2, respectively. PIC is equal to gene diversity in binary marker systems and varies in the range of 0.0-0.5. For determining the overall utility of a given marker system, the marker index, MI, was calculated for IRAP system examined. The average of MI, which is considered to be an overall measure of the efficiency to detect polymorphism, was 14.39 in the rage of 10.06 (3´LTR) to 29.33 (Nikkita) (Table 2). This feature makes the IRAP marker system suitable for fingerprinting or estimating genetic diversity in lemon balm populations.

Population structure and relationships

Analysis of molecular variance showed that most of the genetic variation (76.3%) could be assigned to differences within the populations and 23.7% to variation among them. Analysis of within population variation revealed that Kurdestan, Hamedan-2, Esfahan and Karaj-2 were more heterogeneous populations, whereas Qazvin-1 and Hamedan-1 showed relatively low within population variation (Table 3). Little is known about the ecology and conservation biology of lemon balm, including the levels and distribution of genetic variation within/among populations that are essential for its evolutionary outcomes in a scenario of global warming. But due to wide geographic distribution range, the extremely high levels of genetic variation within populations and low genetic differentiation among populations in a perennial species like lemon balm are expected. It is notable that, the studied populations are widespread in the country and most of them are highly isolated from each other because of the complex topology across sampled geographical regions. The high level of genetic diversity within populations and low genetic differentiation among fragmented populations may prevent threatening the adaptive potentials of *M. officinalis* in a scenario of global warming. Long-term persistence of a species is dependent on the maintenance of sufficient genetic variation within

