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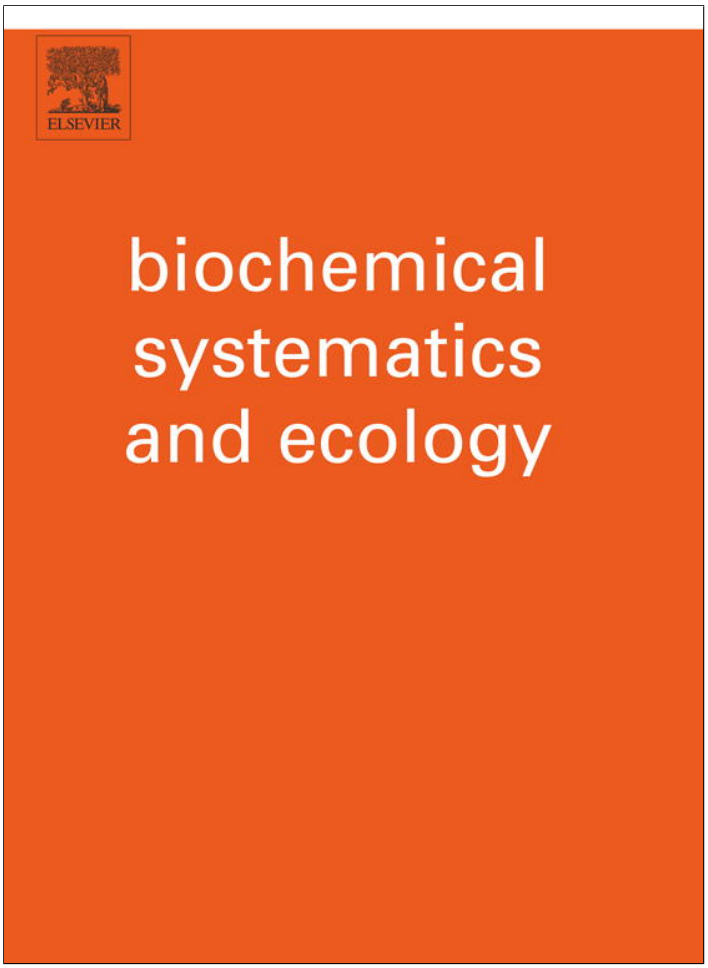

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DNA fingerprinting of *Leonurus cardiaca* L. germplasm in Iran using amplified fragment length polymorphism and inter-retrotransposon amplified polymorphism

Aboozar Soorni [a], Vahide Nazeri [a], Reza Fattahi [a], Abdollah Khadivi-Khub [b,*]

[a] Department of Horticultural Sciences, Faculty of Agriculture, University of Tehran, Karaj, Iran
[b] Department of Horticultural Sciences, Faculty of Agriculture and Natural Resources, Arak University, 38156-8-8349 Arak, Iran

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ABSTRACT

In the present study, the extent of inter and intra-population genetic variation was evaluated in *Leonurus cardiaca* accessions naturally growing in Iran by AFLP and IRAP markers. The fingerprints corresponding to AFLP and IRAP markers revealed high levels of heterozygosity, indicating that *L. cardiaca* is predominantly an out-crossing species. The average percentage polymorphism was detected as 58% and 90.8% on utilizing AFLP and IRAP data, respectively. Gene diversity values within populations varied 0.14 to 0.20 for AFLP and 0.12 to 0.21 for IRAP. The overall levels of genetic variation present in the *L. cardiaca* germplasm in Iran were finally determined by combining the AFLP and IRAP datasets to ensure wide genome coverage. The phenogram depicted that the accessions of Dargaz population were genetically distinct from other populations. Based on AFLP and IRAP analysis, it is concluded that *L. cardiaca* maintains high levels of genetic variation at inter and intra-population level.

1. Introduction

Motherwort (*Leonurus cardiac* L.) is an herbaceous perennial plant in the mint family, Lamiaceae that grows wild in Iran (Mozafarian, 1996). This plant was first described in medicinal literature in the 10th century as a remedy for healing nervous and functional cardiac disorders (Popescu et al., 2009). The names of the plant include the words that show connection with the heart. The English name 'motherwort' is indicative of the other uses of L. *cardiaca.* This herb contains the alkaloid leonurine which is a mild vasodilator and has a relaxing effect on smooth muscles. For this reason, it has long been used as a cardiac tonic, nervine, and an emmenagogue. Among other biochemical constituents, it also contains bitter iridoid glycosides, diterpinoids, flavonoids (including rutin and quercetin), tannins, volatile oils, and vitamin A. *L cardiaca* herbs synthesize flavonoids, alkaloids, iridoids, diterpenoids, cardenolids such as glycosides, tannins and other constituents in lower amounts (Papanov et al., 1998a, 1998b). Traditionally, it is used as a remedy for healing nervous and functional cardiac disorders (Milkowska-Leyck et al., 2002), and now for producing sedative, hypotensive and cardiotonic pharmacological effects components as a superior antispasmodic and nervine. *L. cardiaca* is used for healing cardiac diseases in Germany, France, Russia, Hungary, Bulgaria, Iran and some other countries (Mills and Bone, 2000). *L. cardiaca* is predominantly a womb remedy. A combination of relaxant and uterotonic effects induced by alkaloids (stahydrine, etc.) gives motherwort a useful role in facilitating childbirth. *L. cardiaca* is used to stimulate heart function, especially in conditions when the heart is weak (Mills and Bone, 2000).

