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Genetic structure and relationships among *Melissa officinalis* accessions using AFLP markers

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ABSTRACT

Melissa officinalis, a member of the Lamiaceae family, is one of the most important medicinal herbs that are applied in different fields of industry worldwide. Genetic diversity and structure of 21 *M. officinalis* accessions were examined using amplified fragment length polymorphism (AFLP) markers. For this purpose, 15 primer combinations generated a total of 1592 scorable bands, of which 1402 bands were polymorphic. The primer combination P_{66}/M_{48} showed the highest markers polymorphism values (PIC = 0.28, DI = 0.34, and I = 0.51), while the lowest values (PIC = 0.17, DI = 0.20, and I = 0.32) is obtained from the P_{14}/M_{49} combination. The marker index (MI) values with an average of 18.46 showed high values for both primer combinations E/M and P/M. All of the accessions, based on Neighbor-Net clustering method and structure analysis, are assigned to five clusters; with the maximum genetic dissimilarity of 0.51 belonging to accessions from Gilan (Roodbar and Damash). In addition, the principal coordinate analysis (PCoA) data confirmed the results of the clustering. In this study, the genetic distance was not often related to geographical distance. More likely the relationship among them is due to seed dispersal through human interactions. Our findings indicate that high genetic diversity of *M. officinalis* accessions provides important baseline data and a better understanding of conservation, management, and collection strategies for germplasm of this species.

1. Introduction

Melissa officinalis or lemon balm is an important plant belonging to the family Lamiaceae that is found in East Asia, southern Europe, and North America (Bağdat and Coşge, 2006). To cure diseases, it is common among different nations to grow this plant because of its therapeutic properties in memory improvement. Nowadays, many pharmaceutical properties of lemon balm have been reported including sedative, carminative, anti-microbial, anti-oxidant, neuroprotective, and anti-cancerous (Hosseini et al., 2016; Weitzel and Petersen, 2011; Kim et al., 2010; Öztürk et al., 2010), which are mainly due to the content of essential oil and phenolic acids. The concentration of essential oil and phenolic compounds and biological activities vary within *M. officinalis* accessions, even cultivar and seed company (Boneza and Niemeyer, 2018; Kittler et al., 2018), that diversity is usually created genetically or environmentally. Genetic diversity is the main source of biodiversity (Ramanatha Rao et al., 2002). This issue results from genetic differences among individuals of a species (Acquaah, 2009; Ramanatha Rao et al., 2002). Many methods, such as morphological traits as well as molecular (DNA) and biochemical markers have been employed to assay genetic diversity in plants (Aharizad et al., 2012). Morphological and biochemical traits are influenced by environmental factors. Thus, DNA-based molecular markers (e.g. AFLP) have been developed for genetic diversity in recent decades (Singh et al., 1999). AFLP technique, introduced by Vos et al. (1995), is based on the selective PCR amplification of restriction fragments from a digest of genomic DNA. This technique is a useful tool in evolution and genetic diversity studies in germplasm, because it has proven to be highly reliable and reproducible, generated high multiplex ratio, and does not need knowledge of primary nucleotide sequence (Nyakio et al., 2014; Vos et al., 1995). AFLP technique has been used to assess genetic diversity in many plants such as *Ocimum* spp. (Moghaddam et al., 2011), *Pathoglottis plicata* (Ginibun et al., 2018), *Mentha arvensis, Mentha spicata* and the interspecific hybrid cv. Neerkalka (Shasany et al., 2005), *Olea europaea* (Khaleghi et al., 2017), etc. However, there is no AFLP marker study on these plantpopulations. In *M. officinalis*, genetic diversity has been evaluated using

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morphological traits and biochemical markers (Danaeipour et al., 2016; Aharizad et al., 2012). Also, Kittler et al. (2018) evaluate 28 *M. officinalis* accessions for essential oil and rosmarinic acid contents and report the two different chemotypes. Genetic diversity using molecular markers has been estimated in lemon balm by IRAP (Ghaffariyan et al., 2011), ITS (Heidari et al., 2013), ISSR and RAPD markers (Rahimi and Kordrostami, 2013) on different populations. Each marker explores a part of the differences and similarities within the genome.

