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Original Article

Genetic Diversity Evaluation of Lemon balm (*Melissa officinalis* L.) Ecotypes Using Morphological Traits and Molecular Markers

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Abstract

Genetic diversity of 12 lemon balm ecotypes was evaluated using agronomic traits as well as ISSR and RAPD markers. Results of analysis of variance showed high diversity among the studied ecotypes. Cluster analysis using UPGMA method grouped the ecotypes into three different groups based on the agronomic traits. Twelve ISSR primers created 106 polymorphic bands among the studied ecotypes. An ISSR primer called UBC813 with 16 bands and UBC811, UBC815 and UBC817 primers with 15 bands had the highest number of polymorphic bands and UBC825 with 8 bands had the lowest number of polymorphic bands. In addition, 10 RAPD primers, created 127 polymorphic bands, among them OPA-01 with 22 bands and BB13 and OC4 primers with 19 and 18 bands had the highest number of polymorphic bands. In contrast, OS-03 and OB20 markers had the lowest number of polymorphic bands with 14 and 15 bands, respectively. Polymorphism information content (PIC) value ranged between 0.33 to 0.37 for ISSR markers and 0.31 to 0.37 for RAPD markers. Marker index (MI) value ranged between 1.11 to 4.38 for ISSR markers and 1.85 to 5.28 for RAPD markers. Cluster analysis using UPGMA method based on ISSR and RAPD markers results clustered the studied ecotypes into three and two different groups, respectively, by which the percentage of similarity of the two grouping method was about 50%. Grouping the ecotypes based on both molecular markers and agronomic traits matched each other about 40 to 60%.

Keywords: Cluster Analysis, Genetic Diversity, ISSR, RAPD.

Introduction

Therapeutic effects of medicinal plants are obvious nowadays. Estimation of genetic diversity of related taxa, allows geneticist to understand evolution and exploit wider pools of diversity [1]. In fact, wide range of the genetic diversity is critical for maintaining and development of plant species in different conditions [2]. Molecular markers are abundantly utilized in assessing the genetic diversity and determining the genetic relationships among different plant samples [3]. El Hadj Ali *et al.*, 2012 evaluated genetic diversity of 25 thyme populations (*Thymus capitatus* (L.) Hoffmanns. & Link) using isozyme markers and reported a high degree of genetic similarity within the populations, while a high genetic diversity was observed among them [4]. The populations were divided into four main groups using UPGMA method, while their marker-based grouping indicated a clear relationship with the geographic origination. Belhassen *et al.*, [5] studied mitochondrial genomes of *Thymus vulgaris* L. populations and concluded that there are high

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levels of diversity and polymorphism among thyme mitochondrial DNA. Alamdary *et al.,* 2011 investigated genetic diversity of 13 thyme samples of Iranian indigenous species using 8 RAPD markers and reported 61.86% of polymorphism among the samples [6]. Smolik *et al.,* 2009 studied genetic diversity of several thyme samples using ISSR-PCR and Inter-genic spacers between copies of 5s RNA subunits genes (IGS-PCR) molecular markers [7]. The highest similarity among the samples was observed at level of 77% and the greatest genetic distance was reported to be 0.6931. In a study, Yavari *et al.,* 2012 employed RAPD molecular markers to assess genetic diversity of several wild populations of Azerbaijani *Thymus migricus* (Klokov & Desj.-Shost) in Iran [8]. Their results indicated a high genetic exchange between 5 Azerbaijani thyme habitats.

