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Comprehensive genetic discrimination of *Leonurus cardiaca* populations by AFLP, ISSR, RAPD and IRAP molecular markers

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Abstract *Leonurus cardiaca* is well known for its medicinal importance. In this investigation, genotypic characterization of this species from six eco-geographical regions of Iran was evaluated by four molecular techniques (AFLP, RAPD, ISSR and IRAP). A total of 899 polymorphic fragments were detected by used molecular markers (AFLP = 356, RAPD = 325, ISSR = 113 and IRAP = 105) with an overall average polymorphism of 81.24 %. Genetic variation calculated using Shannon's Information index (I) and Nei's gene diversity index (H) showed high genetic diversity in studied germplasm. Also, analysis of molecular variance showed high genetic variation among (55 %) and within populations (45 %). UPGMA dendrogram constructed from combined data of molecular markers distinguished studied populations in accordance with the results obtained by each marker which all individuals were clearly differentiated into two major clusters. The correlation coefficients were statistically significant for all marker systems with the highest correlation between similarity matrixes of RAPD and ISSR markers ($r = 0.82$). The present results have an important implication for *L. cardiaca* germplasm characterization, improvement, and conservation. Furthermore, the characterized individuals exhibited a great deal of molecular variation and they seem to have a rich gene pool for breeding programs.

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Introduction

Population genetic structure comprises genetic diversity, population variation, gene flow, etc. Genetic diversity of a species is the sum of genetic information within a gene pool. Effective conservation strategies should be based on studies of genetic diversity at various levels [8]. Also, continual advances in crop improvement through plant breeding are driven by the available genetic diversity. Therefore the recognition and measurement of such diversity is crucial to breeding programs [9]. The importance of biogeographical considerations when attempting to conserve populations of diverse organisms is becoming increasingly clear [10].

Motherwort, *Leonurus cardiaca*, is one of many introduced members of the Mint family (Lamiaceae). Iran has developed one of the largest germplasm of this plant in the world [22]. It has foliage with a somewhat distinctive appearance and flowers that are exceptionally hairy [19]. It was introduced as an herb with medicinal properties. Historically, this herb was used to treat menstrual disorders, expel dead fetuses, aid recovery after childbirth, but also to aid miscarriage in unwanted pregnancies [20, 29]. It has been reported that the essential oil of *L. cardiaca* is rich of epi-cedrol, α-humulene, germacrene-D and spathulenol [21].

Since domestication process of medicinal plants is a time consuming process, selection of appropriate genotype can be shorten the process. Recent advances in molecular biology have provided a suite of powerful tools for assessing population differentiation in wild plant species,

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Table 1 Locations of studied populations of *L. cardiaca* in Iran

No.	Population	Province	Sample size	Altitude (m)	Longitude (E)	Latitude (N)
1	Kerman	Kerman	7	2,600	56°50′31″	29°18′36″
2	Dargaz	South-Khorasan	8	2,194	58°42′01″	37°34′42″
3	Taleghan	Tehran	8	1,850	50°45′34″	36°10′27″
4	Khansar	Esfahan	8	2,210	50°17′57″	33°15′50″
5	Sarab	Ardabil	8	1,687	47°31′40″	37°55′59″
6	Sari	Mazandaran	8	2,170	53°12′03″	36°03′37″

and hence exploring processes involved in the formation, evolution and genetic structure of species. With the advances in plant molecular biology, a number of molecular marker types such as amplified fragment length polymorphism (AFLP, [47]), random amplified polymorphic DNA (RAPD, [49]), inter-simple sequence repeats (ISSR, [17]) and inter-retrotransposon amplified polymorphism (IRAP, [13]) have been developed and used extensively in studying genetic diversity, genetic relationship and germplasm management. These molecular markers are powerful tools for characterizing genetic differentiation in nuclear polymorphisms [46]. AFLP markers have significant advantages such as the ability to resolve a large number of loci from a single reaction and the fact that no prior sequence information is required [53]. RAPD offers a cheap and simple DNA-based marker alternative, considering the advantages of small amount of DNA, radioactivity-free procedure, ease and speed of the assay and lack of requirement for DNA sequence information of a species [49]. ISSR is simple and inexpensive technique and is rapidly being used by the research community in various fields of plant improvement [33]. Also, IRAP examines variation in retro transposon insertion sites [13].

Iran has rich *L. cardiaca* (motherwort) germplasm resources [22]. Therefore, categorizing and characterizing of this germplasm is an essential step in the selection and breeding of motherwort populations. More specifically, we investigated the genetic structure of *L. cardiaca* populations in Iran in order to assess the genetic diversity and differentiation of populations at each molecular marker locus; and evaluate the genetic relationships among populations.

