Analysis of genetic diversity between and within Iranian accessions of spinach (*Spinacia oleraceae* L.) by SRAP markers

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ABSTRACT

Spinach (*Spinacia oleracea* L.) is an economically important leafy vegetable crop in many countries. This is the first case study of using SRAP markers to analyze genetic diversity of Iranian spinach accessions. Eight SRAP primer combinations generated 88 scorable bands ranging from 50 to 1000 bp, among which 73 were polymorphic, with an average of 82.9 polymorphic bands per primer combination and average polymorphism information content (PIC) of 0.35. Based on the analysis of SRAP data, a similarity matrix was calculated according to the Dice coefficient. Similarity dendrogram was constructed using UPGMA algoritm. Cophenetic correlation between ultrametric similarities of tree and similarity matrix was found to be high (r=0.85, P < 0.0001), suggesting that the cluster analysis strongly represents the dissimilarity matrix. According to the UPGMA dendrogram, the spinach accessions clustered into two groups. This preliminary study demonstrated that SRAP markers are effective for evaluating genetic diversity between and within spinach accessions.

***Keywords*:** Genetic diversity, *Spinacia oleraceae,* Spinach, SRAP.

INTRODUCTION

The cultivated spinach (*Spinacia oleracea* L.) belongs to the family Chenopodiaceae. It is widely grown as a leafy vegetable both for fresh consumption and for industrial processing in many countries. Spinach is an excellent source of vitamin B and C in the human diet (Siemonsma and Piluek, 1995). It is a cool seasonal vegetable as an important source of minerals that produces a rosette during the vegetative stage (Kansal *et al*. 1981). Genetically, spinach is diploid with 2n =2x=12 chromosomes. It is a dioecious species with separate male and female plants and occasional monoecious plants with both male and female flowers (Ellis and Janick, 1960). The production of spinach has been reported more than 105 thousand tons in Iran in 2013 (Anonymous, 2013).

Despite a substantial progress in the genetic improvement of spinach, genetic variability of the germplasm at the molecular level has not been investigated. Molecular markers are useful for population genetic studies to assess the influence of various factors on genetic diversity and population structure (Liu *et al*. 2006). Among the many types of molecular markers, sequence-related amplified polymorphism (SRAP) has been demonstrated to be a useful tool for population genetic studies (Li and Quiros, 2001; Ferriol *et al*. 2003;

Esposito *et al*. 2007). Ferriol *et al*. (2003) reported that the information given by SRAP markers was more concordant with the morphological variability and the evolutionary history of the morphotypes than the AFLP (Amplified Fragment Length Polymorphism) markers. This may be because the SRAP preferentially amplifies ORFs, which is significant for gene and genetic diversity. The SRAP marker system has been adapted for a variety of purposes in different crops, including map construction, gene tagging and genetic diversity studies (Agarwal *et al*. 2008).

Simple sequence repeats (SSR) (Khattak *et al*. 2007) and target region amplification polymorphism (TRAP) markers (Hu *et al*. 2007) have been used for molecular characterization of spinach accessions. Most of the results obtained by previous studies show that spinach has a high level of variation. The objective of this study was to evaluate the genetic diversity between and within population of *S. oleraceae* grown in Iran. The implications of our study can be useful in future breeding programs of this plant species.

MATERIALS AND METHODS

Plant materials

The fresh seeds of spinach were collected from several provinces of Iran and planted in the greenhouse at Tarbiat Modares University, Tehran. The number, names and the geographic origin of accessions are listed in Table 1. In each of the 10 accessions, 10 individuals were collected randomly with about 5 g of young and clean leaves per plant. In total, 100 samples were collected and immediately placed in sealed plastic bags containing silica gel beads which speeded up the drying process.