Danaeipour *et al.,* 2016 investigated genetic diversity of 20 accessions of lemon balm based of leaf protein [9]. Cluster analysis of the accessions based on protein data produced three clusters. The first cluster comprised of 15 accessions, the second cluster consisted of four accessions and the third cluster contains only Italian accession. The genetic diversity of nine lemon balm populations from different areas of Iran were studied based on morphological, agronomic and essential oils traits by Aharizad *et al.,* 2012 [10]. Populations were classified in four groups using cluster analysis based on Ward's algorithm. High levels of phenotypic variation using Shannon-Weaver diversity index (DI) were found among the countries of origin (DI= 0.95-1.00). Qazvin2 population was more diverse than others (DI = 0.99) whereas populations from Germany and Kurdistan displayed lower diversity indices (DI = 0.96). Ghaffariyan *et al.,* 2011 used 12 Iranian lemon balm ecotypes as well as two populations from Germany and Japan based on inter-retrotransposon amplified polymorphism (IRAP) marker to investigation genetic diversity [11]. Mean of Nei's genetic diversity index and Shannon diversity index were 0.10 and 0.16, respectively. The studied ecotypes were assigned into five groups based on IRAP data using Neighbor-Joining algorithm and p-distance evolutionary distance coefficient. Fourteen landraces of lemon balm with two out group landraces (Basil and Moldavian balm) was investigated by Haidari *et al.,* 2014 to showed genetic diversity based on ITS (Internal Transcribed Spacer) marker [12]. The highest degree of dissimilarity (0.44) was observed from Esfahan and Qazvin landraces. Noroozy *et al.,* 2016 used SRAP marker to evaluate genetic diversity of 20 different *M. officinalis* accessions [13]. Cluster analysis using the unweighted pair-group method with arithmetic averages (UPGMA) produced three groups between their accessions. Genetic similarity ranged between 0.54 to 0.90 for their accessions.

Material and Methods

Plant Material and DNA Extraction

Twelve lemon balm ecotypes were used in this genetic evaluation (Table 1). A total of 233 polymorphic markers were used to detect genetic diversity of the ecotypes. Fresh leaves were used for DNA extraction according to the modified protocol of Murray and Thompson [14]. Quality of the extracted DNA was checked using agarose gel electrophoresis.

Polymerase chain reaction (PCR) was carried out in a total volume of 10 µl per reaction containing 2 µl of template DNA (5 ng / µl), 1 µl 10×PCR buffer, 0.6 µl of forward and reverse primers (5 µM stock concentration), 0.6 µl dNTPs, 0.48 µl of MgCl2 (50 mM), 0.14 µl *Taq* polymerase (5 U/µl) and 4.58 µL of sterile nano-pure H_2O. PCR reaction was performed in a thermal cycler (Applied Biosystems, Germany) at an initial denaturation temperature of 94 °C for 5 min, then 35 cycles of 94 °C for 30 s, 55 °C for 30 s (primer annealing of the most primers), 72 °C for 2 min and final extension at 72 °C for 5 min. PCR products were separated on a 3% agarose electrophoresis.

Genetic Diversity

Clear and reproducible amplified bands were only scored to constitute a data matrix. Amplification products of the ecotypes were manually scored using binary coding system, '0' for absence of a band and '1' for its presence. Number of observed alleles (Na), number of effective alleles (Ne) [15], Nei's gene diversity and Shannon diversity index were calculated using the POPGENE software ver. 1.32 [16]. Polymorphism information content (PIC) was calculated for each marker using equation 1, according to the Anderson *et al.,* [17] method.

$$PIC_j = 1 - \sum_{i=1}^{n} p_i^2 \qquad \text{(Eq. 1)}$$

Where, i= i^{th} allele of j^{th} marker, n= number of alleles at j^{th} marker and p= allele frequency. The PIC value can range from 0 to 1. At a PIC of 1, the marker would have a unlimited number of alleles. At a PIC of 0, the marker has only one allele. PIC value explains diversity within accessions (intra-population diversity) and determines the degree of polymorphism in each locus, a PIC value of less than 0.25 showing low polymorphism, a value between 0.25 and 0.5 average polymorphism and a value higher than 0.5 a very polymorphic locus [18]. Nei's gene diversity was calculated based on Nei formula [19]. Shannon index was calculated based on Lewinton method [20].

Pearson correlation coefficients were used to evaluate relationships between the genetic diversity parameters and number of alleles using SPSS 19. Jaccard's similarity coefficient was used for cluster analysis of the lemon balm ecotypes using UPGMA (Unweighted Pair-Group Method with Arithmetic Mean) method to conclude phylogeny and genetic relationships. Cluster analysis was performed using NTSYS-pc ver 2.02 [21]. Fisher's linear discriminant analysis [22] was applied using SPSS 19 to determine percentage of original cases correctly classified for cluster analysis outputs and predicted group membership.