Materials and methods

Plant material

A total of forty-seven individuals of *L. cardiaca* were collected from six regions of six provinces in Iran. Sampling locations and their geographic coordinates are shown in Table 1. The interval between samples was 300–500 m, whereas the pairwise distance between main regions was 300–600 km. The sampled stands were chosen to provide maximum representation of the ecological conditions of the area.

Molecular analysis

Young leaves were collected, labeled and stored at −80 °C for DNA extraction. Total genomic DNA was extracted from 100 to 200 mg leaf material following the method described by Pirttila et al. [28]. The purified total DNA was quantified by gel electrophoresis and verified by spectrophotometer.

Genotyping was performed using AFLP, RAPD, ISSR and IRAP markers using the protocols described by Vos et al. [47], Khadivi-Khub et al. [14], Shahi-Gharahlar et al. [39] and Du et al. [7], respectively. For AFLP, amplified PCR products were electrophoresed through 4.50 % polyacrylamide gel, while DNA amplification products for RAPD, ISSR and IRAP markers were analyzed by electrophoresis in 1.50 % agarose gels. Initially, three individuals from each of the populations were used to perform a preliminary screening using primers of each molecular marker, to identify those that provided reproducible marker profiles and to exclude those producing a very low proportion of polymorphic bands.

Statistical analysis

We scored all the visible and clear polymorphic bands in the AFLP, RAPD, ISSR and IRAP profiles as 1 and 0 on the basis of the presence and absence of the band. The data was systematically scored along a ladder size. Each polymorphic fragment of AFLP, RAPD, ISSR and IRAP was considered as a different locus corresponding to a unique position in the genome. The genetic similarities according to Jaccard's coefficient were calculated using the SIMQUAL program of the numerical taxonomy multivariate analysis system NTSYS-pc version 2.10 [34] and the dendrograms were constructed through SAHN clustering program using the unweighted pair group method with arithmetic means (UPGMA).

The correlations between the AFLP, RAPD, ISSR and IRAP matrixes were computed by Mantel test [16] using the COPH and MYXCOMP programs. Principal coordinate

analysis (PCOA) was performed based on the variance covariance matrix calculated from the combined AFLP, RAPD, ISSR and IRAP data. Polymorphic information content (PIC) and marker index (MI) were calculated using formulas described by Powell et al. [30]. Ability of the primers to differentiate between the individuals was assessed by calculating their resolving power (Rp), according to Prevost and Wilkinson [31] using the formula $Rp = \sum Ib$, where $Ib = 1 - (2 \times |0.50 - p|)$, and p is the proportion of individuals containing the I band. The resolving power is based on the distribution of detected bands within the sampled individuals. Population genetic parameters such as Nei's gene diversity index (H), Shannon diversity index (I), the percentage of polymorphic bands (PPB) and measures of genetic identity and genetic distance [24] were estimated using Popgen 32 Software (Ver.1.31, [50]). The variation among and within populations based on marker patterns were analyzed by analysis of molecular variation (AMOVA) using GenAIEx version 6.3 [26].

Results

Level of polymorphism and informativeness

A total of 607 amplification fragments were detected by the six AFLP primer combinations, with an average of 101.17 fragments with size of 50–500 bp. Of these, 356 bands were polymorphic, with an average polymorphism of 58.23 % (Table 2). These results agreed with findings of others for high potential of AFLP [1, 2, 23, 32, 37]. Polymorphism detected in this study was higher than studies of Russi et al. [37] and Rahimmalek et al. [32] in *Echinacea* and *Achillea* by AFLP, respectively. This high diversity in this study may be related to studied individuals, different regions and used primers. The number of amplified bands ranged from 80 (for C3 primer pairs) to 126 (for C4 primer pairs). C4 primer pairs showed the highest polymorphic band (72 bands) and C3 primer pairs indicated the least (36 bands). Values of PIC ranged between 0.23 (for C5 primer pairs) and 0.31 (for C2 primer pairs) with an average of 0.27. Primers resolving power (Rp) varied from 17.50 (for C3 primer pairs) to 38.70 (for C2 primer pairs) with an average of 30.76, indicating that C2 primer pairs contain higher genomic information than other primers [18]. Furthermore, MI ranged from 4.21 (for C3 primer pairs) to 14.33 (for C2 primer pairs) with an average of 9.59.

