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Research Article

Assess to Genetic Diversity in Cultivated Forage Pea (*Pisum sativum* var. *arvense* L.) Genotypes Through SSR Markers

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Abstract

Around the world, forage peas (*Pisum sativum* var. *arvense* L.), a significant legume species with high nutritional value, are fed to animals. In the high altitudes of the Eastern Anatolia Region, forage peas have long been grown for animal feeding. Forage peas are becoming less common in local varieties and more readily available in commercial varieties. Thus, the purpose of this study is to use SSR markers to ascertain the genetic diversity and genome size variation of multiple commercial and landrace populations of forage peas. A total of 18 samples and 11 SSR markers, including 11 population and 7 commercial varieties grown in Turkey, were used in this study. It successfully generated 66 polymorphic bands in total, accounting for 89.2% of the samples. These polymorphic alleles ranged between 3 and 11, with an average of 6. Landraces and commercial varieties were the two main groups separated by the genetic diversity dendrogram. The least genetic distance was discovered between Töre and Taşkent varieties, and the maximum was between 1 and 6 landraces. The results of this study suggest that local varieties ought to be taken into account in breeding programs.

Keywords: *Pisum sativum* L., Forage pea, SSR, Genetic diversity

Kültürü Yapılan Yem Bezelyesi (*Pisum sativum* var. *arvense* L.) Genotiplerinin SSR Markırları ile Genetik Çeşitliliğinin Belirlenmesi

Öz

Dünya genelinde, yüksek besin değerine sahip önemli bir baklagil türü olan yem bezelyesi (*Pisum sativum* var. *arvense* L.) hayvanlara yedirilmektedir. Doğu Anadolu Bölgesi'nin yüksek rakımlarında, yem bezelyesi uzun zamandır hayvan yemi olarak yetiştirilmektedir. Yem bezelyesi yerel çeşitlerde daha az yaygın hale gelmekte ve ticari çeşitlerde daha kolay bulunabilmektedir. Bu nedenle, bu çalışmanın amacı SSR belirteçlerini kullanarak birden fazla ticari ve yerel yem bezelyesi popülasyonunun genetik çeşitliliğini ve genom boyutu varyasyonunu belirlemektir. Bu çalışmada, Türkiye'de yetiştirilen 11 popülasyon ve 7 ticari çeşit olmak üzere toplam 18 örnek ve 11 SSR belirteci kullanılmıştır. Örneklerin %89,2'si toplamda 66 polimorfik bant üretmiştir. Bu polimorfik aleller 3 ile 11 arasında değişmekte olup ortalama 6'dır. Yerel çeşitler ve ticari çeşitler, genetik çeşitlilik dendrogramı ile iki ana gruba ayrılmıştır. En az genetik uzaklık Töre ve Taşkent çeşitleri arasında, en fazla ise 1 ile 6 yerel çeşit arasında bulunmuştur. Bu çalışmanın sonuçları ıslah programlarında yerel çeşitlerin dikkate alınması gerektiğini göstermektedir.

Anahtar Kelimeler: *Pisum sativum* L., Yem bezelyesi, SSR, Genetik çeşitlilik

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1. Introduction

Forage pea (*Pisum sativum* var *arvense* L.) is an annual, self-fertilized, diploid (2n = 2x = 14) legume forage crop that is widely grown in cool-season regions. [1,2]. Pea is cultivated for their rich contents of proteins, carbohydrates, vitamins, minerals, and dietary fibers Bastianelli et al. [3] and used in rotation with cereals, increases soil fertility by fixing atmospheric nitrogen [4]. Pea is grown both for grass and for grain and has adapted to high altitude and cool regions in the Eastern Anatolia Region. The local varieties, which are a valuable genetic resource, are grown in summer and have population characteristics [5]. Local varieties are suitable resources that have been adapted to the region grown by the farmers for many years and whose genetic characteristics should be known for breeding studies [6]. Genetic variation is observed within a species or in gene alleles that may occur within or between populations. Therefore, genetic variations within species are very useful for sustainable agriculture and food security [7]. SSRs have provided species-specific allele patterns in plants and can be useful markers for clarifying the genetic similarity and differences between germplasm collections [2, 4]. Most studies of genetic variation in peas mainly rely on morphological features in germplasm collections using selection techniques, but these techniques still have some limitations due to closely related populations and species. The information obtained from the molecular marker technique is widely used for the development of varieties of various species. Therefore, knowledge of genetic variation is a vital feature in the development of new varieties. The purpose of this study is to evaluate the genetic relationship and diversity by using SSR markers of pea varieties and landraces varieties.

