RESEARCH ARTICLE Legume Research- An International Journal, Volume 44 Issue 8: 888-893 (August 2021)

Genetic Relationship in Sainfoin (*Onobrychis viciifolia*) Landraces Cultivated East Anatolia by using RAPD and ISSR Markers

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10.18805/LR-614

ABSTRACT

Background: Sainfoin (*Onobrychis viciifolia*) is a forage crop that yields high in arid and calcareous soils and is cultivated in large areas. There aren't many genetic diversity studies on the varieties of cultured sainfoin. This study was conducted to determine the genetic diversity and the degree of relationship between 23 cultivated landraces and one registered variety.

Methods: To take samples from the populations, seeds were sown in the field in 2014. Samples were taken from the young leaves of the plants and preserved at -80°C in same year. RAPD and ISSR primers were used in the study. The bands obtained as a result of PCR were recorded and the data of both methods were also evaluated by combining them.

Result: In the study, 5 RAPD and 4 ISSR primers were used and a total of 49 bands were obtained. Of 29 bands obtained using RAPD primers, 20 were found to be polymorphic and of 20 bands obtained using ISSR primers, 15 were found to be polymorphic. It was found that there was a very low correlation between the two methods. Using RAPD and ISSR markers and RAPD + ISSR combination, the similarity index among populations was found to be between 0.25-0.95, 0.5-1.00 and 0.45-0.91, respectively. The Nei's genetic diversity index was found to be between 0.3365, 0.2656 and 0.3018 with RAPD, ISSR primers and RAPD + ISSR combination, respectively. Based on the dendrograms obtained using RAPD, ISSR primers and RAPD + ISSR combination, the populations under analysis were classified into 3, 3 and 5 groups, respectively. With this study, the closest populations were identified and a significantly high genetic diversity was detected.

Key words: Genetic diversity, ISSR, *Onobrychis viciifolia*, RAPD.

INTRODUCTION

Sainfoin (*Onobrychis viciifolia*) is a forage plant that thrives in arid and poor soils, prevents erosion, is bloat-safe and has high nutritional value and improves soil fertility (Delgado *et al*., 2008). The cultivated sainfoin is tetraploid genome (2n=4x=28) (Hesamzadeh Hejazi and Ziaei Nasab, 2010; Sepet *et al.,* 2011; Kempf *et al*., 2016) and is a outcrossing species (Hayot-Carbonero *et al*., 2011). Sainfoin is known to have been originated from the Middle East and Central Asia (Shen *et al.,* 2019). It is reported that it was first cultivated in settlements around the Rhine Valley at the end of the sixteenth century (Delgado *et al*., 2008). Although sainfoin has a lifespan of 5-6 years, it dies due to root rot caused by *Bembecia scopigera and Sphenoptera carceli* following two or three years after it is sown (Avcı *et al*., 2014). Although it remains on the stand for two years, it is preferred by farmers as it yields higher than alfalfa, especially in unirrigable and calcareous soils. In addition, due to its positive effect on animal health with its tannin content (CT), in recent years it has become the focus of attention as a forage plant (Kempf *et al*., 2016). Today, sainfoin is cultivated on an area of 153,000 hectares in different regions of Turkey. The sainfoin is an important forage crop in arid and semi-arid regions and is better adapted to areas where alfalfa growth is restricted by environmental conditions (Mohajer *et al*., 2012). Therefore, it is thought that it will be cultivated even more in areas where there is difficulty accessing irrigation water.

Populations that have adapted to the ecological conditions of the region where they are cultivated are referred to as landraces. The widespread use of high-yielding varieties in recent years also entails the protection of landraces facing the danger of extinction. Landraces are important genetic resources for plant breeding and for successful plant breeding, primarily, the genetic diversity of plants must be known (Hejrankesh *et al.,* 2014). However, the identification of existing plant genetic resources has an important place in future genetic studies and the development of approaches (Dos Santos *et al*., 1994; Demdoum *et al*., 2012).

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How to cite this article: Ertuş, M.M. (2021). Genetic Relationship in Sainfoin (*Onobrychis viciifolia*) Landraces Cultivated East Anatolia by using RAPD and ISSR Markers. Legume Research. 44(8): 888-893. DOI: 10.18805/LR-614.

