UDC 575. 630
https://doi.org/10.2298/GENSR2302473J
Original scientific article

EVALUATION OF ACCESSION STRUCTURE AND GENETIC DIVERSITY IN IRANIAN MILK THISTLE (*Silybum marianum* L.) BY ISSR MARKERS

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Jamshidnia M., S. Asgary, M. Rafieian-Kopaei (2023). *Evaluation of accession structure and genetic diversity in Iranian milk thistle (Silybum marianum L.) by ISSR markers.* - Genetika, Vol 55, No.2, 473-490.

The aim of this study was to investigate the genetic variations of Iranian *Silybum marianum* accessions using inter simple sequence repeat (ISSR) markers. Twenty-nine accessions from various Iran regions and a sample from Hungary were selected and evaluated by 19 ISSR primers. A clear banding pattern was produced by 9 primers and a total of 85 repeatable polymorphic bands were detected. ISSR7 and ISSR8 primers identified the most Polymorphic Information Content (PIC) with 100% among primers. In the tested accessions, polymorphism information content (0.45), polymorphic percentage (83 %), and Shannon's information index (0.53) assessed a high level of genetic variation. Based on Jaccards distances, cluster analysis molecular traits with unweighted pair group method with arithmetic mean (UPGMA) method were taken into consideration, and accessions were grouped into nine clusters and confirmed by principal coordinate analysis. Four clusters were identified by a Bayesian structure analysis, and 12 individuals were maintained inside the admixed clusters. According to the findings, ISSR marker system can be considered as a powerful tool for detection of genetic diversity of accessions in *S. marianum*. The results indicate existence of a high variation among Iranian *S. marianum* accessions to start the breeding programs.

Keywords: Asteraceae, Genetic variation, Inter simple sequence repeat, PCR, Silymarin.

INTRODUCTION

Milk thistle (*Silybum marianum* (L.) Gaertner) belonging to Asteraceae family, is native to the Mediterranean region and is widespread throughout the world including Iran (MORAZZONI

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and BOMBAREDELLI, 1995; LENG-PESCHLOW, 1996). Various Milk thistle extracts have been used since ancient times as medicines for diseases related to liver and bile, especially since the 16th century (BURGESS, 2003). It is considered important for its flavonolignans mainly existing in its fruits (achenes). Silymarin is a mixture of flavonolignans consisting of at least silybin, silydianin and silychristin; and 1-4% (w/v) silymarin can be found in dried fruits (MURPHY *et al*., 2000). Strong hepatoprotective and antihepatotoxic activities of silymarin have already attracted considerable pharmacological interest and recent investigations have revealed that silymarin possesses strong antichlosterolaemic property (PREEDY and WATSON, 2011). Moreover, it has been suggested that its extracts can positively affect the possible damages to liver induced by anti-HIV drugs (ANONYMOUS, 2002). The flavonolignans of silymarin can protect the liver against tissue damage and oxidative stress, and to reduce cell death by cytotoxins (RODRIGUEZPEREZ *et al*., 2002; SKOTTOVA *et al*., 2004). In this regard, silydianin and silychristin have the best protection capacities (DVORAK *et al*., 2003). Silybin is the major ingredient of silymarin from the point of view of quantity and therapeutic activity (CACHO *et al*., 1999). In addition to its liver protection property, silybin is useful in human Seepr ostate cancer treatment as far as the interaction of hormone refractory is concerned (ZI and AGARWAL, 1999).

Although Milk thistle is economically and pharmaceutically valuable, negligible research has been done to domesticate and breed this plant (RAM *et al*., 2005). Evaluating the genetic diversity of available ecotypes is necessary to initiate a breeding program. Regarding to specific breeding purposes, identification and accessions classification of the subsets of core accessions would be carried out by analyzing the genetic diversity (MOHAMMADI and PRASANNA, 2003; KULUS, 2018). In Iran, despite the existence of Milk thistle in various geographical regions, almost no reports regarding the extent of its genetic diversity can be detected.

DNA-based markers and other molecular techniques give us useful tools to genetically analyze its diversity and population structure, comprehensively. Inter simple sequence repeat (ISSR) marker system was developed by ZIETKIEWICZ *et al*. in 1994 and used to describe genetic differences or similarities in various plant species (HENAREH *et al*., 2016; ABBASI *et al*., 2017; DASH *et al*., 2019; LEMA-RUMIŃSKA *et al*., 2019; SAFARI *et al*., 2019; JAMSHIDNIA *et al*., 2020). In ISSR, two inverted microsatellites delimit each band corresponding to a DNA sequence. ISSR markers are a quick and easy way to handle like RAPDs and, due to the length of their primers, they possess the reproducibility of simple sequence repeats (SSR) markers. In this method, amplification needs no genomic sequence information, and it results in patterns that are multilocus and highly polymorphous (RUKAM, 2010). Moreover, it is involved longer (16-18 nucleotides) primers, which encode microsatellite elements that amplify DNA segments' intra-microsatellite repetitions (GUPTA *et al*., 1994; RUKAM, 2010). ISSR is like RAPD since it is a dominant marker, but it has more robustness in repeatability and is highly variable. Such characteristics render ISSR superior to other accessible marker systems in studying genetic variations in individuals that are closely related and in classification of crop cultivars (HADIAN *et al*., 2014; SINGH *et al*., 2014). This method has been employed to find out genetic diversity and polymorphism of DNA in a large number pistachio germ cells originating from seven countries along with the RAPD and AFLP markers (KAFKAS *et al*., 2002).