2. Materials and methods

2.1.Plant Material

Eleven populations and seven pea varieties were utilized as study materials (Table 1). Forage pea seeds germinated in 0.7% (w/w) water agar gel (pH 6.8). To initiate seedlings, they were kept in a temperature-controlled growth chamber at 25/27 °C (day/night) with a 16-hour light and 8-hour dark cycle for 8 days. After about two weeks, plant leaves from all populations were collected for DNA extraction.

Table 1. The Number, Id, Types and Origin of forage pea varieties and populations

Number	ID	Type	Origin	Resource/Registrant
1	Population 1	Landrace	Turkey	Yerlisu/Erzurum
2	Population 2	Landrace	Turkey	Bayburt
3	Taşkent	Variety	Turkey	Selçuk University
4	Töre	Variety	Turkey	Namık Kemal Unv.
5	Population 5	Landrace	Turkey	Arıbahçe/Erzurum
6	Population 6	Landrace	Turkey	Altınbulak/Erzurum
7	Population 7	Landrace	Turkey	Altınbulak/Erzurum
8	Population 8	Landrace	Turkey	Umudum/Erzurum
9	Population 9	Landrace	Turkey	Kumluyazı/Erzurum
10	Ulubatlı	Variety	Turkey	Uludağ University
11	Özkaynak	Variety	Turkey	Selçuk University
12	Population 12	Landrace	Turkey	Yusufeli/Artvin
13	Population 13	Landrace	Turkey	Mülkköy/Erzurum
14	Kirazlı	Variety	Turkey	Uludağ University
15	Population 15	Landrace	Turkey	Ortadüzü/Erzurum
16	Ürünlü	Variety	Turkey	Uludağ University
17	Livioletta	Variety	Turkey	Maro Tarım
18	Population 18	Landrace	Turkey	Kars

2.2. SSR

Total genomic DNA was purified following CTAB (cetyltrimethylammonium bromide) method [9] with minor modifications. Eleven primer pairs detecting SSR loci in the forage pea genome were used in the amplification reactions [2, 9]. PCR reactions were applied in a 50 µL volume consisting of (200 ng) 1 µL genomic DNA, 4 µL of dNTPs, 5 µL of 10x buffer, 0,5 µL BioVan Taq pol (5U), 1 µL each primer (Table 2) and 37,5 µL of ddH_2O. The cycling conditions for the PCR reaction were as follows; 95^0C for 5 min, 94^0C for 1 min, 58^0C for 45 s, and 72^0C for 1 min and 72^0C final extension for 38 cycles. The PCR products were electrophoresed and visualized using a 2% (w/v) agarose gel. Fragment sizes were determined by DNA ladder (Thermo Scientific™ , SM0314).

2.3. Marker Data Analysis

The data were scored as the presence of band (1) and absence of band (0) for the PCR-SSR results. We utilized STRUCTURE software [10] to ascertain the population structure of forage pea varieties and populations, defining the potential number of populations (K) as ranging from 1 to 10. The optimal K value [12] was found by applying the ad hoc procedure [10] and Evanno's [11] method. Using the Principal Component Analysis (PCA) software, we examined the genetic variation in pea populations as a second approach. We then used GenAlEx to plot the populations according to the first to principal coordinates. Using the software POWERMARKER V3.25, we constructed a neighbor-joining dendrogram and identified the genetic distance matrix [14]. Next, we drew dendrograms using DENDROSCOPE [15]. Using the software POWERMARKER V3, we performed genetic diversity analyses by calculating allele numbers, major allele frequency, gene diversity, and polymorphic information content parameters. We also assessed the number of polymorphic bands and rates [16].