Submitted: 04-02-2021 **Accepted:** 22-05-2021 **Online:** 27-07-2021

There are many DNA-based methods used in identifying the genetic variation and in phylogenetic studies. Considering the laboratory facilities, since they involve non-radioactive substances, RAPD and ISSR are methods that can be used to conduct research easily under limited conditions (Kumar *et al.,* 2009). In addition to offering a new and practical approach in taxonomic and phylogenetic comparisons, the ISSR method is also used as a mapping tool for many organisms (Zietkiewicz *et al*., 1994; Semagn *et al*., 2006). RAPD and ISSR methods are used because they exhibit high polymorphism, do not require prior knowledge and are rapid and inexpensive (Bornet *et al*., 2002; Semagn *et al*., 2006). Sanghani *et al.* (2015) used SSR, RAPD and ISSR markers to determine the genetic diversity in *Vigna radiata* genotypes and found that RAPD markers are effective in determining the genetic relationship between populations. RAPD and ISSR primers are very useful in determining the relationship of the sainfoin and determining its genetic difference (Nosrati *et al*., 2012; Archer *et al*., 2013; Rasouli *et al*., 2013; Hejrankesh *et al*., 2014; Zarrabian and Majidi, 2015; Sharifi, 2016). Shen *et al*. (2019) determined the genetic diversity of sainfoin collected from 5 different regions, using EST-SSR markers on 8 plants per region and reported a high level of genetic diversity among individuals. With this study, the aim was to facilitate the selection of parents for breeding programs by identifying the genetic diversity and the degree of relationship among cultivated sainfoin varieties before starting breeding.

MATERIALS AND METHODS

Plant material

A total of 23 different sainfoin landraces obtained from 21 different sites at the Eastern Anatolia Region and the Lütfibey variety cultivated by the Eastern Anatolia Agricultural Research Institute were used as the material of this study (Table 1). To take samples from the populations, seeds were sown in the field in 2014. For DNA isolation, thirty plants leaves from each population were taken and stored at -80°C. The laboratory phase of the study, which included DNA extraction and PCR, was carried out in the Laboratory of the Field Crops Department of Van Yüzüncü Yıl University in same year.

Methods

Genomic DNA was extracted from young leaves of each genotype following the method by Doyle and Doyle (1987) with minor modifications. DNA concentration was determined by spectrophotometer at 260 nm. The measurement of the OD 280 nm was used to define the content and the ratio OD260/OD280 was between 1.8 and 2.0 (Touil *et al.,* 2008). The 20 µl master mix for the RAPD and ISSR analysis

Table 1: Data on the sainfoin varieties cultivated.

Number	Province/Town	Village/Central	Latitude	Longitude	Altitude
1	Van/Özalp	Centre	38 39 32	43 59 21	2008
2	Van/Central	Erçek	38 39 15	43 39 03	1823
3	Van/Çaldıran	Yukarıyanıktaş	39 13 53	43 52 38	2203
4	Van/Çaldıran	Kılavuz	39 11 41	43 54 47	2091
5	Van/Başkale	Barış	38 01 17	43 59 04	2290
6	Van/Muradiye	Central	38 59 41	43 46 05	1708
7	Van/Muradiye	Açıkyol	38 58 24	43 42 25	1697
8	Van/Erciş	Central	39 04 24	43 26 48	1917
9	Van/Erciş	Dinlence	39 01 29	43 12 21	1910
10	Van/Çatak	Alacayar	38 01 36	43 09 40	1813
11	Van/Çatak	Uzuntekne	38 09 34	43 06 71	2249
12	Van/Çatak	Alacayar	38 01 36	43 09 40	1813
13	Van/Gevaş	Yuva	38 19 36	42 54 34	1820
14	Van/Gevaş	Abalı	38 16 58	43 12 29	1837
15	Van/Gevaş	Yemişlik	38 17 51	42 55 45	1789
16	Van/Gevaş	Atalan	38 17 46	43 08 55	1746
17	Van/Saray	Sırımlı	38 40 25	44 12 42	2159
18	Van/Saray	Dolutaş	38 36 58	44 07 24	2214
19	Van/Saray	Sırımlı	38 40 27	44 13 53	2159
20	Van/Gürpınar	Yukarıkaymaz	38 19 09	43 24 06	1750
21	Van/Gürpınar	Yukarıkaymaz	38 18 58	43 23 35	1750
22	Van/Gürpınar	Bozyiğit	38 23 22	43 34 40	1919
23	Bitlis/Hizan	Central	38 13 40	42 25 78	1446
24*	Erzurum/Central	Central	39 56 73	41 63 12	1712

* cv. Lutfi Bey developed by the Institute of Agricultural Research in Eastern Anatolia.

consist of 10x buffer, 0,2 mM of each dNTP, 50mM $MgCl_2$, 5 mM of primer, 1 U Taq polymerase, sterile water and 1 µl (30 ng) of DNA for PCR reaction (Yıldız *et al.,* 2011).