MOHAMMADI *et al*. (2011) assessed the molecular diversity of *S. marianum*, collected from seven provinces of Iran using AFLP markers. However, no comprehensive studies have

been reported yet on the genetic diversity of different Milk thistle populations in Iran. In a study, the molecular diversity of 8 populations of *S. marianum* collected from southwest Iran was evaluated using the Start Codon Targeted (SCoT) marker (RAFIZADEH *et al*., 2018). Information about genetic diversity in Milk thistle accessions can have a vital function in developing new views for breeding plans. The present study aimed to evaluate the patterns and levels of genetic diversity and to get information regarding the population structure of the *S. marianum* germplasm in Iran, by using ISSR markers in order to make selective breeding of this plant easy. The results, which were obtained from this research, will give appropriate information for further accession. Investigations of *S. marianum* and activities related to their management.

MATERIAL AND METHODS

Plant materials

The analyzed plant materials consisted of Twenty-nine accessions of *S. marianum* L., which were collected from various regions of Iran and an introduced variety, Budakalaszi (Hungary), were studied (Table 1). Young leaves of the plants were collected and stored at -80°C for experiments.

Table 1. List of S. marianum accessions used for genetic diversity using ISSR marker in this research

No	Name	Province	Geographical region	Latitude	Longitude	Altitude (m)
1	Manjil	Gilan	North	36° 44' N	49° 26' E	396
2	Rudbar1	Gilan	North	36 49' N	49° 24' E	1020
3	Rudbar2	Gilan	North	36 48' N	49°26' E	1225
4	Sari	Mazandaran	North	36° 33' N	53° 03'E	400
5	Fereydounkenar	Mazandaran	North	36° 40' N	52° 31' E	40
6	Juybar	Mazandaran	North	36° 41' N	52° 31' E	7
7	Gorgan	Golestan	North	36° 50' N	54° 25' E	200
8	Moghaan	Ardabil	Northwest	39°24' N	47°30' E	45
9	Anjirlou	Ardabil	Northwest	39° 10' N	48° 07' E	150
10	Angut	Ardabil	Northwest	39°03' N	47°44' E	614
11	Mehmandoost	Ardabil	Northwest	38° 25' N	48° 29' E	1471
12	Ghara Aghaj	Ardabil	Northwest	39° 01’ N	47° 40' E	600
13	Ziveh	Ardabil	Northwest	38°53' N	47°34' E	1276
14	Khoruslu	Ardabil	Northwest	39°03' N	47° 44' E	1351
15	Gharachilar	East Azerbaijan	Northwest	38° 52′ N	46° 32′ E	420
16	Miraseghloo Kalateh	East Azerbaijan	Northwest	37°36′ N	47°00′ E	256
17	Borazjan	Bushehr	South	29°16' N	51°13' E	70
18	Andimeshk	Khuzestan	South	32° 27' N	48° 52' E	200
19	Hamideih	Khuzestan	South	31° 28' N	48° 26' E	0
20	Ahvaz	Khuzestan	South	31° 19' N	48°40' E	17
21	Izeh	Khuzestan	South	31°49' N	49°52' E	824
22	Khuzestan	Khuzestan	South	31° 30' N	49°00' E	9
23	Ghaemieh	Fars	South	29° 36' N	51° 39' E	800
24	Nour Abad	Fars	South	27°24′ N	53°00′ E	980
25	Jolge khalaj	Lorestan	South	33° 17' N	47° 48' E	780
26	Markazi	Markazi	Center	34° 03' N	50° 29' E	1718
27	Isfahan	Isfahan	Center	32° 39' N	51° 40' E	1590
28	Najafabad	Isfahan	Center	32° 38' N	51° 21' E	1642
29	Rafsanjan	Kerman	Center	30°30' N	56°05' E	1469
30	Budakalaszi	Hungary	Europe	47°29' N	19°2' E.	111

Genomic DNA extraction

Total genomic DNA was extracted from young leaves using a modified cetyltrimethyl ammonium bromide (CTAB) method of MURRAY and THOMPSON (1980). The quantity and quality of DNA were determined by measuring absorbencies at A260 and A280 using a UV spectrophotometer (Bio-Tek, PowerWaveXS, USA), and by comparing band intensity with λ DNA (Fermentas, Germany) of known concentrations using a 0.8% agarose gel electrophoresis. In this experiment, the DNA was diluted to 40 ng ml^{-1}.

Analysis of ISSR

Nineteen ISSR primers were tested for PCR; of which nine with low missing data (null alleles) and amplified scoreable polymorphic DNA bands were chosen for analysis (Table 2). Amplification was done in a 15 µL volume containing 2.5 mM of each 1 U Taq DNA polymerase (Cinnagen, Iran) and dNTP, 40 ng of genomic DNA, 10 pmol of primer and 1.5 mM of $MgCl_2$. Amplification of the sample was started with initial denaturation of two min at 94°C, followed by 35 cycles of two min denaturation at 94°C, one min annealing at 49–56°C, one min extension at 72°C, and five min at 72°C (Table 2). The products of PCR were separated in 1.5% agarose gel (w/v) at 75 voltages in 1X TBE buffer for 90 to130 min. Then the gels were stained with gelred at a final concentration of 1X and their sizes were determined by comparing to a 100 bp DNA ladder (Fermentas, Germany).