3. Results and Discussion

From the forage pea marker databases, we selected 11 SSR markers transferability present on the genomes of *P. sativum* (Table 2). The analyses of the markers in chosen pea vaireties and populations were carried out via agaraose gele electrophoresis analysis (Figure 1).

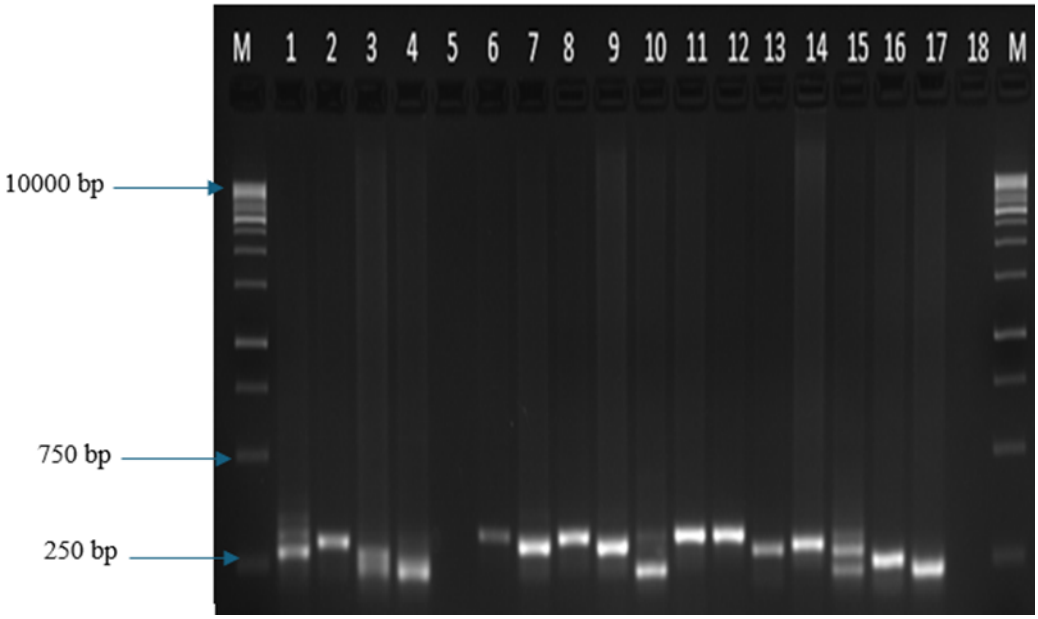

Figure 1. SSR _PCR experiments' agarose gel electrophoresis demonstrating primer 5 amplification. Fragment sizes were determined by DNA ladder (Thermo Scientific™ , SM0314)

When we look at the population structure analysis results, the optimal K value was found as 2 based on the ad hoc procedure and the Evanno method [11] (Figure 2a, b, and Figure 3).

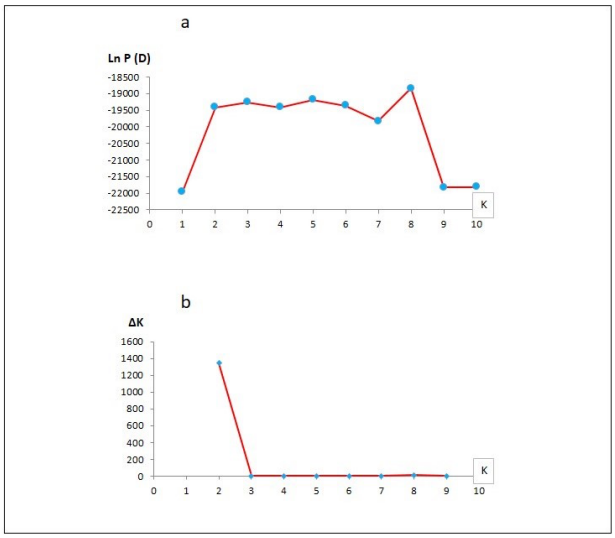

Figure 2. *ad hoc* procedure described by Pritchard et al. (2000) (a) and (ΔK) method developed Evanno et al., 2005 (b) for determining optimal value of K. Both of these procedures showed optimal values of K= 2 for forage pea varieties.