* Corresponding author. Tel./fax: +98 8612762087.
E-mail addresses: akhadivi@ut.ac.ir, a-khadivi@araku.ac.ir (A. Khadivi-Khub).

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Since domestication process of medicinal plants is a time consuming process, selection of appropriate genotype can be shorten the process. Various methods have been employed to evaluate genetic diversity. In plants evaluation, genetic diversity is the basic step for breeding programs (Gichuki et al., 2003). Breeders are discovering genetic relationships among genotypes and related phenotypic information (Klocke et al., 2002). Iran has rich resources of motherwort germplasm (Mozafarian, 1996). Therefore, categorizing and characterizing of this germplasm is an essential step in the selection and breeding of motherwort populations. Thus, efficient use of genetic resources in plant breeding programs requires deeper knowledge about genetic diversity.

No studies have been carried out on the genetic diversity of *L. cardiaca* in Iran and we could not find any molecular report about this species, but Yu et al. (2009) and Chen et al. (2009) evaluated genetic variation of related species, *Leonurus japonicus* in China by AFLP (amplified fragment length polymorphism) and ISSR (inter-simple sequence repeat) markers, respectively. In present study, AFLP and IRAP (inter-retrotransposon amplified polymorphism) analyses were used to evaluate the genetic diversity of *L. cardiaca* populations, with the aim of using them in breeding programs as well as for conservation management of this germplasm in Iran.

2. Materials and method

2.1. Plant material and genomic DNA extraction

Samples of motherwort (*Leonurus cardiaca* L.) were collected from six natural region of Iran (Fig. 1). The distance between sampled individuals of each population was at least 250 m. A total of 47 accessions of six populations were evaluated in this

Fig. 1. Pictures of *L. cardiaca* collected from different regions of Iran.

work. The passport information of the studied accessions is shown in Table 1. Genomic DNA was extracted from young leaves, following the method described by Pirttilä et al. (2001).

2.2. AFLP evaluation

We essentially followed the AFLP protocol developed by Vos et al. (1995) with minor modifications. DNA was digested with *EcoRI* and *MseI* restriction enzymes. Digestion was performed for 4 h at 37 °C in a final volume of 17.5 μl containing 10 mM Tris-Ac, 10 mM MgAc, 50 mM KAc, 5 mM DTT, 25 U *EcoRI* (Pharmacia), 4U *MseI* (New England Biolabs) and 250 ng of genomic DNA. Two linkers, one for the *EcoRI* sticky ends and the other for the *MseI* sticky ends, were ligated for 3 h at 37 °C to the digested DNA by adding 2.5 μl of a mix containing 2.5 pmol *EcoRI* linker, 25 pmol MseI linker, 8 mM ATP, 10 mM Tris-HAc, 10 mM MgAc, 50 mM KAc, 5 mM DTT and 0.85 U T4 DNA ligase (Pharmacia). This ligation product was diluted five-fold. A first preselective PCR amplification was performed using EcoRI + A and MseI + C primers in a 50 ml mix of 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 30 ng of each primer, 1U *Taq* DNA polymerase (GibcoBRL) and 5 μl of diluted ligation product. The reaction was carried out in a thermocycler (iCycler, Bio Rad Co., USA) and the samples were subjected to 28 amplification cycles with three steps of 30 s at 94 °C, 60 s at 56 °C and 60 s at 72 °C. The preamplification products were diluted 10-fold and used as starting material for the selective radioactive amplification. For selective amplification, *EcoRI* and *MseI* primers with three and two additional nucleotides, respectively, were used (Table 2), the first one being ^{33}P-labeled using T4 polynucleotide kinase. The PCR reaction was performed in a 20 μl volume of 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 5 ng [^{33}P] *EcoRI* primer, 30 ng MseI primer, 1 U Taq DNA polymerase (GibcoBRL) and 5 μl of diluted preamplified DNA. The selective amplification was carried out using the following cycling parameters: 11 cycles of 30 s at 94 °C, 30 s at 65 °C, 60 s at 72 °C, in which the annealing temperature was lowered by 0.7 °C per cycle, followed by 24 cycles of 30 s at 94 °C, 30 s at 56 °C and 60 s at 72 °C. The PCR products, in which an equal volume of load buffer (98% formamide, 10 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol) had been added, were denatured 5 min at 94 °C and immediately placed on ice. In all, 2 μl of each sample was loaded on a polyacrylamide gel (4.5% acrylamide/bisacrylamide 20:1, 7.5 M Urea and 0.50 TBE) and were run at 95 W for 2 h. After electrophoresis, gels were dried on a standard slab gel drier for 2 h and exposed for 5 days to an X-ray film. Each analysis was performed twice for each individual. Only intense, polymorphic and reproducible bands were taken into account to generate a binary matrix 0–1, where 1 and 0 denote, respectively, the presence and the absence of a band.