DNA extraction

Total genomic DNA of samples was extracted according to the CTAB method (Uzun *et al*. 2009). Quantities of DNA samples were determined using a Nanodrop (1000 Spectrophotometer). Qualitative evaluations of DNA were checked on the agarose gel and the 260 to 280 nm ratio as well. Genomic DNA samples were diluted to a final concentration of 20 ng/µl with 1× TE (10 mM Tris-HCl, 0.1 mM EDTA, pH=8.0) buffer and stored at -20ºC prior to the polymerase chain reaction (PCR) amplification.

SRAP analysis

SRAP analysis was carried out based on described by Li and Quiros (2001) with minor modifications. PCR reactions were performed in a final volume of 20 µl containing 0.2 mM dNTPs, 1× PCR buffer, 0.05 U Taq DNA polymerase, 0.33 µM forward and reverse primers, 45 ng templates DNA and dH_2O up to final volume. The PCR program was as the following: 3 min at 95°C, 5 cycles of 94°C for 1 min, 35°C for 1 min, 72°C for 1 min, 35 cycles of 94°C for 1 min, 47°C for 1 min, 72°C for 1 min, and a final extension of 3 min at 72°C. The PCR products were separated on a 10% polyacrylamide gel.

Table 1. Geographic origin of spinach accessions used in this study.

Locality	Latitude	Longitude	No. of samples
Ardebil	37° 06′	47° 17′	10 (1 to 10)
Khorasan	37° 23′	57° 54′	10 (11 to 20)
Fars	29° 37′	52° 32′	10 (21 to 30)
Hamedan	34° 48′	48° 31′	10 (31 to 40)
Yazd	31° 53′	54° 21′	10 (41 to 50)
Tehran	35° 19′	51° 39′	10 (51 to 60)
Esfahan	32° 38′	51° 40′	10 (61 to 70)
Lorestan	33° 29′	48° 21′	10 (71 to 80)
Zabol	31° 01′	61° 29′	10 (81 to 90)
Zanjan	36° 40′	48° 28′	10 (91 to 100)

Data analysis

Each band was scored as present (1) or absent (0) and pairwise dissimilarity of genotypes with different dissimilarity functions was calculated by DARwin 5 (Dissimilarity Analysis and Representation for Windows) software Package Version 5.0.158 (Perrier *et al*. 2003). GenAlex software (version 6.4; Peakall and Smouse 2006) was used to calculate dissimilarity matrix and cluster analysis. Percentages of polymorphic loci (P), observed number of alleles (Ao), effective number of alleles (Ae) and gene diversity (He) were analyzed. GenAlex software was used to estimate the relative genetic differentiation (Gst) between populations.

The dissimilarity matrix was used to construct a dendrogram using the unweighed pair group method arithmetic average (UPGMA) algorithm to determine genetic relationships among the accessions studied. The dendrograms were evaluated by estimating cophenetic correlation for the dendrogram and comparing it with the similarity matrix, using Mantel's matrix correspondence test (Mantel, 1967). MEGA 5.05 software (Tamura *et al*. 2011) was used to analyze the unweighed pair group method arithmetic average (UPGMA) on the

Table 2. Some information on amplified fragments of SRAP primers used in this study.

Primers	Total fragments	Polymorphic bands	Polymorphic level (%)	PIC
Me1-Em1	9	8	88.88	0.33
Me1-Em5	8	6	75.00	0.34
Me2-Em3	12	9	75.00	0.29
Me3-Em2	9	7	77.77	0.40
Me4-Em3	9	6	66.66	0.43
Me5-Em4	10	9	90.00	0.27
Me4-Em2	14	12	77.85	0.37
Me5-Em3	17	16	94.11	0.32
Total	88	73	Mean (82.9)	Mean (0.35)

10 spinach accessions.

For each amplified fragment in a primer combination, the frequencies of presence and absence of bands were calculated with Excel software according to Smith *et al*. (1997), and polymorphism information content (PIC) values were obtained using $PIC=1-\Sigma_{i=1}^{n} f_i^2$ function,

where f_i^2 is the frequency of the i^{th} allele. The average PIC values for all fragments in a primer combination was calculated by this function and represented as PIC value of the primers. PIC indicates an estimate of the discriminatory power of a locus by taking into account, not only the number of alleles that are expressed but also the relative frequencies of those alleles.