Table 2. Primer sequences used for PCR amplification of S. marianum and DNA fragment analysis

Primer	**5′-3′** sequence	Annealing temperature (°C)	Total amplified bands	Number of polymorphic bands	Percentage of polymorphic (%) bands	Polymorphism information content (PIC)
ISSR7	$(GA)_8C$	50	6	6	100	0.50
ISSR8	$(CT)_8G$	51	15	15	100	0.41
ISSR9	$(AG)_8C$	49	12	9	75	0.44
ISSR12	$(GA)_8A$	50	12	10	83	0.46
ISSR15	$(AC)_8G$	54	13	9	69	0.50
ISSR16	$(TG)_8A$	52	11	8	72	0.48
ISSR17	$(AC)_8C$	51	11	8	72	0.48
ISSR18	$(ATC)_6T$	56	14	12	85	0.46
ISSR19	$(ATC)_6C$	50	9	8	88	0.30
Total			103	85		4.03
Average			11.44	9.44	83	0.45

Data analysis

Distinct polymorphic ISSR band profile in each gel was scored as present (1) or absent (0). Principle Coordinate Analysis (PCoA) and cluster analysis were conducted by Numerical Taxonomy and Multivariate Analysis System (NT-SYS-pc) ver. 2.02 (ROHLF, 1998). The polymorphic information content (PIC) was calculated by the following formula introduced by ANDERSON *et al*. in 1993: PICi=2fi (1-fi), where fi is the percentage of amplified ith. Genetic

similarities among ecotypes were calculated according to a previously defined method (JACCARD, 1908), using the similarity of qualitative data (SIMQUAL) routine. The proposed dendrogram was constructed using Arithmetic mean clustering procedure of Ntsys ver. 2.02 software and the Unweighted Pairgroup Method (UPGMA). The accessions were classified into five main groups according to the geographical distribution; that is, North (Gilan, Mazandaran, Golestan Provinces), Northwest (Ardabil, East Azarbaijan Provinces), South (Bushehr, Khuzestan, Lorestan and Fars Provinces), Central regions (Markazi, Isfahan and Kerman Provinces) of Iran (ISN, ISNW, ISS and ISC, respectively) and a sample from Hungary (Buda). AMOVA with 1,000 permutations was done for all population comparisons in order to divide all genetic variation into separate components among 2.39% and within 97.61% groups. Analysis of Molecular Variance (AMOVA) within each group was calculated using ver. 3.1 of ARLEQUIN software. The molecular variance analysis was performed for four geographic groups and a foreign group (30 accession samples). POPGENE 32 software was used to compute the percentage of polymorphic loci, observed number of alleles (*Na*), expected heterozygosity (*He*), effective number of alleles (*Ne*) and Shannon's information index (*I*). The Genetic Distance (GD) among genotypes was calculated and the phylogenetic relationship was reconstructed using MEGA version 4 (TAMURA *et al*., 2007). The Mantel test was performed to check correlation between the geographical distance and the genetic distance of the studied ecotypes using Genalex software (ver 6.5; PEAKALL and SMOUSE, 2006).

Structure 2.3.4 software was used to analyze the population structure and to estimate numbers of genetically distinct accessions (*K*) (PRITCHARD *et al*., 2000). STRUCTURE analyses were run twenty times with subgroup numbers ranging from two to ten. The optimal value of *K* was determined based on delta *K* (EVANNO *et al*., 2005) using STRUCTURE HARVESTER (EARL and VONHOLDT, 2012). A genotype was assigned to a group if more than 75 % of its genome fraction value was derived from that group.

RESULTS

Out of the 19 primers that were used for PCR, nine produced polymorphic and repeatable bands. The nine selected ISSR primers resulted in 85 polymorphic bands, which was 83% of 103 scoreable polymorphic DNA fragments (bands; Figure 1) from genomic DNA of 30 accessions having fragment sizes from 200-1500 base pairs (bp). Eighteen bands were monomorphic in all samples and were excluded from the analysis. The bands varied from 6 (primer ISSR7) to 15 (ISSR8), and they had an average of 9.44 polymorphic bands per primer (Table 2). All 30 ecotypes were clearly distinguished by the ISSR data. The upper and lower vague bands were deleted due to the weakness and lack of consistency in amplification.

Polymorphism Information Content (PIC) values with a mean of 0.45 were determined as marker index to clarify the informativeness of the utilized markers. Values with the highest (0.50) and lowest (0.30) PIC were related to ISSR7 and ISSR15 primers and ISSR 19 primers (Table 2).

The genotypes similarity matrix utilized in analyzing the ISSR revealed that the similarity ranged from 0.07 to 0.80. Among the accessions that were investigated, the maximum genetic distances and the lowest similarity coefficient (0.07) were observed between Juybar from the

north and Ghaemieh from the South of Iran. Andimeshk and Rudbar2 accessions revealed the lowest genetic distance and the highest similarity coefficient (0.80) (Supplementary Table 1).

The ISSR dendrogram was divided into 9 molecular clusters (I-IX) and I, II, IV, V, VI, VII, VIII groups were divided to many other subgroups (Figure 2).