Results and Discussion

Results of Analysis of variance based on a randomized complete block design (Table 2) showed significant differences among the studied ecotypes at 1% probability level which implies existence of high genetic diversity among the studied ecotypes. Aharizad *et al*. [10] also observed significant differences between several lemon ecotypes representing genetic variation among the lemon balm ecotypes which was consistent with the results of present study.

Table 1 Geographical origins and code number of lemon balm.

Code	Collection region	Code	Collection region
G1	Tehran-Damavand	G7	kerman
G2	Ardabil	G8	Esfahan- YazdAbad
G3	Esfahan-Najafabad	G9	South Khorasan-sarbisheh
G4	Qazvin-1	G10	East Azerbaijan
G5	Fars	G11	Qazvin-2
G6	Hamedan	G12	Gilan- Lahijan

Cluster Analysis Based on Total Traits

To obtain an idea about the extent of the similarities and differences among the studied ecotypes based on the studied traits, cluster analysis was performed using different methods such as average distance between and within groups, the closest and furthest neighbors and Ward minimum variance method and their grouping results were compared. Since UPGMA method (Euclidean distance criterion) presented the best results in grouping of the studied landraces, therefore, only the results of this method were reported (Fig. 1). By cutting the dendrogram at cutting point 5, three groups were created which included 6, 3 and 3 ecotypes, respectively. The first group included landraces No: 2, 3, 7, 10, 11 and 12, respectively. Second group included landraces No: 1, 5 and 8 and finally third group contained landraces No: 4, 6 and 9 respectively. Maximum distance was observed between Isfahan-Najafabad and Qazvin – Yek landraces. Minimum distance was observed among Isfahan-Najafabad and East Azerbaijan landraces.

Table 2 Analysis of variance of the studied traits on twelve lemon balm ecotypes

S.oV.	df	MS Days to 50% flowering	Plant height	Stem diameter	Number of branches per plant	Internode Length	Leaf lenghth	Leaf width	N. nodes	Fresh weight	Dry weight	Essential oil percentage
Rep	2	0.083^{ns}	0.575^{ns}	0.006^{ns}	0.361^{ns}	0.75^{ns}	0.0002^{ns}	0.0004^{ns}	0.003^{ns}	57.27^{**}	109.91^{**}	0.0000004^{ns}
Treat	11	23.39^{*}	144.39^{**}	0.578^{**}	42.76^{**}	11.27^{**}	0.727^{**}	0.334^{**}	0.223^{**}	2379.97^{**}	2419.71^{**}	0.0032^{**}
Error	22	0.841	0.464	0.002	1.149	1.022	0.0006	0.0002	0.0002	9.037	8.81	0.000004
CV		0.97	0.82	1.16	2.25	5.14	0.43	0.27	0.43	0.063	2.43	1.19

^{ns} and **: Non-significant and significant at 1% probability levels, respectively

Table 3 ISSR and RAPD markers characteristics used to study twelve lemon balm ecotypes