2.3. IRAP evaluation

Twenty-eight IRAP primers were tested for formal amplification. IRAP amplification was performed in thin-walled microcentrifuge tubes using thermocycler (iCycler, Bio Rad Co., USA). Reaction volume of 20 μL was composed of 2 μL 10× buffer (100 mmol L^{-1} Tris–HCl pH 9, 500 mmol L^{-1} KCl, 20 mmol L^{-1} $MgCl_2$ and 0.1% Triton X-100), 1 unit *Taq* polymerase, 200 μmol L^{-1} of each dNTP, 30 nmol L^{-1} primer and 20 ng DNA template. Each reaction mixture was overlaid with 10 μL mineral oil. A negative control reaction, in which DNA template was omitted, was included in every PCR run in order to ensure that no self-amplification or DNA contamination occurred. The amplification program was as follows: 4 min at 94 °C, 1 min at 36 °C and 2 min at 72 °C for one cycle; 1 min at 94 °C, 1 min at 36 °C and 2 min at 72 °C for 40 cycles; and 1 min at 94 °C, 1 min at 36 °C and 10 min at 72 °C for one cycle. Amplified PCR products were electrophoresed through 1.5% agarose (Roche Co., Germany) stained with ethidium bromide in TBE buffer and photographed under UV light, by a Gel Doc system (UVP, Bio Doc Co., USA).

2.4. Data analysis

The level of genetic distance (GD) between pair of genotypes was estimated using pairwise comparison (Nei and Li, 1979). Each AFLP and IRAP considered as a character and the presence or absence of the allele was scored in binary code (present = 1, absent = 0). Dendrograms were constructed using UPGMA (unweighted pair group method based on arithmetic average) based on the similarities between genotypes estimated by Dice's coefficient (Nei and Li, 1979). The representativeness of the dendrogram was evaluated by estimating cophenetic correlation for the dendrogram and comparing it with the similarity matrix by Mantel's matrix correspondence test (Mantel, 1967) using MxComp module in NTSYS-pc ver 2.1 (Rohlf, 2000). The

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

Population no.	Location	Sample size	Province	Altitude (m)	Longitude (E)	Latitude (N)
1	Kerman	7	Kerman	2600	E56° 50′ 31/49″	N29°18′ 36/71″
2	Dargaz	8	South Khorasan	2194	E58° 42′ 1/47″	N37° 34′ 42/91″
3	Taleghan	8	Alborz	1850	E50° 45′ 34/51″	N36°10′ 27/80″
4	Khansar	8	Isfehan	2210	E50° 17′ 57/65″	N33° 15′ 50/63″
5	Sarab	8	Ardabil	1687	E47° 31′ 40/24″	N37°55′ 59/28″
6	Sari	8	Mazandaran	2170	E53° 12′ 3/58″	N 36° 3′ 37/78″

Table 2
AFLP primers, total number of bands, polymorphic bands and the average percentage of polymorphic bands in 47 *L. cardiaca* accessions.

AFLP primers	Sequence	Total bands	Polymorphic bands	Polymorphism (%)	RP value	PIC	MI
C1	E ACA M CAA	94	49	52.1	29.1	0.30	7.66
C2	E ACA M CAC	100	68	68	38.7	0.31	14.33
C3	E AAG M CAC	80	36	45	17.5	0.26	4.21
C4	E AGC M CAT	126	72	57.1	34.5	0.26	10.69
C5	E AAG M CAG	99	69	69.7	32.38	023	11.06
C6	E ACT M CTG	108	62	57.1	32.38	0.27	9.61
Total		608	355	–	–	–	–
Average		101.1	59.1	58	30.8	0.27	9.54

RP: Resolving power.
PIC: Polymorphic information content.
MI: Marker Index.

result of this test (r) is a cophenetic correlation coefficient (CCC) indicating how well the dendrogram represents similarity data. Bootstrap analysis with 1000 replicates was performed using FreeTree software (version 0.9.1.50, Pavlicek et al., 1999) to obtain the confidence of branches of the UPGMA tree and validation of the dendrogram. Trees were viewed using Tree View program, version 1.6.6 (Page, 1996). The bootstrap analysis estimates the probability values (P values) for each cluster (the probability of a true cluster at each edge of the dendrogram). Ability of the primers to differentiate between the accessions was assessed by calculating their resolving power (Rp), according to Prevost and Wilkinson (1999) using the formula $R_p = \sum I_b$, where $I_b = 1 - (2 \times |0.5 - p|)$, and p is the proportion of the 47 accessions containing the I band. Polymorphic information content (PIC) and marker index (MI) were calculated using formulas described by Powell et al. (1996). Population genetic parameters were estimated using POPGENE (V. 1.31) (Yeh et al., 1999) to determine Nei's genetic diversity (H) and Shannon's information index (I) among populations. AMOVA was calculated within and among the six populations studied.