Because of the commercial importance of this plant, the demand for its utilization has increased and overexploitation of resources has endangered it (Ghaffariyan et al., 2011). Given that genetic diversity is essential for human present and future (Ramanatha Rao et al., 2002) the current study was undertaken to assess the genetic diversity and population structure among 21 accessions of *M. officinalis* using AFLP to provide genetic data and a theoretical basis for the conservation and germplasm management of this species.

2. Material and methods

2.1. Plant materials

Accessions of *M. officinalis* seeds including 4 exotic accessions and 16 accessions of Iran were collected from wild habitats by Medicinal Plants Research Center, Shahed University, Tehran (Table 1). The seeds, in a completely randomized design with three replications, were grown in a greenhouse in 2013.

2.2. DNA extraction

Total genomic DNA was extracted by phenol-chloroform-isoamyl alcohol method using CTAB buffer (Ghaffariyan et al., 2012), according to the following protocol: 1 g of bulked fresh leaf from each accession was grounded in liquid nitrogen using pre-chilled mortar and pestle to obtain a fine powder, and then homogenized in 10 mL of 60 °C CTAB extraction buffer (100 mM Tris-HCl, 1.4 M NaC1, 20 mM sodium EDTA, 2.0% CTAB, 2.0% PVP and 2.0% β-mercaptoethanol). Tubes were incubated at 60 °C for 60 min and mixed. After 5 min incubation at room temperature, chloroform-isoamyl alcohol was added (24:1, v v^{-1}) and mixed gently. Thereafter, tubes were centrifuged for 15 min at 8, 000 rpm and the supernatant was transferred to a new tube. Next, DNA was precipitated with cold isopropanol and discarded supernatant. The precipitate of DNA was washed two times with ethanol 70%, air dried, and dissolved in TE. Next, tubes containing DNA were incubated in 1 μL RNase at 37 °C for 60 min, was added with phenol-chloroform-isoamyl alcohol (25:24:1), mixed gently and centrifuged for 10 min at 8, 000 rpm. Finally, DNA was precipitated with cold isopropanol, washed with ethanol 70%, and dissolved in TE. The quantity of the DNA was estimated using NanoDrop spectrophotometer (DeNovix DS-11, USA) and its quality was checked on 1.8% agarose electrophoretically.

Table 1
Melissa officinalis accessions used in this study.

code	Collection region	code	Collection region	code	Collection region
1	Alborz -Karaj	8	Qazvin-Herif	15	Esfahan-Najafabad
2	Esfahan-PakanBazr	9	Hamedan A	16	Kordestan
3	Qazvin-Hir	10	Esfahan-Esfahan	17	Hamedan B
4	Alborz -Malard	11	Hungary	18	Gilan- Roodbar
5	Shiraz	12	Italy	19	England
6	Gilan -Damash	13	Germany	20	North Khorasan
7	Alborz- Karaj-Shahrak	14	Japan	21	Ardabil

2.3. AFLP analysis

The AFLP procedure was performed with some modifications as described by Vos et al. (1995). Total genomic DNA (500 ng) was digested using 1 U *EcoRI* and *MseI* (or *PstI* and *MseI)* endonuclease mixture in a total volume of 20 μL for 2 h at 37 °C. Successful digestion was tested by electrophoresis on 1.8% agarose gels. Then, 10 μL ligation solution containing 5 pM *EcoRI* (or *PstI*) adaptor, 50 pM *MseI* adaptor (Table 2), and 1 U T4 DNA ligase was added to each double-digested sample and incubated for 3 h at 37 °C. The resulting DNA (template DNA) was then diluted to 1:20. The adapter-ligated DNA was pre-amplified using universal primers (E000, P000, and M000). The PCR reaction was performed using the following temperature profile: 1 cycles of 60 s at 94 °C, 25 cycle of 30 s at 94 °C, 60 s at 56 °C, 60 s at 72 °C, and 1 cycle of 120 s at 72 °C. The pre-amplification product was diluted in a ratio of 1:200 and was used as a template for the selective amplification, which involved the use of +3 and + 2 primer (Table 2). The following cycle profile was applied to ensure the optimal primer selectivity: 1 cycle of 120 s at 94 °C, 12 cycles of 30 s at 94 °C, 30 s at 56–65 °C, 60 s at 72 °C, where the annealing temperature was lowered by 0.7 °C per cycle. Next, 24 cycles were performed for 30 s at 94 °C, 30 s for 56 °C, and for 60 s at 72 °C, followed by 1 cycle of 120 s at 72 °C. The amplification products were mixed with 80% formamide loading buffer in a ratio of 1:2, heated up to 94 °C for 5 min, and cooled on ice. They were loaded and run on 6% polyacrylamide gels (Sigma-Aldrich company) (Sambrook and Russell, 2001).