Primers		No. of Polymorphic bands	Total bands	% Polymorphism	PIC	EMR	MI	Shannon	Nei	Ne
ISSR	UBC811	12	15	80	0.33	9.6	3.2	0.62	0.43	1.76
	UBC812	9	14	64.29	0.36	5.79	2.08	0.66	0.47	1.89
	UBC813	14	16	87.50	0.36	12.25	4.38	0.66	0.47	1.88
	UBC814	8	12	66.67	0.30	5.33	1.61	0.56	0.38	1.64
	UBC815	10	15	66.67	0.32	6.67	2.15	0.60	0.41	1.71
	UBC816	7	10	70	0.36	4.9	1.77	0.66	0.47	1.9
	UBC817	10	15	66.67	0.35	6.67	2.37	0.65	0.46	1.87
	UBC823	7	11	63.64	0.37	4.45	1.66	0.69	0.50	1.98
	UBC824	6	10	60	0.37	3.6	1.33	0.68	0.49	1.95
	UBC825	5	8	62.50	0.35	3.13	1.11	0.65	0.46	1.86
	UBC826	8	11	72.73	0.34	5.82	1.98	0.63	0.44	1.79
	UBC876	10	14	71.43	0.31	7.14	2.21	0.57	0.39	1.66
Mean		8.83	12.58	69.34	0.34	6.28	2.15	0.64	0.45	1.82
RAPD	OB20	9	15	60	0.34	5.4	1.85	0.63	0.44	1.8
	OH-04	11	17	64.71	0.36	7.12	2.56	0.66	0.47	1.9
	OA12	14	18	77.78	0.36	10.89	3.87	0.66	0.46	1.87
	BB13	17	20	85	0.37	14.45	5.28	0.67	0.48	1.93
	SA-R	10	16	62.5	0.34	6.25	2.10	0.62	0.43	1.77
	CS-56	12	16	75	0.31	9	2.82	0.58	0.40	1.72
	OC4	15	19	78.95	0.37	11.84	4.34	0.68	0.48	1.94
	OS-03	9	14	64.29	0.36	5.79	2.08	0.66	0.47	1.9
	OPA-01	17	22	77.27	0.36	13.14	4.7	0.66	0.47	1.88
	OPC-04	13	18	72.22	0.35	9.39	3.32	0.65	0.46	1.88
Mean		12.7	17.5	71.77	0.35	9.33	3.29	0.65	0.46	1.86

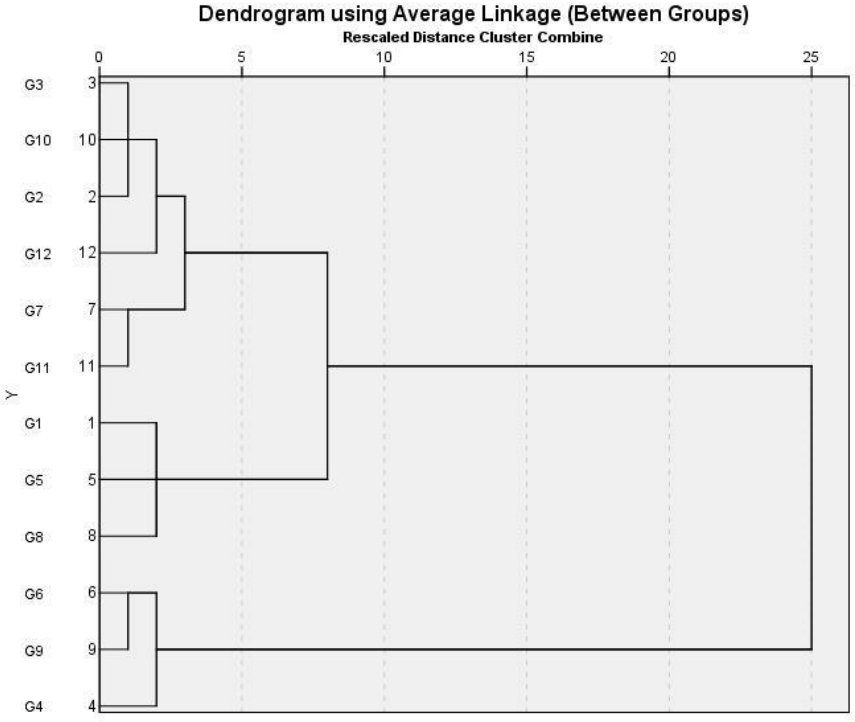

Fig. 1 Grouping twelve lemoan balm ecotypes based on morphological traits using UPGMA method. The symbols for the ecotypes is presented in Table 1.