3. Result

3.1. AFLP analysis

The level of polymorphism of the six AFLP primers studied was investigated in the 47 *Leonurus cardiaca* accessions. The parameters of variability analyzed are presented in Table 2. A total of 608 amplification fragments were detected, with an average of 101.33 fragments/AFLP and fragment sizes that ranged from 80 to 126 with size of 50–500 bp (Fig. 2). These results agreed with finding of others reported high potential for AFLP (Russi et al., 2009; Rahimmalek et al., 2009). In present study, 356 bands were polymorphic, with an average polymorphism of 58% and 59.1 bands per primer that were higher than study of Russi et al. (2009) and Rahimmalek et al. (2009) in *Echinacea* and *Achillea*, respectively. This high diversity in this study may be related to studied accessions, different regions and used primers. This result confirmed that the AFLP technique is effective, economical and combines the reliability of restriction fragment length polymorphism (RFLP) and the power of PCR (Vos et al., 1995). The number of amplified bands ranged from 80 (for C3 primer) to 126 (for C4 primer). C4 primer showed the highest 72 polymorphic bands with 57.14% polymorphism. Primers resolving power (Rp) was varied from 17.5 (for C2 primer) to 38.7 (for C3 primer) with an average of 30.8. This showed that C3 primer contain high genomic information than other primers (Milbourne et al., 1997).

The pair-wise genetic similarity computed based on the proportion of shared fragments ranged from 0.06 to 0.70 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 accessions. 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,

Table 3
List of the IRAP primers used in the study and Level of polymorphism and informativeness obtained in 47 accessions of *L. cardiaca*.

IRAP primers	Total bands	Polymorphic bands	Polymorphism (%)	RP value	PIC	MI
3′LTR-3′LTR	16	15	93.7	8.4	0.93	13.1
3′LTR-LTR6150	11	10	90.9	4.3	0.62	5.6
LTR6150-Sukkula	16	14	87.5	6.8	0.82	10.7
Sukkula-Sukkula	18	16	88.8	7.9	0.89	12.7
Sukkula-Nikita	15	12	80	4.4	0.91	8.7
Sukkula-3′LTR	14	12	85.7	6.2	0.93	9.5
5′LTR2-Sukkula	12	12	100	2.9	0.97	11.7
LTR6150-LTR6150	14	14	100	8.2	0.91	12.7
Average	14.5	13.1	90.8	–	0.88	10.6
Total	116	105				