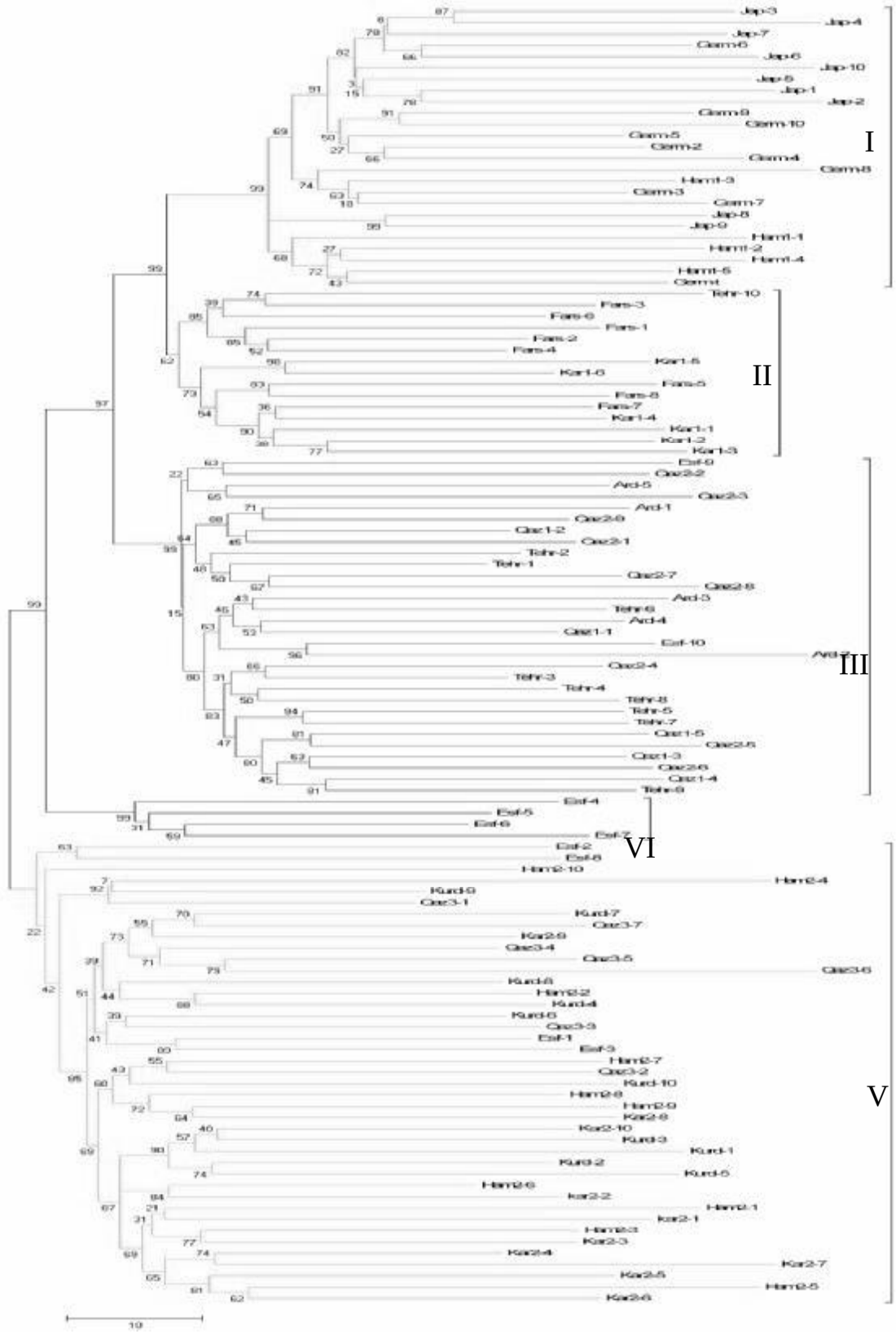

Figure 2. Neighbor-joining dendrogram of all 115 individuals of lemon balm (*Melissa officinalis* L.) sampled from 12 Iranian, a German and a Japanese populations based on P-distance evolutionary distance matrix illustrates five well-defined clusters. The values are shown above branches with 1000 bootstrap replicates.

and among populations (Allendorf and Luikart 2007; Habel *et al*. 2011).

The relationships among all the individuals are shown in the NJ-dendrogram based on the p-distance evolutionary distance coefficient (Figure 2). It revealed five well-defined clusters that were mainly consistent with the population geographical regions. The only exception was that distribution of Esfahan population individuals among other populations. Similar pattern was found in PCoA analysis (Figure 3). Two first principal coordinates explained 65.36% of the total molecular variation.

Table 3. The average number of fragments amplified (P_M) in each population using IRAP markers and within lemon balm (*Melissa officinalis*) populations variation

Population	P_M	Variance	Population	P_M	Variance
Ardebil	8.8	132.4	Kurdestan	9.3	344.4
Esfahan	9.2	316.7	Qazvin-1	8.1	111.2
Fars	7.9	194.37	Qazvin-2	8.9	253.3
Hamedan-1	9.2	116.4	Qazvin-3	9.2	202.9
Hamedan-2	9.8	337.9	Tehran	8.4	253.2
Karaj-1	7.6	142.0	Germany	8.5	248.1
Karaj-2	9.6	305.3	Japan	8.9	285.7

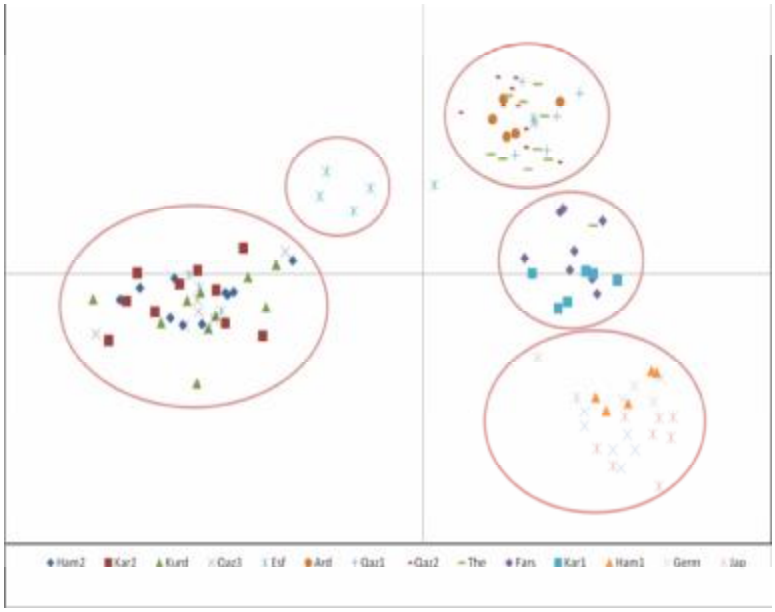

Figure 3. PCoA results showing scatter plot of 115 individuals of lemon balm (*Melissa officinalis* L.) sampled from 12 Iranian, a German and a Japanese populations based on IRAP markers. The first coordinate explains 41.09 % of the total variation, the second and the third explain 24.28 % and 10.07 % of the total variation, respectively.