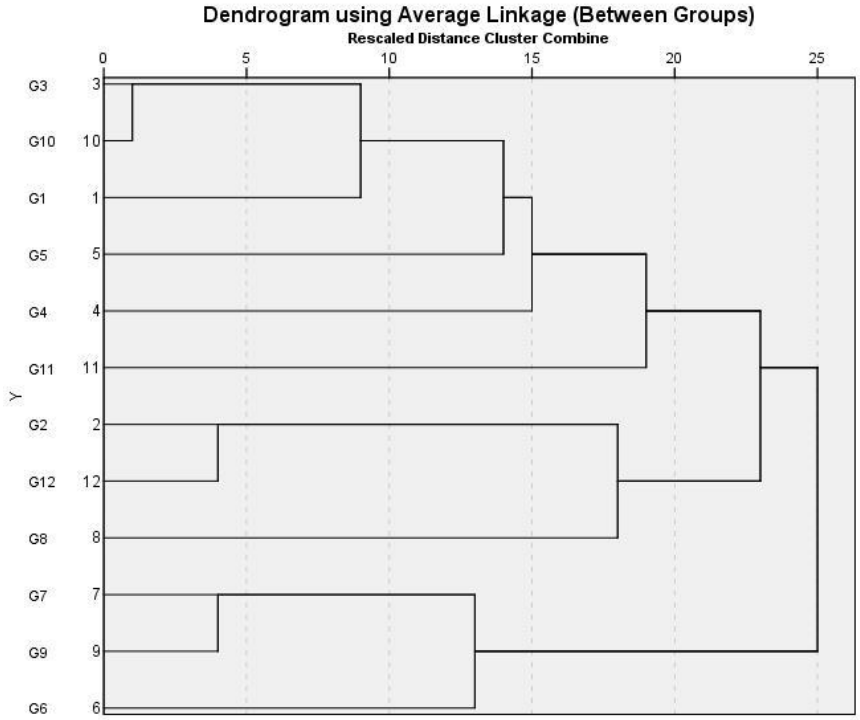

Fig. 2 Dendrogram of 12 lemon balm ecotypes using simple matching genetic distance and UPGMA clustering method based on ISSR data.

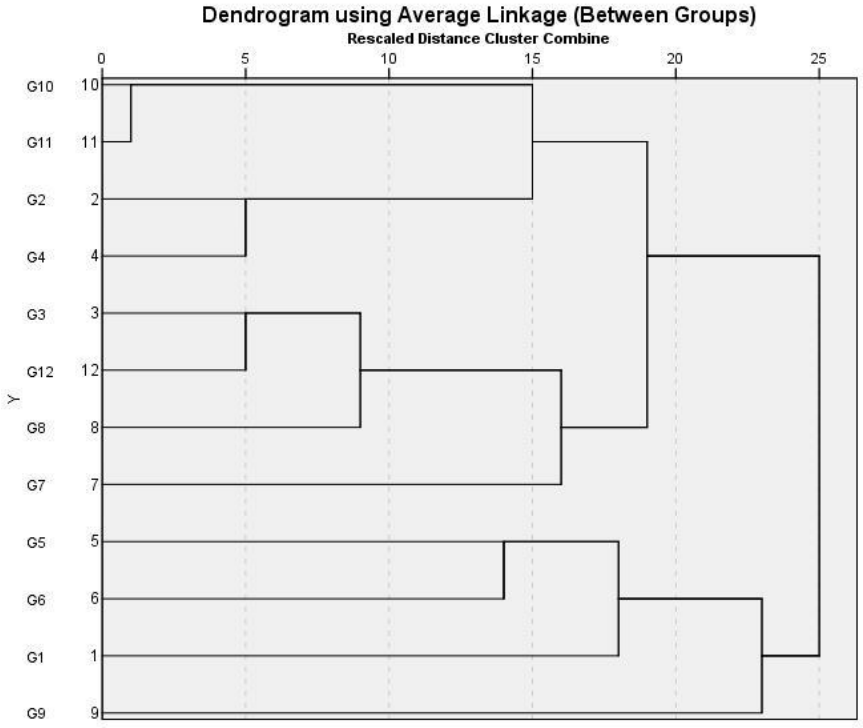

Fig. 3 Dendrogram of 12 lemon balm ecotypes using simple matching genetic distance and UPGMA clustering method based on RAPD data.