Five RAPD and four ISSR primers were selected from the previous studies on *Medicago sativa* L. (Denghan-Shoar *et al.,* 1997; Gherardi *et al.,* 1998; Mengoni *et al.,* 2000; Paredes *et al.,* 2002; Touil *et al.,* 2008; Tucak *et al.,* 2008; Petolescu and Nedelea, 2009; Ertus *et al.,* 2014). RAPD amplification was performed in thermo-cycler starting with 4 min. of denaturation at 94°C followed 35 cycle of 1 min at 94°C, 1 min at for annealing (Table 2) and 2 min. at 72°C and final extension of 6 min. at 72°C. The RAPD and ISSR fragments were separated by 1.5% agarose-gel electrophoresis with TAE 1X buffer at 90V for 3 hours and visualized with ethidium bromide. Gels were photographed under UV light to score band were analyzed in binary form for absence (0) or presence (1). Monomorphic bands were excluded from data analysis.

Similarity index coefficients of the genetic distance among the genotypes were formed in accordance to the similarity coefficient of Jaccard by SİMQUAL program and dendrograms with package software NTSYSpc-2.0 according to non-weighted arithmetical average similar group method (UPGMA) (Rohlf, 1997). Nei genetic diversity index were determined using package software POPGENE (Nei, 1973; Yeh *et al.,* 1997; Labate, 2000). The correlation between RAPD and ISSR markers were calculated by means of Mantel test (Mantel, 1967).

RESULTS AND DISCUSSION

RAPD and ISSR amplification

Genetic diversity and degree of genetic relationship of 23 landraces collected from 21 different locations in the Eastern Anatolia region of Turkey and one registered variety were determined using RAPD and ISSR markers. With the 5 RAPD markers used in the study, a total of 29 bands ranging between 500-1500 bp in size were obtained and 20 of them were found to be polymorphic. All of the 8 bands obtained using RAPD-3 primer, which yielded the highest number of bands, were polymorphic (100%) whereas only one of the three bands obtained using RAPD-1 primer, which yielded the lowest number of bands, was polymorphic (33.3%). Thus, the percentage of polymorphism ranged from 33.3% to 100%. Nosrati *et al.* (2012) reported the percentage of polymorphism with RAPD markers as 66.67%-84.62%. Hejrankesh *et al.* (2014) reported a percentage of polymorphism between 75.5%-83.89% with RAPD primers among 10 different sainfoin landraces. Similar to other studies, RAPD primers had a high percentage of polymorphism. A total of 20 bands ranging between 450-1350 bp in size were observed with ISSR primers and 15 of them were found to be polymorphic. Of the bands obtained using ISSR-3 primer, which gave the highest number of bands, 7 were polymorphic (87.5%), whereas only 1 of the bands obtained using ISSR-1 primer, which gave the lowest number of bands, was polymorphic (33.3%) (Table 3). Thus, the percentage of polymorphism was found to be between 33.3%-83.3%. According to the similarity matrix, there is a low correlation among RAPD and ISSR markers (r=0.063).

Relationship degree and genetic diversity

According to the Jaccard similarity coefficient, using RAPD markers, the closest populations were found to be Bozyiğit and Hizan varieties with a similarity coefficient of 0.95 and the most distant populations were found to be Atalan and Lütfibey varieties with a similarity coefficient of 0.25. According to the results obtained with ISSR markers, the

Table 2: Characteristic of RAPD primers.

Primer (RAPD)	Sequences 5′ → 3′	Annealing temperature (°C)	Number of total bands	Number of polymorphic bands	Percentage of polymorphic bands (%)	Size (bp)
RAPD-1	TGCTCTGCCC	36	6	2	33.3	600-1300
RAPD-2	CGTCTGCCCG	36	6	5	83.3	650-1500
RAPD-3	GTGCGTCCTC	36	8	8	100	550-1350
RAPD-4	CAAACGGCAC	36	3	2	66.7	1000-1300
RAPD-5	GGGCATCGGC	36	6	3	50.0	500-1200
Total			29	20		

Table 3: Characteristic of ISSR primers.