The relationships among populations were assessed using cluster analysis based on Neighbor-Joining algorithm and Nei's distance coefficient (Figure 4). In this grouping, populations were assigned into three distinct groups. Qazvin-1, Qazvin-2, Tehran, Ardebil, and Fars were grouped in cluster I. Cluster II consisted of Hamedan-1, Karaj-1, Germany and Japan populations. In cluster III, Hamendan-2, Esfahan, Qazvin-3, Karaj-2 and Kurdestan populations were placed. Grouping of populations based on IRAP data was according to their ecological specifications.

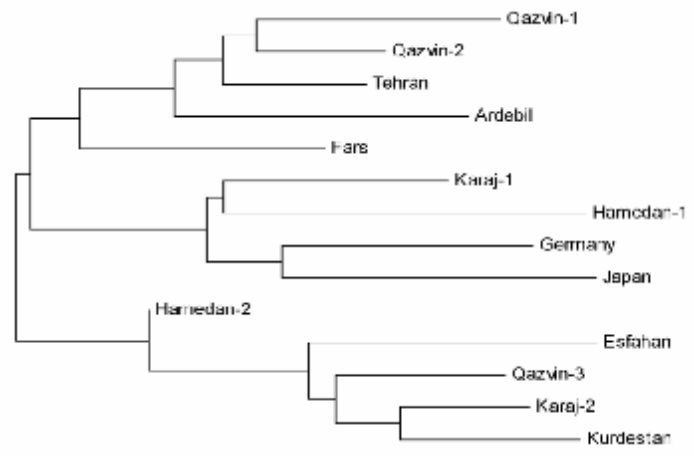

Figure 4. Dendrograms depicting genetic relationships among 14 populations based on IRAP data using Neighbor-Joining algorithm and Nei distance coefficient.

To our best knowledge, no investigation has been conducted on the application of DNA markers in *M. officinalis* studying the analysis of genetic diversity and population structure. In the current study, thus, we aimed to analysis pattern of genetic diversity and population differentiation in this species using retrotransposon based markers for its potential for population conservation and breeding program. Assessment of genetic diversity provides an efficient and effective way to estimate genetic variation and to delineate phenetic relationships among accessions. Molecular markers with a higher multiplex ratio, such as IRAPs, have been used to screen a wider region of the genome and to estimate relationships among and within populations (Kalendar *et al.* 1999; Iwamoto *et al*. 1999; Pearce *et al*. 2000; Baumel *et al*. 2002; Teo *et al*. 2002; Guo *et al*. 2006; Branco *et al.* 2007; Alavi-Kia *et al.* 2008; Smýkal *et al*. 2011). A large potential lies in their ability to identify the structure of genetic diversity within and among accessions, which can be relevant for the optimization of collections, planning of seed

regeneration, and successful implementation of pre-breeding approaches. The results obtained in this study confirm that the use of IRAP markers allowed to unequivocally fingerprint each of the populations examined and to effectively detect genetic variation among the 115 genotypes. In addition, IRAP revealed a large number of polymorphic DNA fragments with an average of 54.62 markers for each primer combination used. An average PIC value of 0.28 demonstrated the relatively good discriminatory power of the markers identified, and suggesting that considerable variation is detectable with IRAP markers. The large proportion of variation residing at the within population level suggests that there would be enough variation at the population level to select parents to generate new synthetic or mapping populations. This could lead to develop well-characterized populations to select parents contributing good adaptation, persistence and secondary metabolites. In the long term though, and to avoid exhausting the variability existing at the within population level, it would be advisable to monitor the levels of genetic diversity available and to introgress valuable alleles from other populations, to prevent the loss of complementary gene interactions due to inbreeding. The distribution of genetic diversity within and among populations is a function of the rate of gene flow among the individuals within a population and among the populations. The extent of gene flow in a species depends on the distribution of the habitats it occupies, on the size and degree of isolation of its populations, and on the movement of pollen and seeds between populations (Mariette *et al*. 2002; Albaladejo *et al.* 2008; Dinesh *et al*. 2010). In the lemon balm, each population proved to be heterogeneous at a large number of loci. This result can be explained considering the high rate of free pollinations within population, a low gene flow due to local isolation of the populations, and/or large effective population sizes. Therefore, each sampled population is actually a mixture of a large number of distinct genotypes that casually intercross at each generation sharing a common gene pool, which belongs to the populations because of local adaptation, which may be ascribed to a combination of climatic conditions and agronomic practices.

Acknowledgements

This work was supported by Center of Excellence in Cereal Molecular Breeding, University of Tabriz, Tabriz, Iran.

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