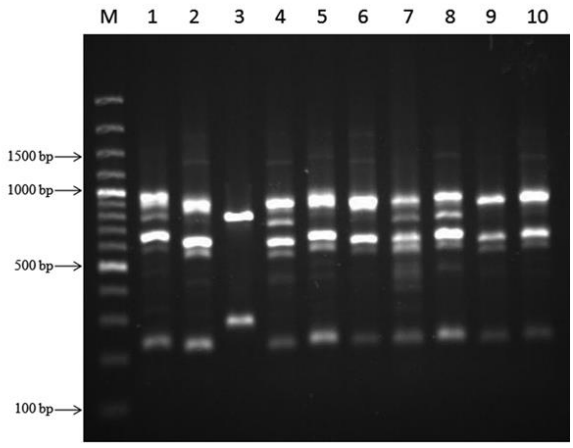

Figure 1. Illustration of the bands patterns of 10 *S. marianum* accessions revealed by ISSR 18 primer on the 1.5% agarose gel electrophoresis M: DNA ladder, 1 Najafabad, 2 Nour Abad, 3 Ghaemieh, 4 Kerman, 5 Borazjan, 6 Rudbar 1, 7 Sari, 8 Andimeshk, 9 Moghaan, 10 Rudbar 2

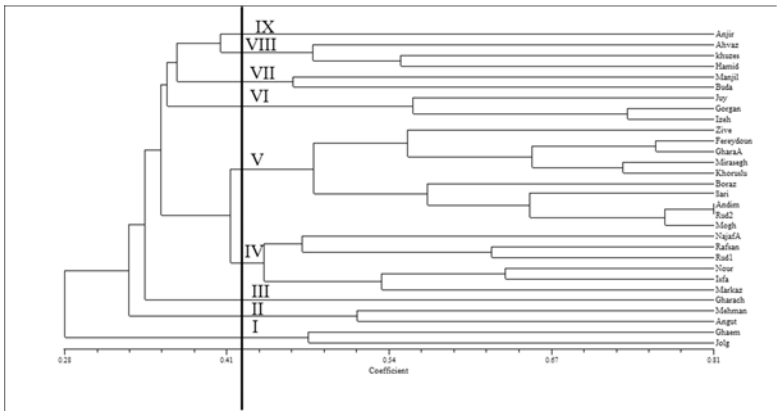

Figure 2. Molecular relationships among 29 local accessions and one foreign (Buda) variety of Milk thistle (*S. marianum*) using UPGMA

Based on genetic similarity matrix (Figure 3), principal coordinate analysis (PCoA) was carried out in order to achieve a better understanding of genetic relationships in *S. marianum* accessions. This analysis categorized the genotypes into nine groups in agreement with constructed dendrogram, and the findings in PCoA were the same as those illustrated by the UPGMA dendrogram. One more, the three accessions (Sari, Borazjan and Ziveh) were positioned outside of the cluster V, which is indicative of possible introgression.

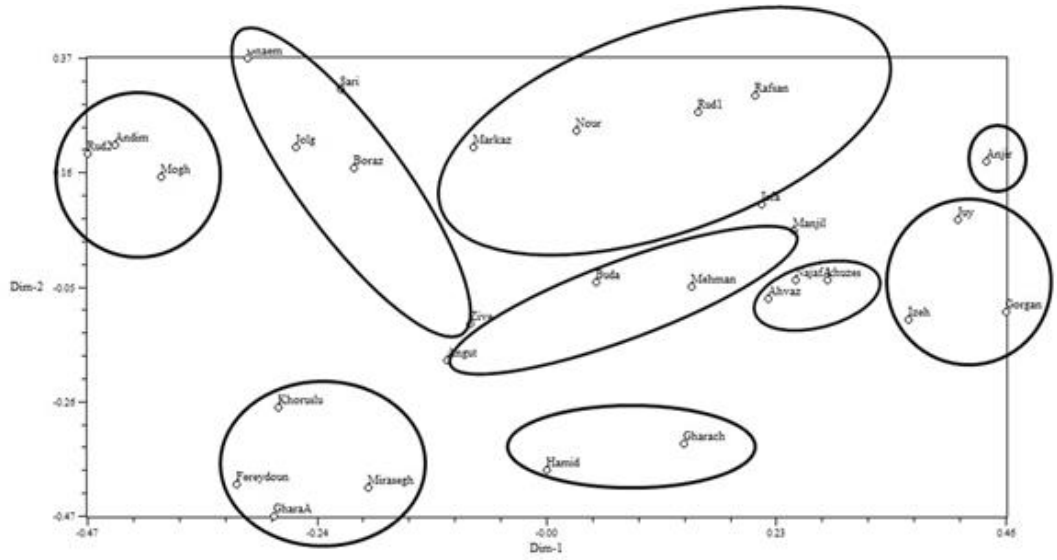

Figure 3. Relationships among the 29 local accessions and one foreign variety of Milk thistle (*S. marianum*) visualized by ISSR markers based on PCoA.

The results of AMOVA showed that the most important variation was related to within-population contribution (F_{ST} = 0.0239) (Table 3). Table 4 shows the effective number of alleles obtained (Ne), Nei's gene diversity index (h) and Shannon's information index (I). The average genetic variations calculated at each gene loci in the studied groups of the North, Northwest and South were higher than the genetic diversity mean of the gene loci in other groups, which could indicate a high genetic diversity in the northern, northwestern and southern groups (Table 4).