Among 60 examined RAPD primers, 28 primers detected polymorphism bands among individuals. A total of 362 bands were produced that 325 were polymorphic with an average of 11.61 polymorphic bands per primer (Table 2). The highest polymorphic bands were observed by TIBMBC-02 primer (21 bands) and the least by TIBMBC-12 (five fragments). Also, the observed average polymorphism was 89.02 %. Six RAPD primers including TIBMBA-20, TIBMBC-11, TIBMBC-17, TIBMBD-03, TIBMBE-04 and TIBMBE-20 showed 100 % polymorphism. Values of PIC ranged between 0.15 (for TIBMBE-04) and 0.38 (for TIBMBB-03) with an average of 0.26. Resolving power (Rp) values varied from 1.20 (TIBMBD-12) to 7.90 (TIBMBA-20) with an average of 4.62. Furthermore, the mean value for MI was 2.70 and ranged from 0.94 (for TIBMBC-12 primer) to 5.52 (for TIBMBE-19 primer).

From a total of 17 arbitrary ISSR primers, 11 ISSR primers were chosen for PCR amplification and generated 86.84 % polymorphism, corresponding to 113 polymorphic fragments of a total of 126 bands scored (Table 2). The number of bands per primer varied from 5 (by ISCS-11 primer) to 17 (by ISCS-07 and ISCS-14 primers) with an average of 11.45 bands per primer. The total of polymorphic bands ranged from 3 (by ISCS-11) to 17 (by ISCS-07) with an average of 10.27 bands per primer. The amplicon size ranged from 240 to 2700 bp. The percentage of polymorphism ranged between 60 % (by ISCS-11) and 100 % (by ISCS-07, ISCS-13 and ISCS-16). The Rp value ranged from 0.40 (for primer ISCS-11) to 5.60 (for primer ISCS-05) with an average 3.33. Values of PIC ranged between 0.11 (by ISCS-11) and 0.36 (by ISCS-16) with an average of 0.22. Furthermore, MI ranged from 0.54 (for ISCS-11 primer) to 4.13 (for ISCS-05 primer) with an average of 2.48 (Table 2).

From prescreening assays with three individuals using 28 IRAP primer pairs, eight primer pairs generated bright polymorphic amplification products and were used in further analysis. The total number of detected bands was 116 with range of 11 (by 3′LTR–LTR6150) to 18 (by Sukkula–Sukkula). The total of separable bands were 105 and ranged from 10 (3′LTR–LTR6150) to 16 (Sukkula–Sukkula) with an average of 13.13 polymorphic bands per primer pairs. The observed average polymorphism was 90.85 % and two primers including LTR6150–LTR6150 and 5′LTR2–Sukkula, showed 100 % polymorphism. The mean PIC value was 0.87, ranging from 0.62 (by 3′LTR–LTR6150) to 0.97 (by 5′LTR2–Sukkula). Seven primer pairs had a PIC value higher than 0.80. Because of the high polymorphic value of the studied bands, we were able to distinguish genetic variation within and among populations. The mean value for MI was 10.59 and ranged from 5.60 (by 3′LTR–LTR6150) to 13.10 (by 3′LTR–3′LTR). The mean of Rp value was 6.14 with range of 2.90 (for 5′LTR2–Sukkula) to 8.40 (for 3′LTR–3′LTR).

Level of molecular diversity and variance

Heterozygosity and molecular variance were calculated for AFLP, RAPD, ISSR and IRAP markers individually. Nei's

Table 2 List of molecular marker primers (AFLP, IRAP, ISSR and RAPD) used and informativeness obtained in studied individuals of *L. cardiaca*