2.4. Statistical analysis

Because AFLP is a dominant maker, the presence or absence of every single fragment was coded by 1 or 0, respectively, and scored for a binary data matrix. Percentage of polymorphic loci (%P), observed number of alleles (Na), effective number of alleles (Ne) (Kimura and Crow, 1964), Nei's gene diversity (H) (Nei, 1973), and Shannon's index (I) (Lewontin, 1972)were estimated for each primer combination of all accessions using the software POPGENE (version 1.32). Polymorphism information content (PIC) (Botstein et al., 1980)and genetic diversity (DI) were calculated using the software Power Marker (version 3.25). Marker index (MI) was used to assess the utility of a marker for detecting genetic variation, according to Powell et al. (1996). A model-based Bayesian clustering method was used to assign populations structure by the software STRUCTURE (version 2.3). The analysis was performed as described in Chenet al. (2010). On the basis of P-Uncorrected distance, the Neighbor-Netmethod with 1,000 bootstraps was applied to construct a dendrogram using programs of Split Tree (version 4.13.1) (Huson, 1998). To illustrate the genetic relationships among accessions,

Table 2
DNA sequences of pre-amplification and selective amplification primers for amplified fragment length polymorphism (AFLP) analysis.

Code	Sequence (5′-3′)
E_{000}	GACTGCGTACCAATTC
M_{000}	GATGAGTCCTGAGTAA
P_{000}	GACTGCGTACATGCAG
E19	GACTGCGTACCAATTC + GA
E64	GACTGCGTACCAATTC + GAC
E50	GACTGCGTACCAATTC + CAT
E38	GACTGCGTACCAATTC + ACT
M15	GATGAGTCCTGAGTAA + CA
M48	GATGAGTCCTGAGTAA + CAC
M49	GATGAGTCCTGAGTAA + CAG
M42	GATGAGTCCTGAGTAA + AGT
P14	GACTGCGTACATGCAG + AT
P86	GACTGCGTACATGCAG + TCT
P66	GACTGCGTACATGCAG + GAT

principal-coordinate analysis (PCoA) was performed in the software GenAlEx (version 6.2).

3. Results

3.1. AFLP marker properties

In this study, 15 primer combinations, 10 *EcoRI/MseI*, and 5 *PstI/MseI* primer combinations were used to assay 21 accessions of *M. officinalis*. The primer combinations demonstrated a total of 1592 scorable bands, ranged in the molecular weights from approximately 100 to 3000 base pairs, of which 1402 bands were polymorphic (Fig. 1). Among all of the accessions, the average percentage of polymorphic bands was 90%, with the highest and lowest percentages of polymorphic being respectively 99.12 and 70%, was obtained from the E_{64}/M_{48} and P_{14}/M_{49} combinations, respectively (Table 3). The primer combination P_{66}/M_{48} showed the highest PIC values (0.28), DI (0.34) and I index (0.51) while the lowest PIC values (0.17), DI (0.20), and I index (0.32) were obtained from the P_{14}/M_{49} combination. The MI values with an average of 18.46 showed high values for primer combinations E/M and P/M so that both markers efficiently estimated polymorphism in fragments amplified. The average of the observed number of alleles per primer combination ranged from 1.7 to 1.99, with the lowest value being for the P_{14}/M_{49} primer combination and the highest value for the E_{64}/M_{48} and E_{38}/M_{49} primer combinations. Also, an effective number of alleles was observed from 1.34 for P_{14}/M_{49} to 1.58 for P_{66}/M_{48}. To assay appropriate and equal distribution of alleles in the genome, the proportion of the observed number of alleles to an effective number of alleles was calculated. This proportion was lowest for P_{66}/M_{48} and highest for E_{50}/M_{15} and E_{38}/M_{49} among all of the accessions.