Table 3. AMOVA among and within five Silybum marianum groups

Source of variation	*df*	Sum of squares	Variance components	Percentage of variation	*p* value	F_{ST}
Among group	4	19.738	0.10628 Va	2.39	0.26	0.0239
Within group	21	108.529	4.34114 Vb	97.61	<0.001	
Total	25	128.267	4.44742			

Table 4. Group properties of Silybum marianum from the North (ISN), Northwest (ISNW), South (ISS) and Central (ISC) regions of Iran and a sample from Hungary (Buda) based on the 85 ISSR markers.

Property	Group name					Total
	ISN	ISNW	ISS	ISC	Buda	
Sample size	7	7	7	3	1	25
Percentage of polymorphic loci	81.18	81.18	81.18	50.59	0	100
Observed number of alleles (Na)	1.82±0.38	1.81±0.39	1.81±0.39	1.50±0.50	1±0	2±0
Effective number of alleles (Ne)	1.53±0.34	1.52±0.33	1.53±0.34	1.40±0.42	1±0	1.6±0.22
Nei's gene diversity (h)	0.30±0.17	0.30±0.17	0.31±0.17	0.22±0.22	0±0	0.36±0.12
Shannon's Information index (I)	0.45±0.23	0.45±0.24	0.45±0.24	0.32±0.32	0±0	0.53±0.15

The estimated GD indicated divergence among the accessions (Supplementary Table 2). The largest GD (8.329) was found between Gorgan from the Golestan province in North and Ghaemieh accessions (Supplementary Table 2), while the smallest genetic distance (0.124) was obtained between Rudbar 2 and Andimeshk, and (0.126) between Rudbar 2 and Moghaan (Supplementary Table 2). The overall average distance was 1.310 using MEGA 4. The largest GD (0.4353) was found between Budakalaszi and Northwest *S. marianum* accessions of Iran, and (0.4327) between Budakalaszi and Central *S. marianum* accessions of Iran (Table 5) The smallest distance (0.1004) was obtained between *S. marianum* accessions of North and South regions of Iran (Table 5). Mantel test showed a low correlation between genetic distance and geographic distance (r = 0.21, p = 0.01).

Table 5. Nei's genetic identity and genetic distance (above and below diagonal, respectively) among five geographical groups of Silybum marianum based on the DNA marker data

Group	ISN	ISNW	ISS	ISC	Buda
ISN	****	0.8591	0.9045	0.8547	0.6954
ISNW	0.1518	****	0.8778	0.8144	0.6471
ISS	0.1004	0.1304	****	0.8792	0.6741
ISC	0.1570	0.2053	0.1288	****	0.6488
Buda	0.3633	0.4353	0.3944	0.4327	****

* ISN, ISNW, ISS and ISC are related to the North, Northwest, South and Central regions of Iran, respectively and a sample from Hungary (Buda).

The number of accessions that were genetically distinct (*K*) was determined by employing STRUCTURE program in order to study the structure of accession and degrees of admixture. Bayesian information collection from the 9 ISSR primers revealed that the highest value of ΔK among the 30 accessions was $K = 4$ (Figure 4); hence, $K = 4$ was selected for ultimate analysis of accession structure. STRUCTURE analysis was carried out for $K = 4$ with 4 clusters (Red [A], green [B], blue[C] and yellow [D]) for *S. marianum* accessions (Figure 5). Mixed accession ancestry was observed among the 4 clusters; and cluster A in Figure 5 had the accessions of South, North and Central regions. Cluster B had accessions from North, Northwest, South and Central regions; and cluster C consisted of the accessions of North and Northwest, while the remaining accessions from the South and North were assigned to cluster D. Cluster A comprised four accessions, and cluster B included six accessions. Cluster C consisted of four accessions 'Ghara Aghaj', 'Miraseghloo Kalateh', 'Fereidounkenar' and 'Khoruslu', while four accessions were assigned to cluster D. The most mixture was noticed in clusters B and C. Accessions with probabilities of membership <0.75 were assigned to an admixed group. In addition, 18 accessions were assigned to clusters, and 12 accessions were kept in the admixed clusters (Figure 5).

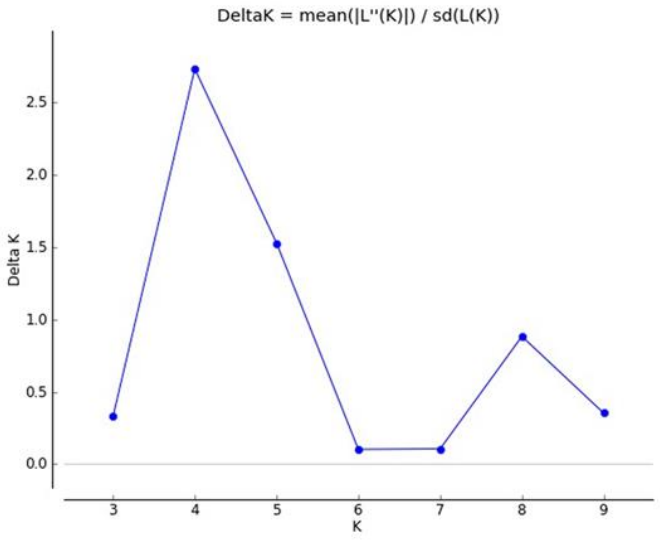

Figure 4. Plot of ΔK, for detection of an optimal K ($K = 4$) for the analyzed data.