Primer pairs	TB	PB	PP (%)	R*p*	PIC	MI	Primer	TB	PB	PP (%)	R*p*	PIC	MI
AFLP							RAPD						
C1	94	49	52.13	29.10	0.30	7.66	TIBMBA-02	17	16	94.12	7.00	0.27	4.05
C2	100	68	68.00	38.70	0.31	14.33	TIBMBA-03	12	10	83.33	3.40	0.22	1.81
C3	80	36	45.00	17.50	0.26	4.21	TIBMBA-06	13	11	84.62	4.20	0.27	2.48
C4	126	72	57.14	34.50	0.26	10.69	TIBMBA-08	17	15	88.24	6.80	0.29	3.80
C5	99	69	69.70	32.38	0.23	11.06	TIBMBA-09	13	10	76.92	3.80	0.32	2.44
C6	108	62	57.41	32.38	0.27	9.61	TIBMBA-10	12	10	83.33	5.70	0.37	3.11
Total	607	356	–	–	–	–	TIBMBA-14	11	9	81.82	2.30	0.28	2.03
Mean	101.17	59.33	58.23	30.76	0.27	9.59	TIBMBA-15	9	8	88.89	2.80	0.21	1.53
							TIBMBA-20	19	19	100.00	7.90	0.23	4.33
IRAP							TIBMBB-03	19	16	84.21	5.00	0.38	5.12
3′LTR–3′LTR	16	15	93.75	8.40	0.93	13.10	TIBMBB-04	10	9	90.00	4.60	0.25	2.02
3′LTR–LTR6150	11	10	90.91	4.30	0.62	5.60	TIBMBB-05	13	12	92.31	4.50	0.24	2.68
LTR6150–Sukkula	16	14	87.50	6.80	0.82	10.70	TIBMBC-02	22	21	95.45	7.40	0.20	4.00
Sukkula–Sukkula	18	16	88.89	7.90	0.89	12.70	TIBMBC-03	18	17	94.44	6.60	0.25	4.01
Sukkula–Nikita	15	12	80.00	4.40	0.91	8.70	TIBMBC-10	7	6	85.71	2.40	0.22	1.15
Sukkula–3′LTR	14	12	85.71	6.20	0.93	9.50	TIBMBC-11	8	8	100.00	2.60	0.24	1.90
5′LTR2–Sukkula	12	12	100.00	2.90	0.97	11.70	TIBMBC-12	6	5	83.33	2.30	0.23	0.94
LTR6150–LTR6150	14	14	100.00	8.20	0.91	12.70	TIBMBC-17	11	11	100.00	3.90	0.25	2.80
Total	116	105	–	–	–	–	TIBMBC-20	14	10	71.43	4.50	0.31	2.18
Mean	14.50	13.13	90.85	6.14	0.87	10.59	TIBMBD-03	14	14	100.00	6.60	0.18	2.54
							TIBMBD-05	10	7	70.00	3.90	0.21	1.02
ISSR							TIBMBD-12	7	6	85.71	1.20	0.25	1.30
ISCS-01	7	6	85.71	2.40	0.26	1.79	TIBMBE-02	12	10	83.33	4.20	0.28	2.81
ISCS-02	8	6	75.00	3.00	0.31	2.50	TIBMBE-03	11	10	90.91	3.30	0.33	2.97
ISCS-05	15	14	93.33	5.60	0.28	4.13	TIBMBE-04	20	20	100.00	7.00	0.15	2.97
ISCS-07	17	17	100.00	3.80	0.14	2.32	TIBMBE-17	10	9	90.00	5.60	0.21	1.73
ISCS-08	16	14	87.50	4.60	0.21	3.41	TIBMBE-19	18	17	94.44	6.50	0.34	5.52
ISCS-09	9	6	66.67	2.30	0.25	2.28	TIBMBE-20	9	9	100.00	3.40	0.25	2.27
ISCS-11	5	3	60.00	0.40	0.11	0.54	Total	362	325	–	–	–	–
ISCS-13	9	9	100.00	2.90	0.19	1.68	Mean	12.93	11.61	89.02	4.62	0.26	2.70
ISCS-14	17	16	94.12	4.20	0.17	2.90							
ISCS-15	14	13	92.86	3.00	0.18	2.46							
ISCS-16	9	9	100.00	4.40	0.36	3.24							
Total	126	113	–	–	–	–							
Mean	11.45	10.27	86.84	3.33	0.22	2.48							

TB total bands, *PB* polymorphic bands, *PP* polymorphism percentage, *Rp* resolving power, *PIC* polymorphic information content, *MI* marker index

gene diversity (H) values for AFLP, RAPD, ISSR and IRAP were 0.18, 0.09, 0.08 and 0.15, respectively. Similarly, the Shannon's information indices (I) were 0.26, 0.13, 0.13 and 0.22 for AFLP, RAPD, ISSR and IRAP markers, respectively (Table 3). Effective number of alleles (Ne) for AFLP, RAPD, ISSR and IRAP were 1.30, 1.15, 1.16 and 1.28, respectively.

Genetic variation within populations using Shannon's Information index (I) and Nei's gene diversity index (H) showed the highest genetic diversity within Sarab population (H = 0.20, I = 0.30) and the least within Taleghan population (H = 0.14, I = 0.21) by AFLP markers. The H and I indexes at the population level ranged from 0.05 and 0.08 (in the Sari population) to 0.10 and 0.15 (in Taleghan and Sarab populations) based on RAPD data, while in the case of ISSR analysis, within each population, the H and I indexes varied from 0.06 and 0.10 (for the Khansar population), to 0.10 and 0.15 (for the Sarab

Table 3 Genetic variation and polymorphic features estimated using AFLP, RAPD, ISSR and IRAP markers in studied six populations of *L. cardiaca*