3.2. Genetic distance

Neis' genetic distance (Dice) generated from the binary matrix varied from 0.10 to 0.51, with an average of 0.26 among the accessions of *M. officinalis*. The minimum genetic dissimilarity of 0.10 is observed between the accessions collected from Qazvin-Herif and Alborz-Karaj-Shahrak while the maximum genetic dissimilarity (0.51) belongs to accessions from Gilan (Roodbar and Damash).

3.3. Cluster analysis and population structure

Dominant markers such as AFLP are usually ambiguous in representing a band obtained from an amplified fragment on one or both chromosomes, in the diploid genotypes. Therefore, the software STRUCTURE 2.3 was used to obtain the correct estimate of the number of clusters (subpopulations). Also, the rate of change of the log probability (ΔK) between successive values of K was calculated as proposed by

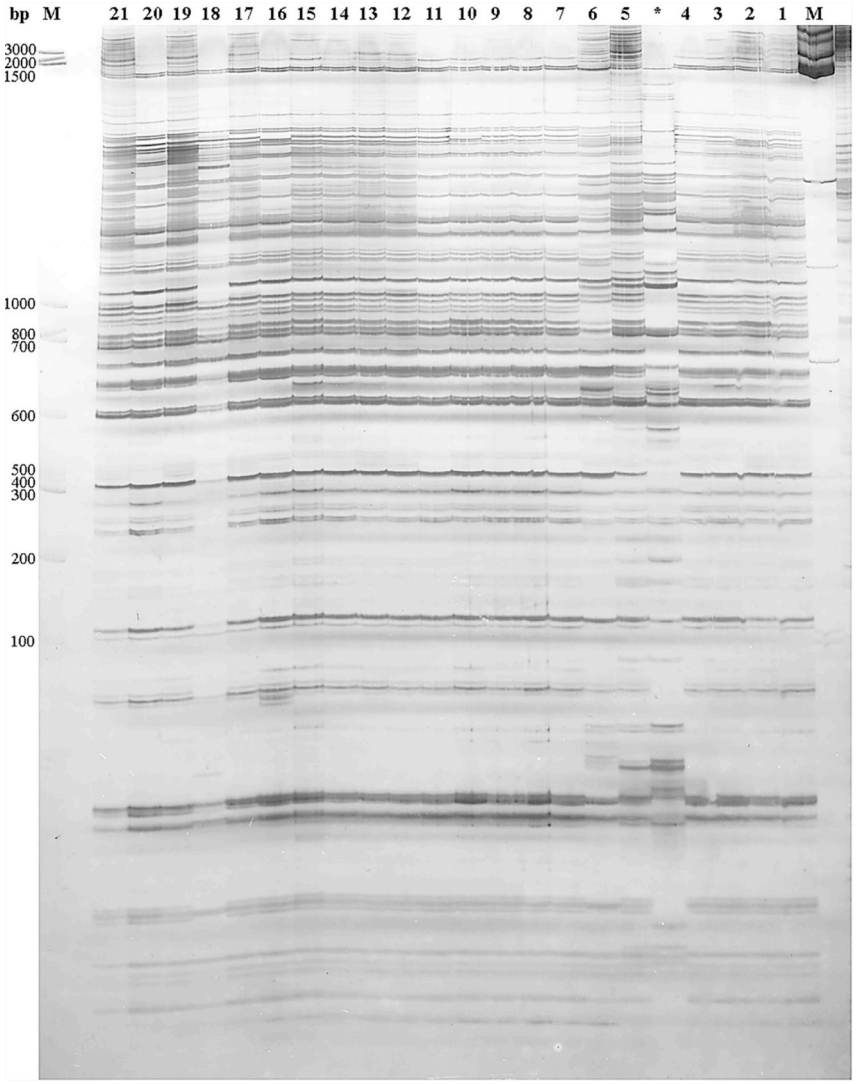

Fig. 1. AFLP fingerprint pattern in lemon balm accessions using primer combination P14/M49. Codes for accessions are listed in Table 1. M: 100 bp marker.