RESULTS

SRAP analysis

Eighty-eight fragments were obtained from eight SRAP primer combinations ranging from 50 to 1000 bp, and were used for the analysis. The number of bands per primer ranged from 8 to 17 with a mean value of 11. The maximum number of fragments and also the highest level of polymorphism were found in Me5-Em3. Ratio of polymorphic SRAP markers was 82.9%. The PIC values for the eight primer combinations ranged from 0.27 (Me5-Em4) to 0.43 (Me4-Em3), with a mean of 0.35 (Table 2).

Genetic diversity and differentiation

Genetic indices for 10 accessions of spinach based on SRAP markers are summarized in Table 3 (10 individuals per accession). The percentage of polymorphic bands (PPB), observed number of alleles (Ao), effective number of alleles (Ae), Nei's gene diversity (He) and Shannon's information (Ho) were 73.56, 1.54, 1.47, 0.26 and 0.28, respectively. Genetic diversity varied among 10 accessions with PPB values ranging from 63.01% in Ardebil and Esfahan accessions to 83.56% in Yazd accession with an average of 73.56%. Four indices of Ao, Ae, He and Ho also indicated that Yazd accession had the greatest variation (1.72, 1.53, 0.30, and 0.45, respectively), while Ardebil and Esfahan accessions showed the least variation. The genetic variations of other accessions were observed between Ardebil and Yazd accessions (Table 3).

Table 3. Population genetic parameters for 10 accessions of spinach based on SRAP markers.

Accessions	PPB (%)	Ao	Ae	He	Ho
Ardebil	63.01	1.41	1.40	0.23	0.34
Khorasan	75.34	1.56	1.49	0.28	0.41
Fars	76.71	1.56	1.47	0.27	0.40
Hamedan	76.71	1.60	1.46	0.26	0.39
Yazd	83.56	1.72	1.53	0.30	0.45
Tehran	75.34	1.54	1.51	0.28	0.42
Esfahan	63.01	1.42	1.40	0.22	0.33
Lorestan	72.6	1.54	1.43	0.25	0.37
Zabol	73.97	1.56	1.47	0.27	0.40
Zanjan	75.34	1.54	1.50	0.27	0.41
Mean	73.56	1.54	1.470	0.26	0.28

Note: (PPB) Percentage of polymorphic band; (Ao) Observed number of alleles; (Ae) Effective number of alleles; (He) Nei's gene diversity; (Ho) Shannon's Information index.

Based on the analysis by GenAlex software on the genetic differentiation of spinach in 10 accessions, the major genetic variation originated from intra accessions

Table 4. Analysis of molecular variation of different *Spinacia oleracea* L. accessions.

Source of variation	DF	SSD	MSD	Absolute proportion	Proportion %
Accession	9	335.68	37.298	2.702	21
Individual	90	924.6	10.273	10.273	79
Total	99	1260.28		12.976	100

Table 5. Nei genetic similarity matrix among pair-wise comparisons for spinach accessions based on SRAP markers.

Accessions	A	K	F	H	Y	T	E	L	Zb	Zj
Ardebil (A)	1.00									
Khorasan-shirvan (K)	0.93	1.00								
Fars-Zarghan (F)	0.91	0.94	1.00							
Hamedan (H)	0.88	0.94	0.93	1.00						
Yazd (Y)	0.90	0.92	0.93	0.92	1.00					
Tehran-Varamin (T)	0.81	0.87	0.87	0.88	0.87	1.00				
Esfehan-Rahnan (E)	0.78	0.85	0.81	0.86	0.83	0.93	1.00			
Lorestan-Khoramabad (L)	0.77	0.87	0.83	0.87	0.83	0.92	0.95	1.00		
Zabol (Zb)	0.83	0.89	0.84	0.88	0.85	0.92	0.93	0.93	1.00	
Zanjan (Zj)	0.80	0.85	0.86	0.86	0.86	0.92	0.90	0.91	0.93	1.00

accounted for 79% of the total variance, while only 21% of variations occurred between accessions (Table 4). The highest and the lowest heterozygosities were found in Yazd and in Ardebil accessions, respectively. However, according to the Nei genetic diversity index, Yazd accession had the highest mean value (0.305) and Ardebil accession had the lowest (0.233).