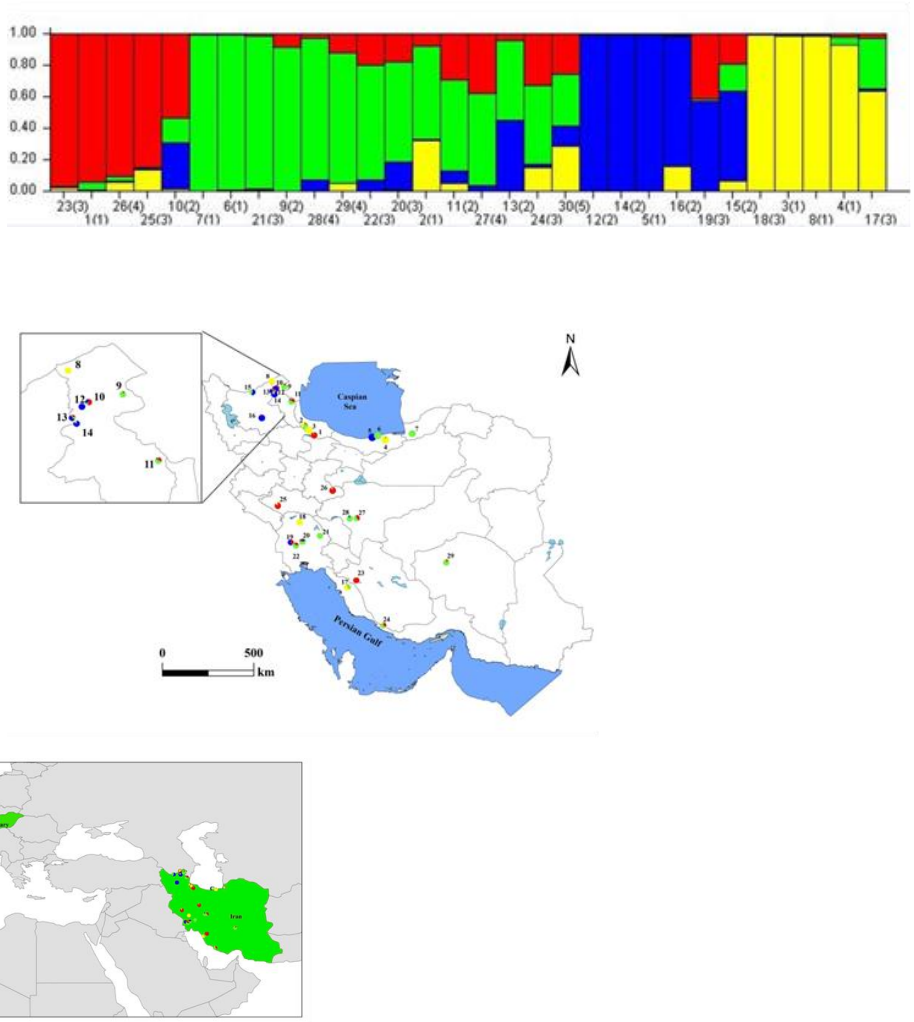

Figure 5. Distribution map of the examined accessions of *S. marianum* (color of each accession is related to genetic cluster in the bar plot). STRUCTURE plot of 30 *S. marianum* accessions with $K = 4$ clusters. (Red, Green, Blue and Yellow colors are related to the cluster A, cluster B, cluster C and cluster D, respectively). Extent of admixture in an individual is shown by segments of each vertical line. (1) North, (2) Northwest, (3) South, (4) Center and (5) Buda show groups.

Budakalaszi, an introduced variety from Hungary was assigned to an admixed group. Rudbar1 showed admixturing, while Rudbar2 was assigned to cluster D. Based on these results, classification of the 30 *S. marianum* accessions partly agrees with the results of NJ clustering.

DISCUSSION

ISSR analysis is mostly used in order to discover relationships among species in subgeneric groups, as has previously been done in *Diplotaxis* DC. (MRRTIN and SANCHEZ-YELAMO, 2000), *Oryza sativa* L. (JOSHI *et al*., 2000), *Allium* groups (HAO *et al*., 2002; MUKHERJEE *et al*., 2013) and *Tolpis* (ARCHIBALD *et al*., 2006). This method was useful in trying to find genetic differences in *S. marianum* genome. The efficiency of a molecular marker technique mostly depends on quantity of polymorphism that is able to find out among genotypes under investigation (TATIKONDA *et al*., 2009). High levels of polymorphism were seen with nine ISSR primers from the 29 accessions, which were collected in twelve provinces in Iran and one variety that was introduced. All studied ecotypes were polymorphic in this research, the average level of PIC was evaluated at 0.45. According to MOHAMMADI *et al*. (2011), the average level of PIC (0.35) was calculated by AFLP analysis of *S. marianum*. Start Codon Targeted (SCoT) marker system was utilized to investigate the genetic variability of eight *S. marianum* accessions in Iran (RAFIZADEH *et al.*, 2018). The SCoT marker system's polymorphism information content value was 0.43.

There was an evaluation of accession differentiation in the analyzed germplasm based on genotyping data achieved for all nine primers by employing a neighbor-joining-based phenogram. Most accessions were grouped according to their geographical locations. In the ISSR dendrogram, congruity between geographical distance and genetic distance is smaller compared to the AFLP analysis, which reported that genetic distance between *S. marianum* accessions was also in good agreement with geographical distance (MOHAMMADI *et al*., 2011). *S. marianum* accessions of 12 provinces were studied in the ISSR dendrogram in comparison to the 7 studied provinces in the AFLP analysis which identified well differences between accessions from wide geographical distances by ISSR analysis rather than the previous studies. The dendrogram that was produced by Jaccard's similarity coefficients on the basis of ISSR (Figure 2) classified the 30 tested accessions into nine groups. A large genetic distance was discovered among some of the accessions, which may act as a valuable genetic resource identifying promising parental material in *S. marianum* breeding programs.