Table 3
Summary of gene diversity information of fifteen AFLP primer combinations among 21 lemon balm accessions.

primer combination	NT	NP	P%	PIC	MI	DI	I	H	Na	Ne	Na/Ne
E64/M48	114	113	99.12	0.23	26.28	0.28	0.44	0.28	1.99	1.45	1.37
E50/M42	80	77	96.25	0.23	17.04	0.28	0.43	0.28	1.96	1.45	1.35
E19/M48	86	82	95.35	0.22	17.55	0.27	0.42	0.27	1.95	1.42	1.38
E38/M48	95	80	86.32	0.21	14.76	0.25	0.39	0.25	1.86	1.41	1.32
E19/M15	114	108	94.74	0.22	22.60	0.26	0.41	0.26	1.95	1.40	1.39
E19/M49	94	89	94.68	0.23	19.77	0.28	0.44	0.28	1.95	1.46	1.33
P66/M49	76	58	76.32	0.19	8.50	0.23	0.36	0.23	1.76	1.38	1.28
P66/M48	111	107	96.4	0.28	28.39	0.34	0.51	0.34	1.96	1.58	1.24
E38/M49	84	83	98.81	0.22	17.92	0.26	0.41	0.26	1.99	1.40	1.42
E38/M42	78	71	91.03	0.23	15.01	0.28	0.43	0.28	1.91	1.46	1.31
P14/M48	163	125	76.69	0.19	18.18	0.23	0.36	0.23	1.77	1.39	1.27
P14/M49	180	126	70	0.17	14.78	0.20	0.32	0.20	1.70	1.34	1.27
E50/M15	69	65.00	94.2	0.20	12.40	0.24	0.38	0.24	1.94	1.37	1.42
E50/M48	85	82.00	96.47	0.26	20.47	0.32	0.48	0.32	1.96	1.54	1.28
P66/M15	163	136	83.44	0.20	23.24	0.25	0.39	0.25	1.83	1.42	1.29
Mean		93.60	90.0	0.22	18.46	0.27	0.41	0.27	1.90	1.43	1.33
Total	1592	1402									

NT: number of Total bands; NP: number of polymorphic bands; P%: percentage polymorphic loci; PIC: polymorphism information content; MI: marker index; DI: genetic diversity; I: Shannon's index; H: Nei's gene diversity; Na: observed number of alleles; Ne: effective number of alleles; Na/Ne: proportion of observed number of alleles to effective number of alleles.

Evanno et al. (2005). The highest modal value of ΔK was at K = 6, which possibility was six subpopulations for studied accessions (Figs. 2 and 3). The clusters obtained from STRUCTURE analysis were confirmed by the results from Neighbor Net-tree (Fig. 4) and PCoA analysis (Fig. 5). The Neighbor-Net clustering method with the highest cophenetic correlation coefficient (r = 0.99), indicating a good fit between the dendrogram clusters and the similarity matrices, produced five clusters. Also, population structures were considered in this clustering. The first cluster contained 8 accessions from Iran (Kordestan, Esfahan-Najafabad, Qazvin-Hir, Alborz-Mallard, North Khorasan, Hamedan A, Alborz-Karaj-Shahrak, and Qazvin-Herif) and 1 accession from Hungary. The second cluster included Hamedan B and Esfahan-Esfahan accessions from Iran and three countries of Italy, Germany, and Japan. Two Iranian accessions (Ardabil and Gilan-Roodbar) were just assigned in the third cluster. The fourth cluster contained one accession from England and two accessions of Shiraz and Gilan-Damash from Iran, with high geographical distance together showed a high genetic similarity and same genetic structure. Finally, Alborz-Karaj and Esfahan-PakanBazr accessions formed the fifth cluster. The results of the principal coordinate analysis (PCoA) corresponded approximately to those obtained through the cluster analysis. The first and second principal component accounted for 27 and 22% of the total variation, respectively, and accordingly distributed in four groups. The accessions belonging to the fifth cluster in dendrogram were distributed in PCoA 1 and PCoA 2. The PCoA analysis revealed a high scattering for Ardabil, Gilan-Roodbar, Shiraz, Gilan-Damash, and England while having a higher distance compared with other accessions.