Population	AFLP			RAPD			ISSR			IRAP		
	H	I	PPB %	H	I	PPB %	H	I	PPB %	H	I	PPB %
Kerman	0.17	0.25	44.38	0.08	0.11	22.15	0.09	0.14	29.20	0.16	0.24	39.05
Dargaz	0.19	0.28	49.16	0.10	0.14	25.23	0.09	0.15	30.97	0.12	0.17	27.62
Taleghan	0.14	0.21	42.98	0.10	0.15	26.15	0.09	0.13	24.78	0.21	0.31	52.38
Khansar	0.16	0.24	48.72	0.08	0.12	24.00	0.06	0.10	21.24	0.14	0.21	42.86
Sarab	0.20	0.30	64.89	0.10	0.15	27.08	0.10	0.15	28.32	0.14	0.21	35.24
Sari	0.19	0.29	57.58	0.05	0.08	14.77	0.07	0.11	20.35	0.12	0.18	33.33
Mean	0.18	0.26	51.29	0.09	0.13	23.23	0.08	0.13	25.81	0.15	0.22	38.41

Table 4 AMOVA for studied individuals of *L. cardiaca* based on AFLP, RAPD, ISSR and IRAP markers and combined data

Source	Variation (%)				
	AFLP	RAPD	ISSR	IRAP	Combined data
Among populations	43	70	61	53	55
Within populations	57	30	39	47	45
Total	100	100	100	100	100

population). Also, based on Shannon's Information index (I) and Nei's gene diversity index (H) according to IRAP data, the highest genetic diversity was observed within Taleghan population (H = 0.21, I = 0.31) and the least within Dargaz population (H = 0.12, I = 0.17) (Table 3).

The PPB values calculated for AFLP, RAPD, ISSR and IRAP were 51.29, 23.23, 25.81 and 38.41 %, respectively (Table 3). The PPB at the population level ranged from 42.98 % (for Taleghan population) to 64.89 % (for Sarab population) by AFLP markers. In case of RAPD analysis, the PPB varied from 14.77 % (for Sari population) to 27.08 % (for Sarab population). In the case of ISSR, the PPB at the population level ranged from 20.35 % (for Sari population) to 30.97 % (for Dargaz population). Also, the PPB at the population level ranged from 27.62 % (for Dargaz population) to 52.38 % (for Taleghan population) by IRAP markers (Table 3).

Furthermore, analysis of molecular variance (AMOVA) was performed (Table 4). For AFLP analysis, within-population molecular variation estimated using the AMOVA procedure indicated high genetic variation so that variation among populations was 43 % and within populations was 57 %. For RAPD analysis, the variance within populations accounted for 30 % of the total variance and the variance among populations accounted for 70 % of the total variance. Also, analysis of ISSR showed that the genetic variation was found mainly among populations (61 %), but variance within populations was only 39 %, while for IRAP analysis, the variance contributed among population and within population was 53 and 47 %, respectively (Table 4).

Genetic differences and relationships

Genetic similarity was calculated using the Jaccard's similarity coefficient (J) value for studied populations of *L. cardiaca* considering AFLP, RAPD, ISSR and IRAP markers individually. In the 47 *L. cardiaca* individuals, for AFLP analysis, the genotypes Taleghan6 and Taleghan7 had the highest genetic similarity (0.70), whereas, the least was detected between Taleghan7 and Dargaz1 (0.06), indicating considerable distance and diversity within and between populations. As mentioned above, such high genetic distances are characteristic of AFLPs, which generate notoriously high levels of diversity, especially among distantly related individuals. The distinct advantage of high levels of polymorphisms representing the entire genome as revealed by AFLPs has the potential to generate a more realistic species tree as compared to a particular gene tree. This is especially true among closely related, potentially interbreeding species, where there is a high probability of reticulate evolution occurring. With the use of appropriate statistical tools [53], AFLPs can also be used to analyze genetic structure and differentiation within and among species and populations. For RAPD analysis, Taleghan6 and Taleghan7 showed the highest genetic similarity (0.88), and the least was detected between Khansar3 and Dargaz2 (0.27). For ISSR analysis, the minimum genetic similarity was recorded between Dargaz1 and Khansar3 (0.25) and the maximum was observed between Sari3 and Sari4 (0.94). Based on IRAP markers, the highest genetic similarity was between Sari1 and Sari3 (0.71), while, the least was between Taleghan2 and Dargaz2 (0.21) (similarity matrixes are not shown).