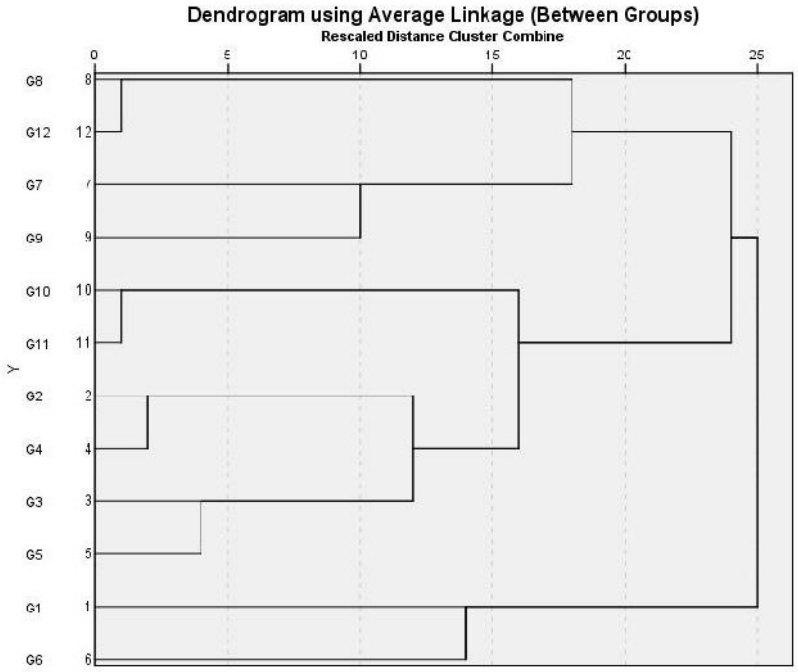

Fig. 4 Dendrogram of 12 lemon balm ecotypes using simple matching genetic distance and UPGMA clustering method based on RAPD and ISSR data.

Morphological variation does not always reflect real genetic variation because of interaction between genotype and environment, and large unknown genetic control of polygenic morphological and agronomic traits. Also, the morphological traits were relatively less efficient for precise discrimination of closely related accessions and analysis of their genetic relationships. Despite this limitation, morphological traits are useful for preliminary evaluation because of their fast and simple application, and can be used as a general approach for assessing genetic diversity among morphologically distinguishable populations. Aharizad *et al*. [10] classified lemon balm populations in four groups using cluster analysis based on traits which was partly consistent with the results of present study.

Number of Observed Bands and Percentage of Polymorphism for ISSR and RAPD Markers

Twelve ISSR and ten RAPD primers were used in this study. ISSR markers produced a total of 151 bands, of which 106 bands were polymorphic and average polymorphic loci per primer was estimated at 8.38. RAPD markers produced a total of 175 bands, of which 127 bands were polymorphic and average polymorphic locus per primer was estimated to be 12.7 (Table 3). Twelve ISSR primers created 106 polymorphic bands among them UBC813 with 16 bands and UBC811, UBC815 and UBC817 primers with 15 bands had the highest number of polymorphic bands and UBC825 with 8 bands had the lowest number of polymorphic bands. Polymorphism percentage in the landraces was obtained 69.34 for ISSR markers. In addition, 10 RAPD primers, created 127 polymorphic among them OPA-01 with 22 bands and BB13 and OC4 primers with 19 and 18 bands had the highest number of polymorphic bands and OS-03 and OB20 with 14 and 15 bands had the lowest number of polymorphic bands respectively. Polymorphism percentage in the landraces was 71.77 for ISSR markers. Solouki *et al.,* [23] evaluated genetic diversity of German chamomile ecotypes using 29 RAPD primers by which, among 369 revealed bands, 314 bands were polymorphic. Pirkhezri *et al.,* [24] evaluated 25 German chamomile ecotypes using 18 primers, RAPD and obtained 220 bands among them 93.1% were polymorphic. The average numbers of total and polymorphic bands were estimated 12.2 and 11.4 respectively. Heidary *et al.,* [25] studied genetic diversity of barberry using 4 AFLP primer combinations and observed 223 band of which 207 bands were polymorphic. Zhang *et al.,* [26] investigated genetic variation among *Glycyrrhiza uralensis* ecotypes in northern China using AFLP

markers. In their study, 50 individuals from five ecotypes were used. Eight primer combinations totally produced 1025 band of which 57% were polymorphic.