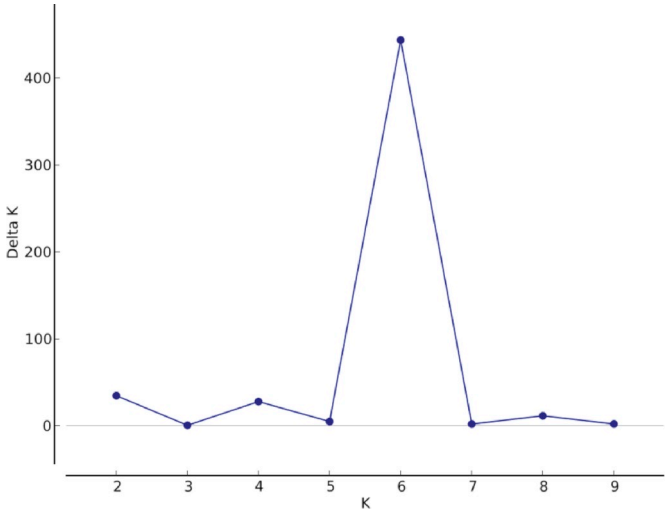

Fig. 2. Estimation of the number of clusters (K) calculated with K values ranging from one to ten for lemon balm accessions.

Generally, the analysis of molecular variance (AMOVA) showed that 1% of the genetic variability is distributed between exotic accessions and Iran accessions; thus, there is a great genetic admixture among accessions.

4. Discussion

Lemon balm as an important medicinal plant is subjected to endanger due to the increased demand to consume and gather it from its natural habitat. In this regard, awareness of genetic diversity provides an opportunity to recognize different genotypes. In the study, 15 primer combinations showed high genetic variation in DNA among 21 Lemon balm accessions (90% polymorphism, MI = 18.46,PIC = 0.22). This percentage of polymorphism washigher than that reported by Rahimi and Kordrostami (2013) using ISSR and RAPD markers for *M. officinalis* (69.34% and 71.77%, respectively) and our previous study on leaf protein pattern of *M. officinalis* (59.09%) (Danaeipour et al., 2016). The high polymorphism in amplified fragments, which are in the form of presence or absence of a band, may be due to the difference in restriction sites, shortage, or increase in nucleotide inside the restriction sites, and deletion or insertion in the joining place of primer or into the amplifiable fragment. The values of polymorphism, MI, and PIC obtained by AFLP markers are compatible with studies on other species using the same marker (Wu et al., 2019; Tangnak et al., 2018). Jones et al. (1997) and Belaj (2003)compared the efficiency and high power of separation of AFLP marker with SSR and RAPD markers and found that the highest amount of MI is related to AFLP (Rahimi and Kordrostami, 2013). However, this method has the potential to segmentize and screen many loci in each test. According to Dice similarity coefficient, the highest genetic distance (0.51) was among accessions from Roodbar and Damash, Gilan (Iran), where this variation is due to the difference in their growth places and altitude in these two regions. The lowest genetic distance was between accessions from Qazvin-Herifand Alborz-Karaj--Shahrak (Iran) such that they are possibly from a common ancestor. The results of similarity coefficient differed with results from proteins, that can be due to the environmental factors.