The UPGMA cluster analysis based on AFLP, RAPD, ISSR and IRAP data revealed genetic relationships among and within populations. The cophenetic correlation coefficient (CCC) indicated high correlation between the similarity matrix of each marker and the cophenetic matrix obtained from the UPGMA dendrogram. CCC value calculated for AFLP, RAPD, ISSR and IRAP were $r = 0.93$, $r = 0.95$, $r = 0.93$ and $r = 0.91$, respectively, indicating a

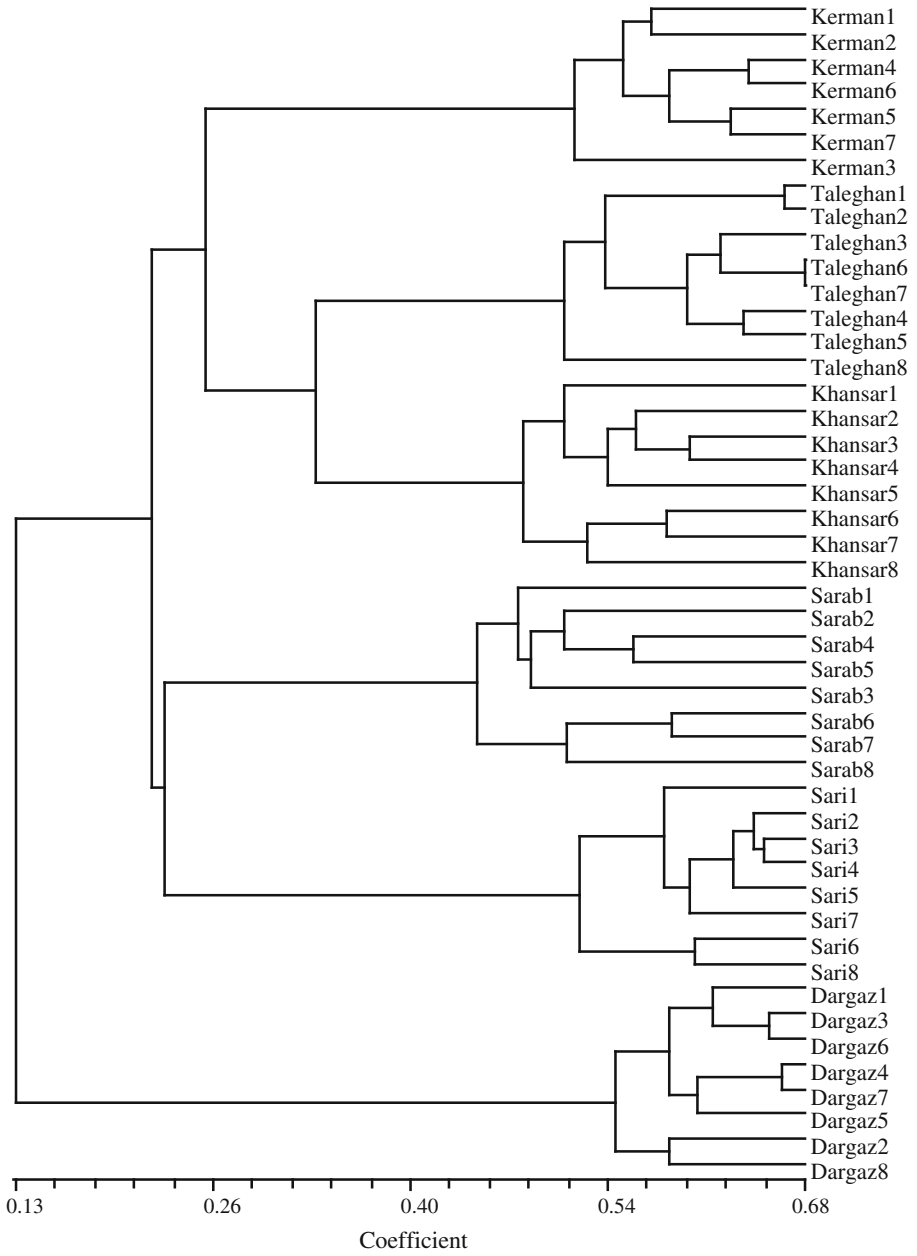

Fig. 1 UPGMA dendrogram produced using Jaccard's coefficient based on combined data of four molecular markers (AFLP, RAPD, ISSR and IRAP) in studied individuals of *L. cardiaca*

good representation of the molecular relationships among genotypes. The cophenetic correlation coefficient is considered to be a very good representative of the data matrix in the dendrogram if it is 0.90 or greater [35].

All molecular markers, AFLP, RAPD, ISSR and IRAP, generated four similar dendrogram topologies which they were highly similar in clustering of individuals and populations (not shown). Thus, for more comprehensive evaluation, we combined all obtained polymorphic fragments by four molecular markers (AFLP = 356, RAPD = 325, ISSR = 113 and IRAP = 105, totally 899 polymorphic bands) and constructed a dendrogram by UPGMA method. Correlation between the genetic similarity matrices was estimated from AFLP, RAPD, ISSR and IRAP markers. The Mantel matrix correspondence test was used to compare the similarity matrix. The correlation coefficients were statistically significant for all marker systems. The highest correlation was found between similarity matrixes of RAPD and ISSR markers ($r = 0.82$) and the lowest between IRAP and ISSR markers ($r = 0.72$), but highly significant.