Primer (ISSR)	Sequences	Annealing temperature (°C)	Number of total bands	Number of polymorphic bands	Percentage of polymorphic bands (%)	Size (bp)
ISSR-1	$(GTG)_3GC$	38	3	1	33.3	800-1100
ISSR-2	$(CA)_6AC$	41	3	2	66.7	550-850
ISSR-3	$(GACA)_5$	55	8	7	87.5	500-1350
ISSR-4	$(AG)_{10}T$	55	6	5	83.3	450-1050
Total			20	15		

highest similarity was between Özalp and Barış, Özalp and Muradiye, Barış and Muradiye and Dinlence and Yemişlik varieties, all with a similarity coefficient of 1.00. However, the least similarity was between Yukariyaniktas and Erciş/ Merkez varieties, with a coefficient of 0.50. According to the results obtained using the combination of RAPD and ISSR primers, Bozyiğit and Hizan were the closest populations with a similarity coefficient of 0.91, while the most distant were found Atalan and Alacayar and Atalan and Dolutaş, with a similarity coefficient of 0.45. Rasouli *et al.* (2013) reported that, with RAPD markers, the Nei's genetic similarity coefficient was between 0.08-0.43 among the landraces. Highly similar populations, which were reported by other researchers, were identified in this study as well.

Genetic diversity among populations was calculated according to Nei's genetic diversity index. According to the findings, it was determined that Nei's genetic diversity index was 0.3365, 0.2656 and 0.3018 when RAPD, ISSR and RAPD+ISSR markers were used, respectively. Avcı*et al.* (2014) reported that the Nei's genetic diversity index among the different species of O*nobrychis*was between 0.2397-0.2916. Nosrati *et al.* (2012) reported that the Nei's genetic diversity index of *Onobrychis viciifolia* species was between 0.2466-0.3186. In their study using RAPD markers, Hejrankesh *et al.* (2014), found that Nei's genetic diversity was between 0.300-0.343 for 10 local varieties of Iran. In a study on 5 populations from Iran, it was reported that Nei's genetic diversity index was between 0.118 and 0.179 with ISSR markers and was between 0.3640-0.44618 with RAPD markers (Nosrati *et al*. 2012; Nosrati *et al*. 2016). Özkan and Bilgen (2018) reported that Nei's genetic diversity index obtained using 10 SSR markers was 0.210 for 5 different sainfoin populations. Shen *et al.* (2019) reported that genetic diversity among sainfoin is high. The findings obtained from the study were in concordance with the findings of other researchers and it was determined that sainfoin has high genetic diversity. Özkan and Bilgen (2018) found that the intrapopulation genetic diversity was high, interpopulation genetic diversity was very low in sainfoin populations. These differences were attributed to the number of populations used by the researchers, the collection of these populations from different regions, as well as the use of different markers.

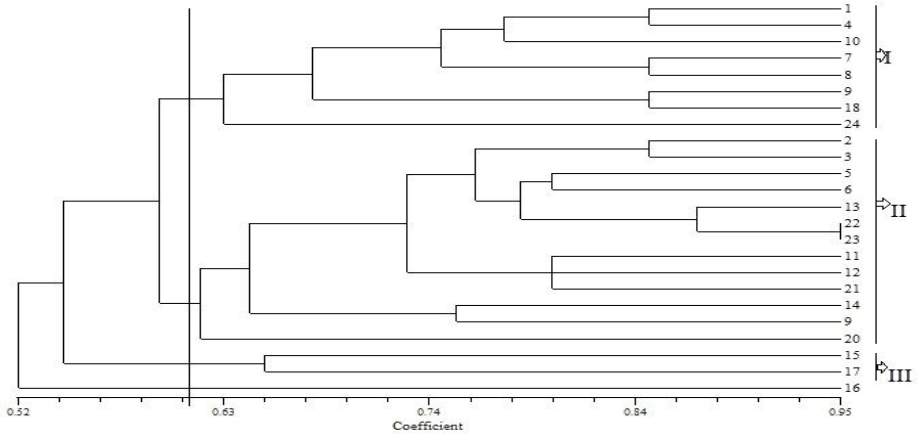

Fig 1: UPGMA dendrogram showing the relationship of *O. viciifolia* based on RAPD markers.