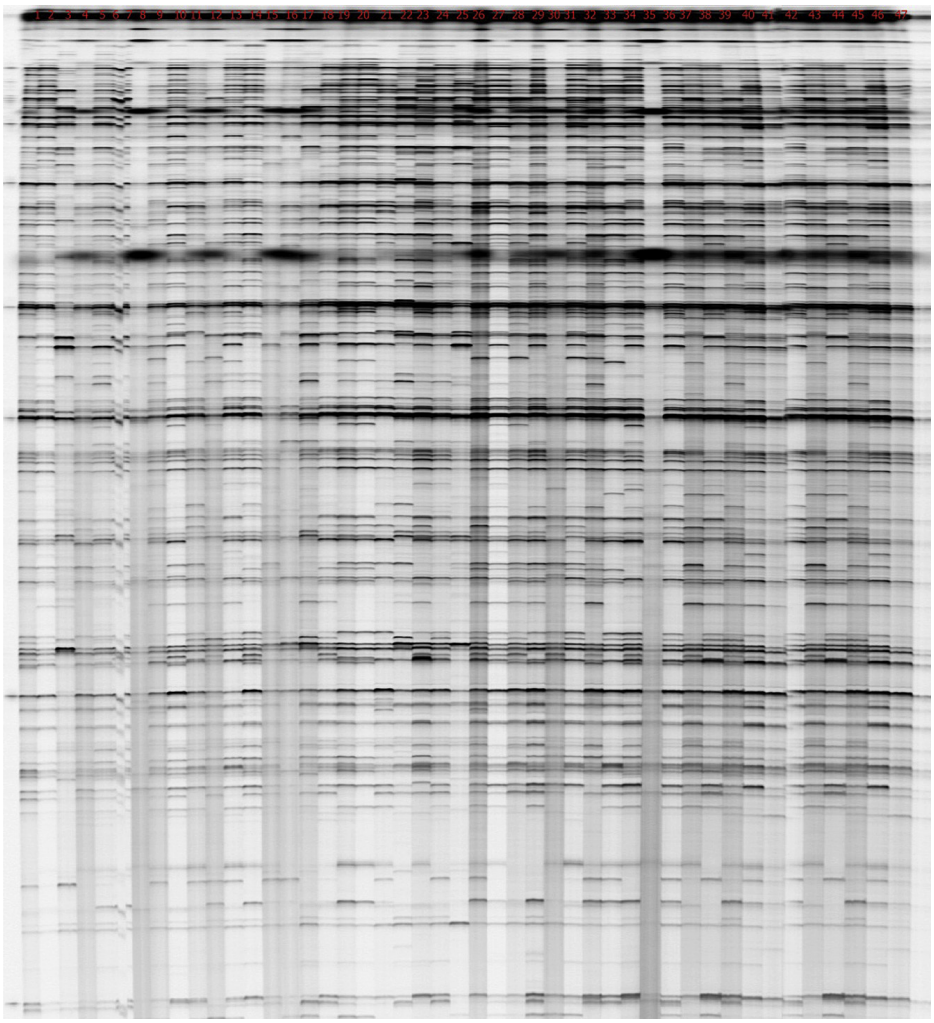

Fig. 2. AFLP profile of the 47 accessions of *L. cardiaca*.

potentially interbreeding species, where there is a high probability of reticulate evolution occurring. With the use of appropriate statistical tools (Yeh and Boyle, 1997), AFLPs can also be used to analyze genetic structure and differentiation within and among species and populations.

The UPGMA cluster analysis revealed genetic relationships among and within populations. The cophenetic correlation coefficient (CCC) indicated high correlation ($r = 0.93$) between the similarity matrix and the cophenetic matrix (obtained from the UPGMA dendrogram), indicating a 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 (Romesburg, 1990). The dendrogram clearly and effectively differentiated all accessions into two major clusters (Fig. 3), so that the bootstrap values strongly support (100%) these two clusters. The first major cluster included accessions of five population included Khansar, Taleghan, Kerman, Sarab and Sari. This first major cluster that is supported in most cases with significant bootstrapping values of higher than 55%, was divided into two main subclusters so that subcluster I consisted of Khansar, Taleghan, Kerman, Sarab populations constructing four subprinciple groups. Subcluster II contained accessions of Sari population. The second major cluster contained accessions of Dargaz population with 100% bootstrapping value. Generally, the accessions were separated according to their populations and collection sites and were placed in same subcluster. Thus, both similarity matrix and cluster analysis indicated high genetic variation within accessions of each population belonging to the different geographic sites.

Genetic variation within populations using Shannon's Information index (I) and Nei's gene diversity index (H) showed the highest genetic diversity within population Sarab ($H = 0.20$, $I = 0.30$) and the lowest in population of Taleghan ($H = 0.14$, $I = 0.21$) (Table 4). 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% so that Dargaz population showed the most variable intra-population diversity, followed by Sari (Table 5). Also the highest genetic distance among population was observed between these two populations (0.29), may indicate a narrow inter-population genetic diversity.