Cluster I (Figure 2) confirm the close relationship of Jolge Khalaj and Ghaemieh similar to the AFLP tree (MOHAMMADI *et al*., 2011), but this close relationship is different from the AFLP tree because they are in the same cluster in the ISSR tree. Cluster II confirms the close relationship of two accessions of Angut and Mehmandoost related to Ardabil province and they are not considered in the AFLP tree (MOHAMMADI *et al*., 2011). Gharachilar accession from East Azerbaijan Province is classified in cluster III in contrast to the result obtained in the AFLP analysis, which grouped with accessions in Gorgan province, Golestan province and Ghaemshahr in Mazandaran province and clustered with the southern accessions, though in a separate sub-cluster.

Cluster IV including central accessions, Markazi, Isfahan, Nour-Abad in subcluster I and Rafsanjan and Najafabad in subcluster II but Rudbar I accession from Gilan province was grouped in this cluster. None of these accessions were classified in the AFLP tree by MOHAMMADI *et al*. (2011) and none of them were studied in the previous analyses. Nevertheless, in AFLP analysis some accessions from north and northwestern of Iran were clustered with the Southern accessions.

Cluster V confirms a close relationship between North, Northwest accessions, but there is some inconsistency between molecular grouping and origin of accession so that Andimeshk and Borazjan of South accessions were grouped in this cluster. In the AFLP dendrogram (MOHAMMADI *et al*., 2011), Andimeshk accession clustered with accession from South of Iran in contrast to the results of the ISSR analysis, which placed close to the north and northwestern accessions. Although Rudbar I and Rudbar II accessions are related to one region (Gilan province), they were classified in different clusters. These findings confirmed high genetic diversity of *S. marianum* in Gilan province.

Cluster VI indicated a close relationship among Juybar (Mazandaran province) and Gorgan (Golestan province) and Izeh (Khuzestan province). It is similar to the result of the AFLP analysis, which placed some accessions from Mazandaran province and the Gorgan (Golestan province) close to the southern accessions.

Cluster VII confirms a close relationship between Budakalaszi and Manjil (Gilan province). It is similar to the result of the AFLP dendrogram, which classified Budakalaszi close to the accessions from North of Iran with the Naharkhuran from Golestan province.

Cluster VIII confirms the close relationship of Hamideih, Khuzestan and Ahvaz from Khuzestan province of south of Iran. It is similar to the AFLP analysis (MOHAMMADI *et al*., 2011), which classified some accessions from South of Iran in one cluster and Hamideih was grouped in this cluster.

In the ISSR dendrogram, Anjirlou from Ardabil Province is grouped in cluster IX. It is in contrast to the result obtained in AFLP analysis, which placed Anjirlou close to the accessions in northwestern provinces (Ardabil and East Azarbaijan) and it is grouped in sub-cluster I.

In this research, the result of ISSR-based dendrogram and PCoA analysis showed that not only there are differences in molecular genetics between accessions collected from one province, but also these differences exist between accessions from different provinces of Iran. Most accessions were grouped according to their geographical locations. Nevertheless, in the present research, congruity between genetic distance and geographical distance decreased with investigation of *S. marianum* accessions of 12 provinces in comparison to the 7 studied provinces by MOHAMMADI *et al*. (2011).

The analysis of 85 polymorphic ISSR markers in Iranian *S. marianum* accessions revealed that high level of genetic diversity persisted in accessions. Genetic diversity was more than that of previously reported for *S. marianum* accessions (MOHAMMADI *et al*., 2011). Considering data analysis, the accessions that were studied had no significant differences. The Fixation index (F_{ST}), which measures the extent of genetic differentiation among subpopulations, may be in the range of 0.0 (no differentiation) to 1.0 (complete differentiation - subpopulations fixed for different alleles). The F_{ST} statistic is a suitable and common way for the comparison of differentiation among two or more subpopulations. In this study the F_{ST} was 0.023. The F_{ST} values between 0 and 0.05 show a slight difference between the groups defined by the researcher (FERRIOL *et al*., 2003). Comparison of the values of variance calculated with F_{ST} indicates that the difference between the introduced groups is not significant and there is little difference among the groups, but the difference within groups is significant. According to the results, 2.39% of the total variation among the groups and 97.61% of it is allocated to the groups. AMOVA showed that within- group genetic variation was higher than genetic differentiation

among groups, which is similar to the AFLP analysis (MOHAMMADI *et al*., 2011), indicative of the fact that within- group genetic variation was higher than genetic differentiation among some *S. marianum* groups. Moreover, RAFIZADEH *et al.* (2018) reported that the variation was displayed by the molecular variance analysis among the populations less than a recorded variation made within the populations. MUMINOVIC *et al*. (2004) reported similar patterns of genetic variation for *Valerian ellalocusta*, and similar patterns of genetic variation were reported for Tunisian fig (*Ficus carica* L.; BARAKET *et al*., 2009) and *Caragana microphylla* (CHEN *et al*., 2009). High variation among intra-group samples can be attributed to processes like gene mutation, recombination of genes and chromosomes, the number of effective individuals and the genetic structure of the populations (FENG *et al*., 2009). Moreover, the greater genetic variation within the group than the intergroup can be related to the nature of the cross-pollinator of this plant, high heterozygosity, and the distribution of this plant in several regions of Iran. The number of polymorphic loci and that of alleles in the accessions from North, Northwest and South are higher than those in other groups, confirming higher polymorphism in northern, northwestern and southern samples. In the present study, the highest genetic variation in *S. marianum* in Iran was within the group. FADHEL *et al*. (2004) considered the great variation within groups to the result of cross pollination and seed distribution.