Polymorphic information content (PIC) is equivalent of genetic diversity and shows resolution of a marker by the number of polymorphic alleles and frequency of the alleles in the studied populations. Polymorphic information content, calculated separately for each primer (Table 3). Polymorphic information content (PIC) ranged from 0.33 to 0.37 and average of polymorphic information content was calculated 0.34 for ISSR markers. Also, polymorphic information content (PIC) ranged from 0.31 to 0.37 and average of polymorphic information content was calculated 0.35 for RAPD markers. The highest PIC for ISSR markers was 0.37 for UBC823 and UBC824 primers and for RAPD markers was estimated 0.37 for BB13 and OC4 primers indicating a high efficiency of the markers in differentiating the landraces used in this research. In order to determine the efficiency of markers in showing the polymorphism, MI and EMR were calculated. Among ISSR markers, the highest amount EMR was calculated for UBC813 (12.25) and the lowest one was observed in UBC825 (3.13). Marker index (MI) ranged from 1.11 to 4.38. Among RAPD markers, the highest amount EMR was calculated for BB123 (14.45) and the lowest one was observed in OB20 (5.40). Marker index (MI) ranged from 1.85 to 5.28. Pirkhezri *et al*. [24] evaluated 25 German chamomile ecotypes using 18 primers, RAPD evaluated number of effective alleles, Nei's gene diversity and Shannon index for different provinces. Maximum and minimum amount for the number of effective alleles was observed in Khuzestan (1.657) and Fars provinces (1.142) respectively. Nei's gene diversity and Shannon index was higher in ecotypes of Khuzestan province (Nei= 0.528; Shannon= 0.364) and less than others in ecotypes of Fars province (Nei= 0.23; Shannon= 0.16).

Number of effective alleles was different among the studied markers. Average number of effective alleles was calculated 1.84 in the populations and ranged between 1.64-1.98. UBC823, and UBC824, BB13, OC4 had the highest number of effective alleles among four studied landraces. Since the number of effective alleles is one of the important criteria in selection of appropriate and useful primers, the primers could be used to investigate the genetic diversity of lemon balm ecotypes for future studies. In order to group the studied ecotypes and assess their relationships by ISSR marker, cluster analysis was performed using simple matching method. Cut-off point of the resulted dendrogram from similarity area of 20 divided the populations into three main groups (Fig. 2). The first group (A) contained 6 ecotypes, whereas, second group (B) includes three ecotypes and third group (C) contained three ecotypes. Cluster analysis was performed using simple matching method for RAPD markers. Cut-off point of the dendrogram from the similarity area of 24 divided the populations into two main groups (Fig. 3). The first group (A) contains 8 ecotypes, whereas, second group (B) included 4 ecotypes. Genetic similarity among the ecotypes varied from 31.1 to 67.9.

Cluster analysis was performed using simple matching method for RAPD and ISSR markers. Cut-off point of the dendrogram from the similarity area of 23 divided the populations into three main groups (Fig. 4). The first group (A) contained 4 ecotypes, whereas, second group (B) included 6 ecotypes and 3rd group included 2 ecotypes. Estimates of genetic similarity among the ecotypes varied from 37.3 to 62.7.

Conclusion

The result indicated that ISSR and RAPD markers could be a powerful tool to assess genetic variability of lemon balm ecotypes. Information about the genetic diversity will be very useful for proper identification and selection of appropriate parents for breeding programs, including gene mapping, and ultimately for emphasizing the importance of marker-assisted selection (MAS) in lemon balm improvement program. Since solitary morphological variation does not reflect total variation which is necessary for breeding new lemon balm ecotypes, newer techniques such as isozyme, protein, molecular markers and quantitative characters must be used in order to provide a complete view about genetic variation of lemon balm ecotypes. In addition, number of ecotypes must be increased with larger agroecological distribution, in order to estimate association between different geographic distances and genetic distance to morphologic variables and

others. In summary, the present study revealed a wide variation among the studied germplasms.

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Conflict of interest

The authors declare that they have no conflict of interest in the publication.

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