Genetic similarity

Genetic similarity is an important index for the estimation of genetic similarities among accessions. The mean genetic similarity between different individuals within a given accession was fairly similar to all cultivars, ranging from 0.77 to 0.95 (Table 5). The minimum coefficient was observed between Ardebil and Lorestan (L), and the maximum coefficient was observed between Esfehan and Lorestan accessions.

Genetic relationships among the spinach accessions

The data obtained from SRAP analyses were used to perform genetic similarity analysis among the 10 spinach accessions. Cophenetic correlation between ultrametric similarities of tree and the similarity matrix was $r=0.85$ with $P < 0.0001$. All accessions used in this study were distinguished. Based on the unweighed pair group method of arithmetic averages (UPGMA) cluster diagram and Dice similarity coefficient, the 10 accessions may be divided by the genetic distance of 0.05 into two groups (Figure 1). Group A consisted of four accessions including Zanjan, Zabol, Lorestan, Esfehan and group B included six accessions, Tehran, yazd, Hamedan, Fars, khorasan and Ardebil. A dendrogram indicated that the genetic relationships among the accessions were not highly associated with the geographic locations in which the germplasms were collected.

PCA analysis

Principal component analysis was performed based on the genetic similarity matrix in order to better understand the relationships between and within accessions. The results were in accordance with the UPGMA cluster analysis with groupings among 10 spinach accessions (Figure 1). The first and second vectors of components accounted for 58.78% and 12.45% of variance, respectively (Figure 2). The groupings within the spinach accessions (100 genotypes) on the PCA were calculated as well, so that the first and second vectors of components accounted for 15.01% and 7.36% of variance, respectively.

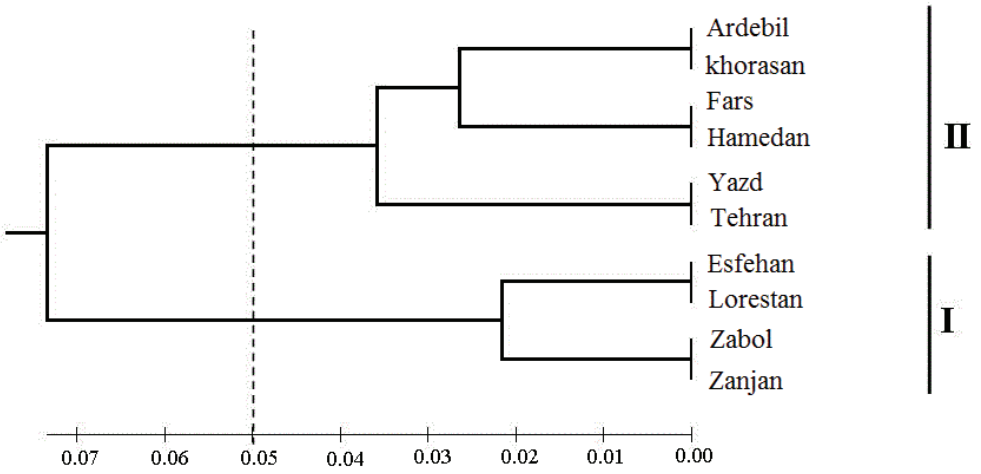

Figure 1. Dendrogram of the 10 spinach accessions using UPGMA method obtained from SRAP markers.