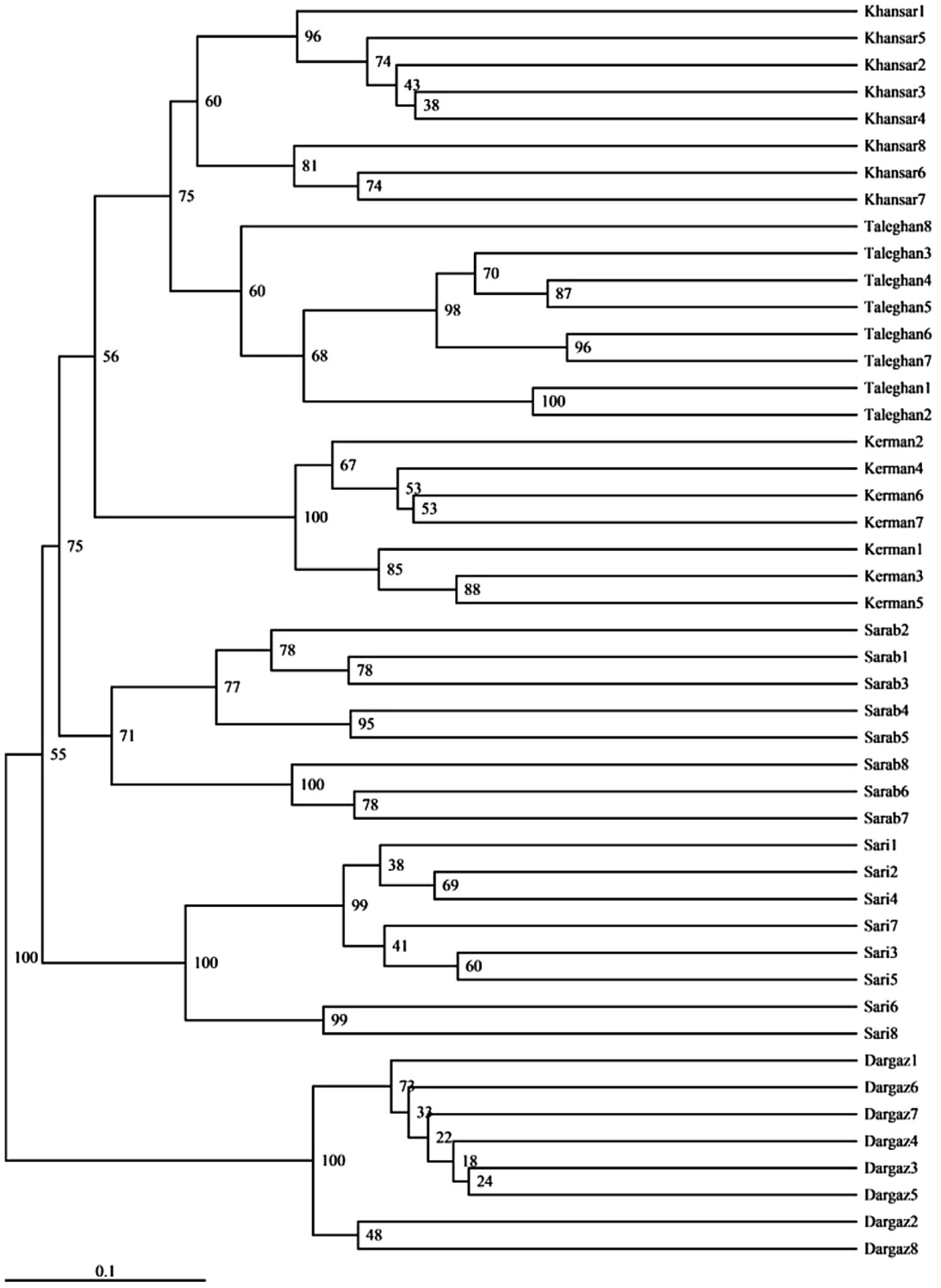

Fig. 3. Dendrogram of 47 studied accessions of *L. cardiaca* generated by the UPGMA method based on AFLP.

3.2. IRAP analysis

From prescreening assays with two *L. cardiaca* accessions using 28 IRAP primers, eight primers generated bright polymorphic amplification products and were used in further analysis. The results of IRAP fingerprinting of 47 studied accessions using eight primers are given in Table 3. These eight primers amplified polymorphic products and showed polymorphism in the all accessions and the total number of bands detected among them was 116 that the number of bands varied from 11 (3′LTR-LTR6150) to 18 (Sukkula-Sukkula) with an average of 14.5 bands per primer with size of 200–800 bp (Fig. 4). The total

Table 4
Genetic parameters of the six populations of *L. cardiaca* based on AFLP and IRAP data.

Population	AFLP			IRAP		
	PBB (%)	I	H	PBB (%)	I	H
Kerman	44.38	0.25	0.17	39.05	0.24	0.16
Dargaz	49.16	0.28	0.19	27.62	0.17	0.12
Taleghan	42.98	0.21	0.14	52.38	0.31	0.21
Khansar	48.72	0.24	0.16	42.86	0.21	0.14
Sarab	64.89	0.30	0.20	35.24	0.21	0.14
Sari	57.58	0.29	0.19	33.33	0.18	0.12

H: Nei's gene diversity index.
I: Shannon diversity index.
PPB: The percentage of polymorphic loci.

Table 5
Results of the AMOVA for six populations of *L. cardiaca*.

Source	AFLP				IRAP			
	df	SS	MS	Variation (%)	df	SS	MS	Variation (%)
Among pops	5	1353.305	270.661	43	5	443.911	88.782	53
Within pops	41	1611.929	39.315	57	41	367.536	8.964	47
Total	46	2965.234		100	46	811.447		100

of separable bands were 105 and ranged from 10 (3′LTR- LTR6150) to 16 (Sukkula-Sukkula) with an average of 13.1 per primer. In present study, the observed average polymorphism was 90.8% that two primers, LTR6150-LTR6150 and 5′LTR2-Sukkula, showed 100% polymorphism. In the germplasm examined, the mean PIC value of the polymorphic bands was 0.88, ranging from 0.62 (3′LTR-LTR6150) to 0.97 (5′LTR2-Sukkula primer), and the mean value for MI was 10.6 ranged from 5.6 (3′LTR-LTR6150) to 13.1 (3′LTR-3′LTR). Seven of eight primer pairs had a PIC value higher than 0.87. Because of the high polymorphic value of the studied band, we were able to distinguish genetic variation among and between populations.