The largest GD between Gorgan accession from the Golestan province in North and Ghaemieh accession from the Fars province in South was shown by DNA marker data. They could be independently introduced from the different geographical regions of Iran following the high geographical distances but the result of Mantel test did not produce a significant correlation between the geographical and the genetic distance of these accessions. This indicates that dispersal occurs in different directions and probably with different mechanisms (sexual and asexual) involved in the dispersal of the accessions.

Milk thistle is predominantly a self-pollinator (HETZ *et al*., 1995). Nevertheless, high levels of diversity within groups can be indicative of the recombination as a result of the overcoming of cross pollination with high fertility. The seeds are capable of being spreading by wind over long distances (kilometers) though this method of dispersal may be highly localized. The tail of seed dispersal distribution might lead to gene flow among groups with significant consequences in homogenization of genetic diversity among groups. Also, localized seed dispersal might lead to significant fine-scale genetic diversity, even in spite of evolutionarily significant rates of inter-populational gene flow (CHUNG *et al*., 2004; TRAPNELL *et al*., 2008), and minimize the effects of isolation and population differentiation in this manner. Also, the pappus might cause an increase in efficient seed exchange, prevent the differentiation of accessions and produce the smallest genetic distance between Rudbar 2 and Andimeshk.

The accession structure was also studied within *S. marianum* germplasms. Accession structure analysis classified the accessions into four groups. This structuring assigned 18 individuals to 4 clusters based on a membership probability threshold of 0.75. The mixed accessions may be attributed to breeding (Budakalazi), resource exchange, domestication history, high level of heterozygosity and gene flow as a result of seed dispersal distribution of *S. marianum*. Similar results were obtained for the plant with the heterozygous nature of the genome such as Chrysanthemums (ANDERSON, 2006; ZHANG *et al*., 2010; ZHAO *et al*., 2010; ROEIN *et al*., 2014). In addition, Rudbar1 showed admixture, while Rudbar2 was assigned to

cluster D (Figure 5). This difference between the two accessions in one region and creation of a mixed accession may be attributed to domestication history, breeding, resource exchange and high heterozygosity.

CONCLUSION

The genetic diversity of different *S. marianum* accessions from 12 provinces of Iran was analyzed by using ISSR molecular markers. The results of this research with more accessions confirmed that Iran is the probable center of origin and diversity of *S. marianum* and helped to understand the genetic variation and evolutionary dynamics of *S. marianum* and to broaden the genetic base for *S. marianum* breeding. This study also showed high efficiency of ISSR markers (83%) in distinguishing various accessions of *S. marianum* from various regions, and revealed the high genetic differentiation of *S. marianum* accessions. Finally, ISSR was proven to be a reliable marker that revealed genetic diversity and that estimated the genetic distance between accessions.

ACKNOWLEDGEMENTS

This study was financially supported by Shahrekord University of Medical Sciences (grant number 946). The authors are grateful to the Institute of Forestry and Rangelands of Iran to provide seeds of eight *S. marianum* accessions for this research.

Received, July 5th, 2022
Accepted April 28th, 2023

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PROCENA STRUKTURE I GENETIČKOG DIVERZITETA KOD IRANSKOG MLEČNOG ČIČKA (*Silibum marianum* L.) ISSR MARKERIMA

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Izvod

Cilj ovog istraživanja bio je ispitivanje genetičkih varijacija uzoraka iranske *S. marianum* korišćenjem ISSR markera. Dvadeset devet primeraka iz različitih regiona Irana i uzorak iz Mađarske je odabrano i ocenjeno pomoću 19 ISSR prajmera. Detektovana je jasna šema traka koju je proizvelo 9 prajmera i ukupno 85 ponovljivih polimorfnih traka. Prajmeri ISSR7 i ISSR8 identifikovali su najpolimorfniji informacioni sadržaj (PIC) sa 100% među svim prajmerima. U testiranim uzorcima, sadržaj informacija o polimorfizmu (0,45), procenat polimorfnosti (83 %) i Šenonov indeks informacija (0,53) procenjuju visok nivo genetsičke varijacije. Na osnovu Žakardovog rastojanja, molekularne osobine klaster analize sa UPGMA metodom su uzete u obzir, a uzorci su grupisani u devet klastera što je i potvrđeno analizom glavnih koordinata. Bajesovom strukturnom analizom identifikovana su četiri klastera, a 12 jedinki je zadržano unutar mešanih klastera. Prema nalazima, ISSR markeri se mogu smatrati moćnim sredstvom za detekciju genetičkog diverziteta uzoraka *S. marianum*. Rezultati ukazuju na postojanje velike varijacije među iranskim uzorcima *S. marianum* za početak programa oplemenjivanja.

Primljeno 5.VII.2022.
Odobreno 28. IV 2023.