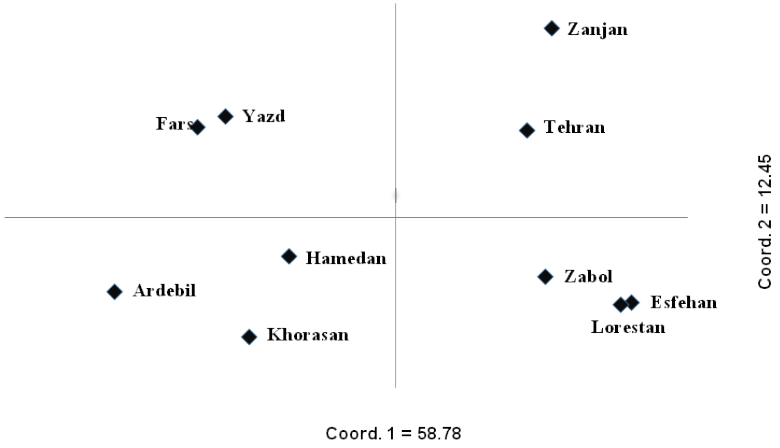

Figure 2. Principal component analysis score plot of 10 spinach accessions (using GenAlEx software) on eight SRAP markers (PC1 and PC2: first and second principal components).

DISCUSSION

It is a common belief that spinach was first cultivated in Iran (Persia). The earliest record of spinach in China occurred in the seventh century and was referred to as ‘‘bo cai’’, meaning ‘‘Persian vegetable’’. It was introduced into China from Nepal in 647A.D. and reached Spain around 1100 A.D. Spinach was grown in Germany in the 13th century, and by the 14th century it was common in European monastery gardens. It was brought to America by the early colonists (Pandey and Kalloo,1993).

To our knowledge, this is the first report describing molecular diversity within and between some Iranian spinach accessions using SRAP markers. The study described in this article shows that SRAP markers are a

powerful and reliable molecular tool for analyzing genetic diversity and relationship in spinach. The spinach accessions used in this study showed a high level of polymorphism.

These results suggest that SRAPs are able to detect a larger level of polymorphism in a more efficient way. Our study also revealed that the genetic diversity of spinach was relatively high in terms of the three genetic diversity measurements (P: 73.56; H: 0.268; I: 0.008). Among the 10 accessions, Yazd accession maintains a higher genetic diversity than the others. In contrast, the level of polymorphism and genetic diversity of Esfahan and Ardebil accessions were found to be lower than the other accessions. Obviously, one option is to conduct crossbreeding with Yazd accession to enhance genetic diversity, and it must be the basis for the improvement of the germplasm resources (Table 3).

Moreover, the genetic similarity matrix and UPGMA (Figure 1) analysis of our data showed a narrow level of genetic diversity among spinach genotypes. In this study, the main goal was the genetic characterization of spinach accessions. This information can be used for the generation of a core collection of spinach by the elimination of redundant accessions and for spinach breeding by identifying useful lines. In this respect, 10 spinach accessions from Iran were studied using eight SRAP marker combinations. A total of 88 fragments were obtained, 73 of which showed polymorphism (82.9%). These numerical data were obtained to determine genetic relationship using UPGMA and Dice methods.

However, the measured relative genetic distances among accessions was not correlated with the geographical distances of places of their origins. This reflects probably both molecular differences and the movement of germplasm. Results of PCA indicated that SRAP markers allowed the evaluation of diversity on the basis of the DNA fragments distributed through the genomes. Obviously, divergence evident in this germplasm warrants additional acquisition of these botanical varieties. In addition to collection management, our information on diversity and relationship in *S. oleraceae* will also be useful for plant breeders to select germplasm samples with the maximum diversity, to choose desirable parents for high yield, quality and resistance to biotic and abiotic stress conditions.

In conclusion, SRAP can be used to establish a foundation for molecular marker assisted breeding of the spinach resource based on our analysis. The genetic diversity shown in this study can also be applied for the identification of differences in intra-species and inter-species. Obviously, further investigations are needed to exploit more accession collections having diverse agronomic traits in breeding improvement programs. These studies can present essential information in revealing genotypic relationship, which may assist breeding planning in the future.

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