To investigate the genetic combinations and genetic background of

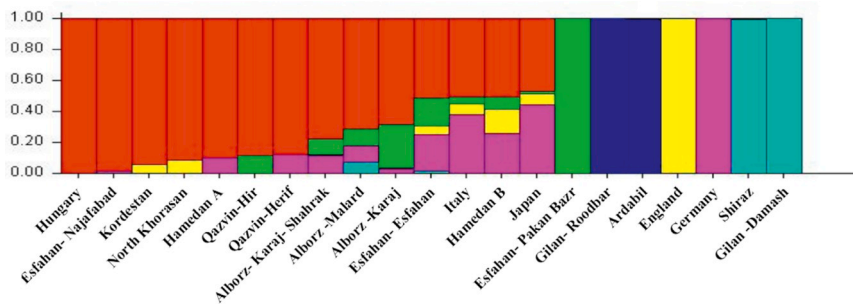

Fig. 3. Estimated population structure of 21 *M. officinalis* accessions using STRUCTURE 2.3. Each accession is represented by a single vertical line, which is partitioned into a maximum of K = 6 differently colored segments. Accessions are labeled below the figure.

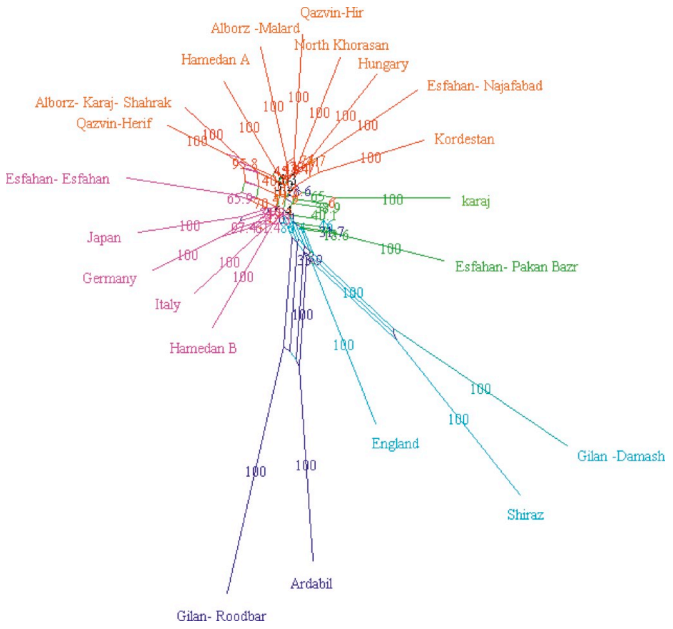

Fig. 4. Dendrogram of 21 lemon balm accessions are revealed by Neighbor Net cluster analysis and P-Uncorrected distance, which are colored on the basis of structure analysis and labeled below the figure.

accessions, population structure was first analyzed and accessions were placed in six probable sub-population (K = 6). Accessions from Damash and Roodbar having the highest genetic diversity had a completely different background, while those from Qazvin-Herifand Alborz-Karaj-Shahrakhaving the lowest genetic diversity had similar genetic background obtained from a genetic combination of other two sub-populations. In addition, 14 accessions of lemon balm were the genetic background of Hungary accession, which shows the genetic combination among these accessions. Thus, final clustering was performed by the analysis of population structure and Neighbor-Net clustering, and accessions with differently geographical distances were placed in the same cluster. Other than accessions from Qazvin-Hir, Alborz-Mallard, Alborz-Karaj-Shahrak, and Qazvin-Herifin the first cluster, there is no general relationship between genetic distance and geographical position. The geographical distance between accessions is very high to show close genetic relations. Also, in general, there is no relation between geographical position and population relatives. These results corresponded to the findings from a study carried out by Ghafarian et al. (2011), who investigated the genetic diversity of 12 Iranian ecotypes and two populations from German and Japan. As with the results of this study, the researchers placed geographically different populations in identical clusters. In the study carried out by Heidary et al. (2013), although populations of different provinces were separated well, provinces with high distance were placed in the same clusters. Additional to DNA markers, the pattern of genetic diversity of Lemon balm has been evaluated on the basis of morphological traits and essential oils content, where the results corresponded to ours (Aharizad et al., 2012).