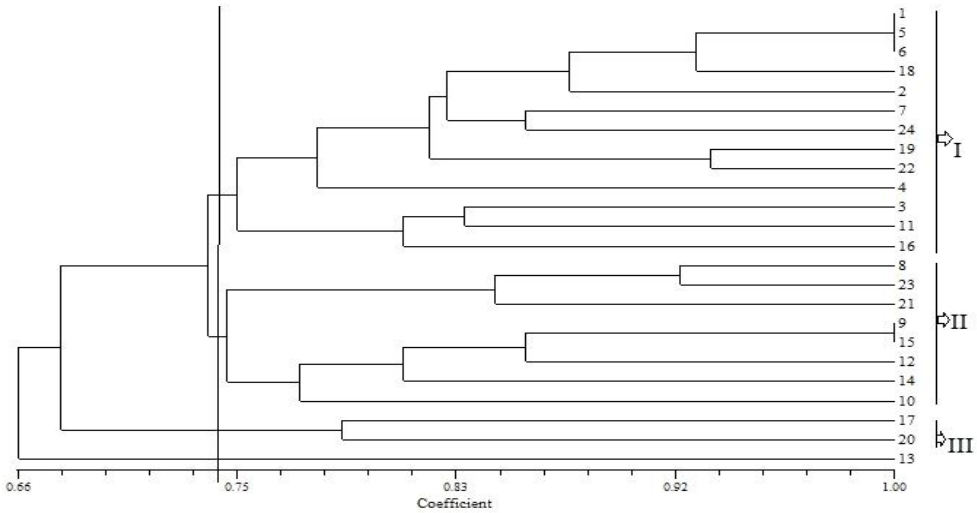

Fig 2: UPGMA dendrogram showing the relationship of *O. viciifolia* based on ISSR markers.

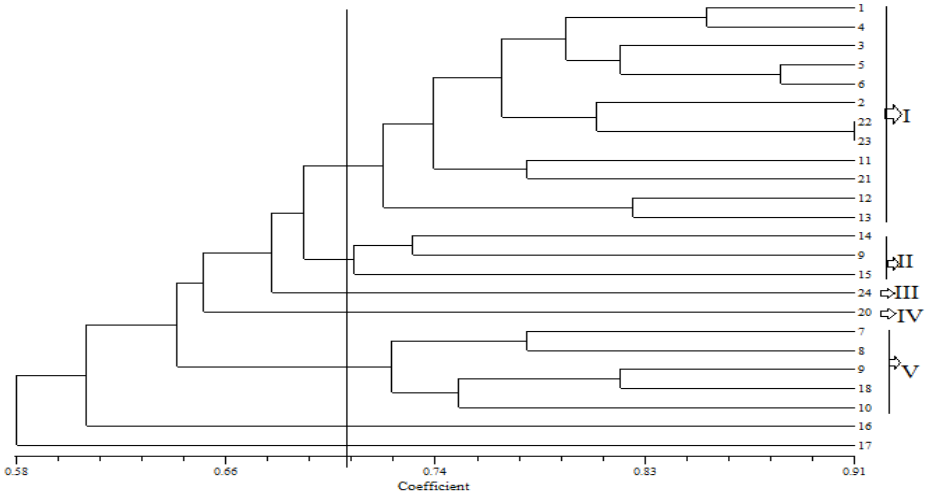

Fig 3: UPGMA dendrogram showing the relationship of *O. viciifolia* based on primers RAPD + ISSR markers.

Cluster analysis

In the dendrogram obtained with RAPD markers, the Atalan variety had a separate branching, Yemişlik and Sirimli were clustered under one group and the other varieties were clustered under two groups (Fig 1). In the dendrogram obtained with ISSR markers, the Yuva population had a separate branching, Sirimli and Yukarikaymaz populations were clustered under one group and the other varieties were clustered under two groups (Fig 2). On the other hand, in the dendrogram obtained with the combination of RAPD and ISSR primers, Yukarikaymaz and Lütfibey varieties were clustered under one group, the other varieties were clustered under three groups, whereas Atalan and Sirimli varieties had their separate branchings (Fig 3). In their study using SSR primers with 32 populations, Kempf *et al*. (2016) reported that the sainfoin populations were clustered under two main groups. Cluster analysis results in Hejrankesh *et al.* (2014) showed that 10 different sainfoin varieties had 3 branchings and in Özkan and Bilgen (2019) showed that 5 different populations had three branching.

CONCLUSION

Identification of the genetic diversity of sainfoin, which is comparable to alfalfa that has been intensively cultivated and yields high in marginal environments, is important. The aim was to identify the genetic diversity and the degree of relationship between 23 sainfoin landraces and one registered sainfoin variety using RAPD and ISSR primers. The high genetic diversity of the cultivated *Onobrychis viciifolia* was attributed to its ploidy level of 2n=4x=28, chromosome structure and allogamous species. In light of the molecular data, it was found that the cultivated sainfoin landraces were significantly rich genetic resources.

The study also demonstrates that the degree of relationship between sainfoin varieties with high genetic diversity can be distinguished much better using RAPD markers. Based on this information, it can be said that RAPD markers will be helpful when selecting parents in the future breeding of sainfoin landraces.

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