Constructed UPGMA dendrogram based on combined data of used four molecular markers distinguished studied populations in accordance with the results obtained by each marker and all individuals were clearly differentiated into two major clusters (Fig. 1). The first major cluster consisted

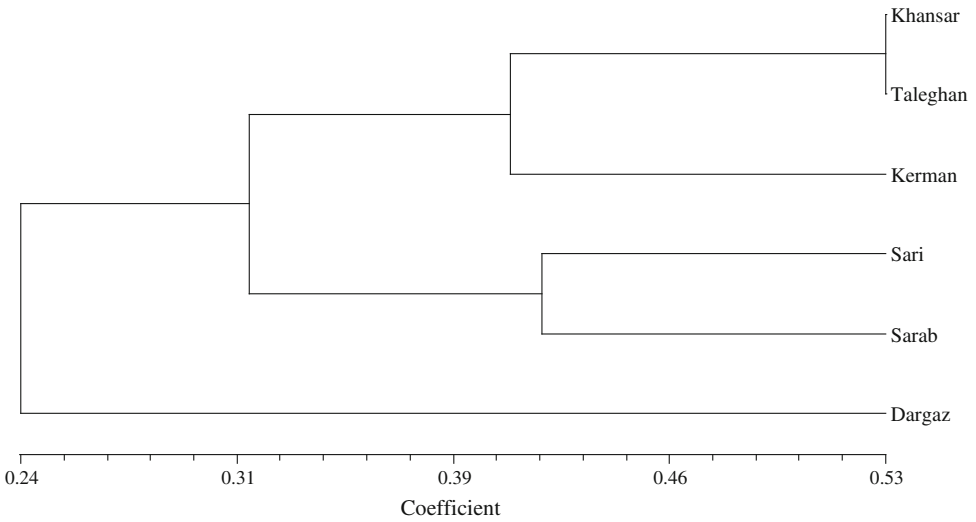

Fig. 2 UPGMA dendrogram for studied populations of *L. cardiaca* based on combined data using Jaccard's similarity

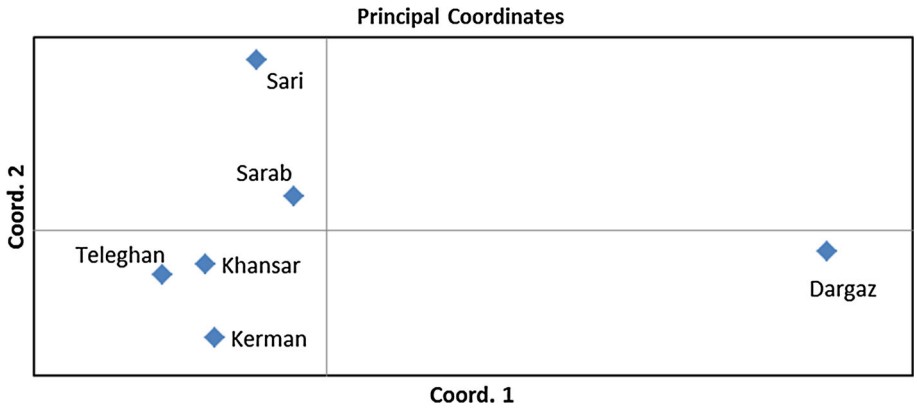

Fig. 3 Biplot of studied six populations of *L. cardiaca* based on the first two principal coordinates using combined data

of individuals of five populations including Khansar, Taleghan, Kerman, Sarab and Sari. This first major cluster was divided into two main subclusters. Subcluster I consisted of Khansar, Taleghan and Kerman populations constructing three subprinciple groups contained individuals of each population. Subcluster II contained Sarab and Sari populations constructing two subprinciple groups consisted of individuals of each population. The second major cluster contained individuals of Dargaz. Generally, the individuals were separated according to their populations and collection sites and were placed in the same subcluster. Thus, a population analysis was performed for more knowledge about interpopulation relationships. Population cluster analysis supported UPGMA dendrogram and distinguished populations accordingly (Fig. 2).

Also, genetic relationship among studied populations was also visualized by performing principal coordinate analysis (PCoA) based on combined data. The first two components accounted for 57.46 % of variation observed among studied populations. Two-dimensional plot generated from PCoA supported the clustering pattern of UPGMA dendrogram and revealed interpopulation relationships (Fig. 3).

Furthermore, AMOVA based on combined data of all used molecular markers (AFLP, RAPD, ISSR and IRAP) indicated high genetic variation among populations (55 %) and within populations (45 %) (Table 4). Thus, similarity matrix, cluster analysis and AMOVA indicated high genetic variation within individuals of each population.