Several populations showed the minimum value of similarity. The maximum genetic similarity between accessions of each population based on polymorphic bands was 0.71, between accessions of Sari population, while, minimum genetic similarity was observed between accessions of Taleghan population. Also the highest genetic similarity among populations was between Taleghan and Khansar and the lowest among Dargaz with Taleghan and Sari.

Based on the results obtained with the IRAP markers, a similarity matrix was used to generate a UPGMA dendrogram (Fig. 5). The cophenetic correlation coefficient between the original similarity matrix and the cophenetic matrix derived from the UPGMA dendrogram was high (0.91), indicating a good fit between the dendrogram and the similarity matrix. UPGMA dendrogram showed two main clusters included cluster I (Khansar, Taleghan, Kerman, Sarab and Sari populations) and cluster II (Dargaz population) with 100% bootstrap. Cluster I was divided to two subclusters with 97% bootstrap. Furthermore, the accessions were separated according to their populations and collection sites and were placed in same subcluster.

Genetic variation within populations using Shannon's Information index (I) and Nei's gene diversity index (H) showed the highest genetic diversity within population Taleghan ($H = 0.21$, $I = 0.31$) and the lowest in population of Dargaz ($H = 0.12$, $I = 0.17$) (Table 4). Analysis of molecular variance (AMOVA) showed that the genetic variation was found mainly among populations (53%), but variance within populations was only 47% (Table 5).

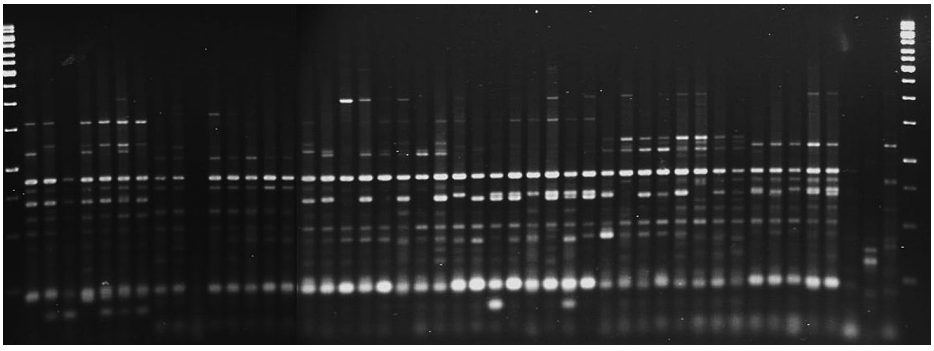

Fig. 4. The profile of amplification by IRAP in 47 accessions of *L. cardiaca*.