There are several explanations for the difference between the geographical distribution of accessions and clustering: first, accessions

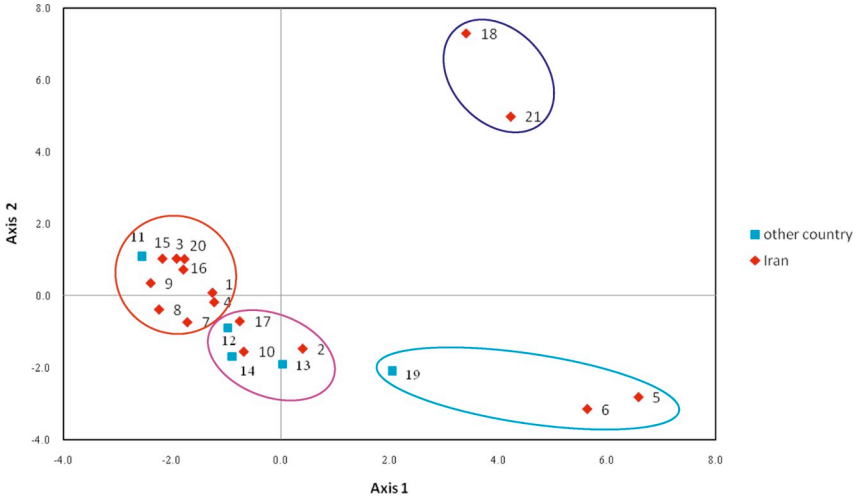

Fig. 5. Relationships among *M. officinalis* accessions revealed by principal coordinate analysis (PCoA). The two principal components accounted for 49% of the total variation, which are colored based on two prior figures (structure and dendrogram).

were gathered from different areas of same provinces with the difference in latitude and geographical region. This issue is considered as the variety of accessions from the same provinces. Second, mating and reproduction system of this herb, as a cross-fertilizer, causes the variety to decrease among accessions and increase within populations of one accession. Third, this discordant result may be due to the complex reactions among different biological characters and evolutionary processes. For example, genetic mutations occurred during species revolution and mutated ones have been well compatible with their natural environment. Fourth, the high genetic relation for accessions from other countries can be attributed to seed or plant exchanges by human among countries that are due to carrying seeds during their trips or migration. However, separation of accessions of different regions must be appropriately measured to prevent the variation in species due to hybrid populations (Ling et al., 2015; Moradkhani et al., 2010).

AFLP shows that, in addition to polymorphisms measurements to recognize and characterize Lemon balm species, it is necessary to combine this molecular approach with agricultural ones to recognize the significant correlation between molecular markers and agricultural parameters. These estimations are necessary to ensure the quality of medicinal herb compounds used in pharmacology and conventional medicine. Moreover, conserving genetic variety is the main focus in conservation and biology and is important to conserve the revolutionary potential of species to bear each environmental change. Therefore, the characteristic of estimating germplasm provides a knowledge base to use sources for managers of a gene bank, plant breeders, and researchers to focus on obtaining ideal genes to resist against diseases, insects, or physiology tolerances.

It is suggested conducting further studies and using primary compounds in order to screen genomes as appropriate sources for the logical development of strategies.

5. Conclusion

This study evaluates the genetic structure and relationships of the *M. officinalis* L accessions based on the powerful AFLP technique. AFLP molecular markers generated a large number of scorable fragments per primer pair that detected high levels of genetic diversity among the *M. officinalis* L accessions. High genetic variation adapts accessions to environmental changes. These results showed that the geographically different populations are placed in identical clusters. Also, in general, there is no relation between geographical position and population relatives. Therefore, *M. officinalis* L accessions from more geographic regions should be collected for conservation and management of lemon balm germplasm.

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Authors' contributions

Conceptualization: Fotokian, M.H; Talei, D. Data acquisition: Danaeipour, Z. Data analysis: Naji, A.M; Danaeipour, Z. Design of Methodology: Talei, D; Naji A. M. Writing and editing: Fotokian, M.H; Danaeipour, Z.

Declaration of competing interest

Authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101416.

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