Discussion

Analysis of genetic relationships in plant species is an important component of plant improvement. It helps to analyze genetic variability of populations and select parental materials for hybridization [14]. The advantage of combining different datasets is a comprehensive taxonomic picture, since each represents a distinct level of taxonomic differentiation and confirmed viewpoint of Sneller et al. [43]. In this study, AFLP, RAPD, ISSR and IRAP markers were used to analyze the genetic differences of *L. cardiaca*,

one of the main medicinal plants in Iran. We also demonstrated the usefulness of the different types of DNA markers for detecting different aspects of genetic variation in *L. cardiaca*. The individuals characterized in this study exhibited a great deal of molecular variation and they seem to have a rich gene pool for breeding programs. The comparative results showed that the four molecular methods detected abundant polymorphisms and they could be applied to identify the plant materials having close genetic relationships. The similar results were also proved in *Citrus* [3] and *Diospyros kaki* [52].

All used molecular markers detected a high PPB among the populations of *L. cardiaca*. These methods detect polymorphism by assaying subsets of the total amount of DNA sequence variation in a genome. In this study, IRAP, ISSR and RAPD markers were found to be more efficient than the AFLP, as they detected 90.85, 86.84 and 89.02 % polymorphism DNA markers, respectively, in *L. cardiaca* as compared with 58.23 % for AFLP. But, the mean R*p* values in AFLP, RAPD, ISSR and IRAP were 30.76, 4.62, 3.33 and 6.14 that was calculated to be more than 5–10 times higher in AFLP than in RAPD, ISSR and IRAP. This confirmed the better discrimination power of AFLP, reported earlier in other plant species [1, 23, 36, 38].

In the present study, high level of genetic variation was detected within and between populations by AFLP and IRAP, so that AFLP and IRAP revealed high intra-population variation (53 and 47 %, respectively) and high inter-population genetic diversity (47 and 53 %, respectively), in accordance with findings of Yu et al. [51] in study of *Leonurus japonicas* by AFLP. While RAPD and ISSR showed low intra-populations variability (30 and 39 %, respectively) and high inter-population diversity (70 and 61 %, respectively), agreeing with findings of Chen et al. [5] in study of *L. japonicus* by ISSR.

To estimate the genetic variation, H based on allele frequency, was selected as a parameter to assess genetic variations within populations for higher plants, when the dominant markers were used for population genetic studies [3, 23, 44]. In this study, the fingerprints corresponding to AFLP, RAPD, ISSR and IRAP markers revealed a high level of heterozygosity, indicating that *L. cardiaca* is predominantly an out-crossing species. We also used H to measure the genetic diversities within populations in conjunction with I, which is based on band phenotypes (i.e. band present/absent) without assumption of Hardy–Weinberg equilibrium. The values of H and I among the six populations were all higher than those within populations for four random markers in this study.

The evolution of varieties in distinct agro-climatic zones demonstrates significant levels of variation in response to the selection pressure in the zones [41]. The genetic differentiation based on four sets of AFLP, RAPD, ISSR and IRAP markers exhibited similar and strong correlations with geographical distance, and the population structure was similar for four types of markers. It has been observed in outcrossing trees such as wild apple [6] oaks [42], and certain other kinds of plant species [25].

The estimation of correlation between matrices revealed that AFLP, RAPD, ISSR and IRAP markers were highly significantly correlated. Researchers have reported the existence of correlation between different molecular marker techniques in various species [2–4, 27, 30, 36]. While, there are several studies on different plants with high correlation between molecular data [12, 15, 40, 48].

Results showed great genetic diversity in studied *L. cardiaca* germplasm and the superiority of all marker system in *L. cardiaca.* UPGMA dendrograms indicated that the Dargaz population is most divergent from the other populations with respect to four markers. The reason for this separation can be related to its geographical background, although it has distinctive morphological characteristics in compression to other populations such as plant, flower and leaf traits. Plants of this population have higher values for lateral inflorescence length, stem height, number of main stems and branches and plant density than others [45].

Conclusion

Genetic variation in our *L. cardiaca* germplasm was high and enough to support the conclusion that the selected material is genetically diverse for the *ex situ* gene conservation plantation. *Ex situ* conservation based on seed harvest from multiple sources must be carried out to capture most of the genetic variability existed among populations [11]. As an important traditional medicinal plant, promoting domestication and cultivation of this wild resource are necessary both to satisfy market demand and protect the wild resource. Successfully cultivation may decrease the harvest of wild populations of *L. cardiaca*, and contribute to the protection of this important medical plant. Finally, the identification and selection of populations that are genetically far distance from others can be used for performing breeding work, so that Dargaz population crosses with other populations can be an excellent option for producing hybrids with desired functionality.

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