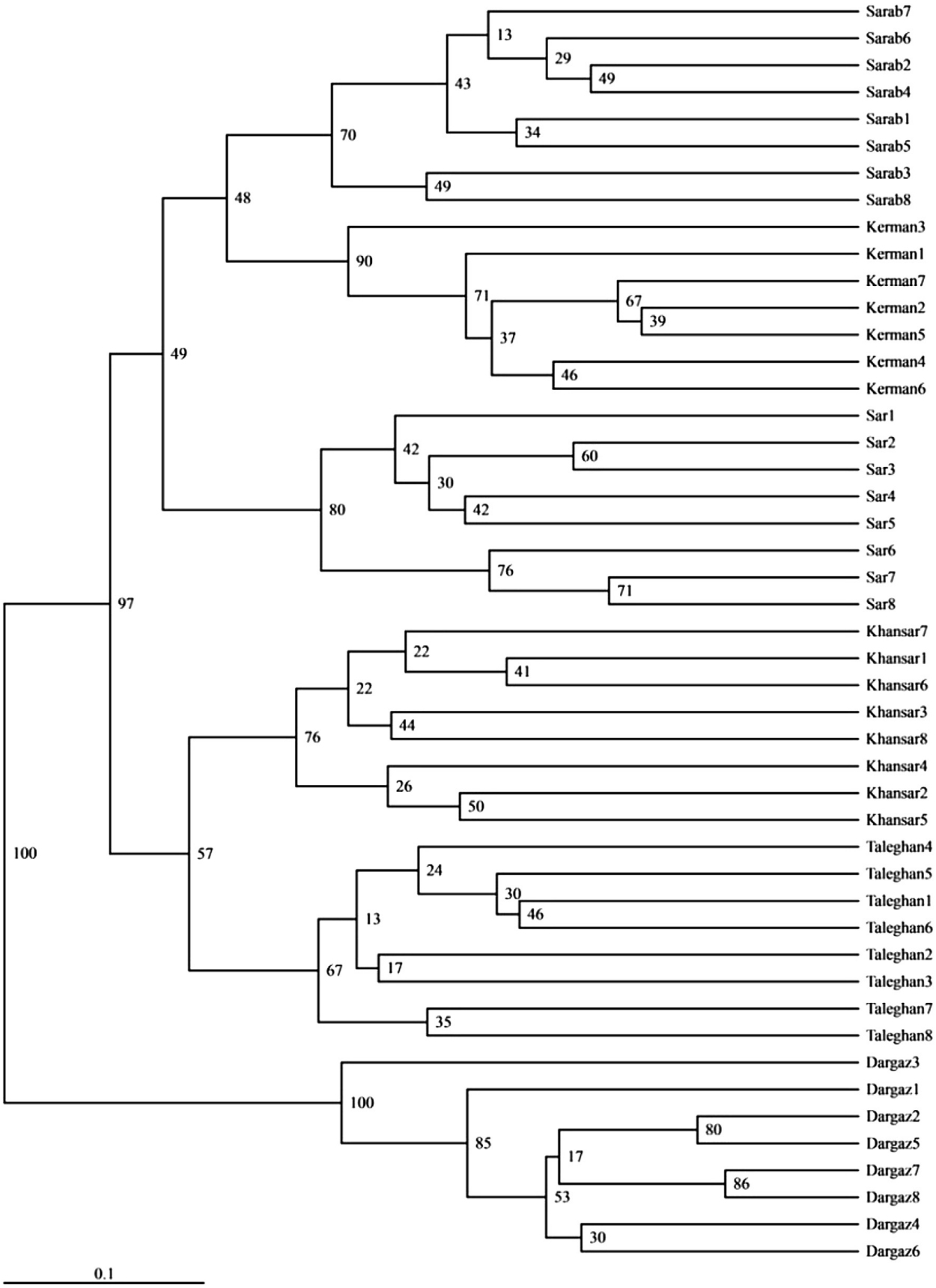

Fig. 5. Diagrams showing the genetic relationships among 47 accessions of *L. cardiaca* generated by the UPGMA methods based on IRAP data.

4. Discussion

The main reason for studying *L. cardiaca* in this research was that although variation of this plant is high in Iran, but there were not enough available data about it. The present study revealed a relatively high level of genetic diversity in *L. cardiaca* based on AFLP and IRAP markers. Totally, 356 and 105 polymorphic bands with 58% and 90.8% polymorphism were detected by AFLP and IRAP in studied species, respectively and also Nei's gene diversity index (H) values within populations varied 0.14 to 0.20 for AFLP and 0.12 to 0.21 for IRAP. While Yu et al. (2009) reported 65 polymorphic bands among accessions of *Leonurus japonicus* by The AFLP. Also, Chen et al. (2009) reported 117 polymorphic bands among *Leonurus japonicus* populations by ISSR marker, confirming the presence of wide genetic variability among our germplasm.

Clustering based on both AFLP and IRAP markers divided accessions in two main clusters, so that the data of these two markers was the same and it seems that these two molecular markers could separate accessions at the same genetic level. UPGMA dendrograms indicated that the Dargaz population is most divergent from the other populations with respect to molecular data. The reason for this separation can be its geographical background, although it has distinctive morphological characteristics in compression other populations such as lateral inflorescence length, stem height, number of main stems and branches and plant density that was higher than others in value (data not shown). For those populations with high levels of genetic variation of different regions, we suggest that their habitats should be protected and the exploitation of wild resources be forbidden. The dendrogram obtained from each dataset indicated that there are close relationships between Khansar and Taleghan populations. Regional climate of Khansar population and possibly exchanging vegetative material makes has been very similarity of with the Taleghan population. Away Kerman population from Sarab population and different climatic conditions the amount of gene exchange between these two populations had reduced, and cause long distance of these two populations has been genetically.

The estimation of correlation between matrices of AFLP and IRAP data ($r = 0.74$) indicated a suitable correlation between these data. AFLP and IRAP markers cover specific regions of the nuclear genome and investigate polymorphism in these specific regions. Therefore, it can be noted that recorded regions by AFLP could have been segregated by IRAP and the similar degrees of among-population differentiation observed that the information about genetic relatedness revealed by AFLP markers has direct correspondence with relatedness information detected IRAP. There are several studies on different plants with good correlation between molecular data (Landry et al., 1994; Shimada et al., 1999; Horvath et al., 2008). Dendrogram based on integrating data of these two molecular markers (AFLP and IRAP) distinguished studied populations in accordance with the results obtained in each. The advantage of these combining different datasets was a comprehensive taxonomic picture, since each represents a distinct level of taxonomic differentiation and confirmed viewpoint of Sneller et al. (1997) in this case.

Finally, because of transferring and polymorphism of most AFLP and IRAP markers, authors suggest that these AFLP and IRAP primers to be sufficient for in *L. cardiaca* populations and related species in most cases. Also, these results will be an important asset to future breeding and operational approaches (for example, evaluation of controlled crosses, establishment and assessment of a seed orchard, clonal propagation and dissemination).

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