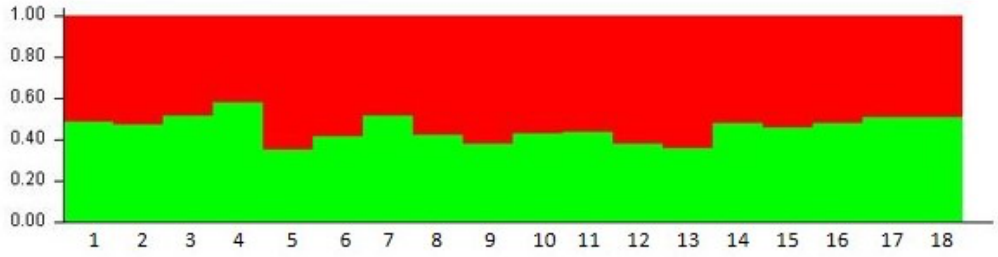

Figure 3. Structure results based on Bayesian inference among 18 individual genotypes analyzed with 11 SSR markers assuming two clusters.

K = 2, for Pea populations. 7 commercial cultivars of forage pea; 3: *Taşkent*, 4: *Töre*, 10: *Ulubatlı*, 11: *Özkaynak*, 14: *Kirazlı*, 16: *Ürünlü*, 17: Livioletta and 11 populations of pea (*Pisum Sativum* L.); 1:Yerlisu, 2: *Bayburt*, 5: Arıbahçe, 6: Altınbulak, 7: Altınbulak, 8: Umudum, 9: Kumluyazı, 12: Yusufeli, 13: Mülkköy, 15: Ortadüzü, 18: *Kars*.

The findings of the STRUCTURE analysis indicated that there were two primary groups: populations and varieties (Figures 2a, b, and 3), but the distinction between the two was not based on genetic content. Generally, genomic differences were in the range of 0,30-0,60 with small deviations (Figure 3). Sharma et al [18], obtained similar results with K= 2 value from cultivar and breeding lines of pea but with sharp separation. Tahir et al. [28], found that pea genotypes were classified into 2 clusters with population Structure Analysis. Ahmad and Kaur [30], reported that the classification of pea accessions into 4 groups depends on model-based population structure analysis.

Table 2. Sequence and melting temperature (T_m) of SSR primers used

Primer No	Primer	Forward sequence (5'-3')	Reverse sequence (5'-3')	T_m
1	PSGAPA1a	GACATTGCCAATAACTGG	GGTTCTGTTCTCAATACAAG	53, 51
2	PSADH1a	GATGTGATAGGCCTAGAACAAGC	CAGTCACACACTACAAGAGATC	58, 61
3	PSMPSAA278b	CCAAGAAAGGCTTATCAACAGG	TGCTTGTGTCAAGTGATCAGTG	58, 58
4	PSMPSAD237	AGATCATTTGGTGTCATCAGTG	TGTTTAATACAACGTGCTCCTC	57, 57
5	PSAD270	CTCATCTGATGCGTTTGGATTAG	AGGTTGGATTTGTTGTTTGTTG	57, 59
6	PSAA456	TGTAGAAGCATAAGAGCGGGTG	TGCAACGCTCTTGGTTGATGATT	57, 60
7	PSMPSAA476e	TAGTTTTGAACTTTGGCCGTAT	CACACCCTAATCTAGGCTATCC	60, 55
8	PSMPSAA473	CAATCGATCAGCAGAGTCCCCTA	AAGCTCACCTGGTTATGTCCCT	60, 60
9	PSP4OSGa	CAACCAGGCCATTATACACAAACA	GGCAATAAAGCAAAAGCAGGA	53, 57
10	AA430902	CTGGAATTCTTGCGGTTAAC	CGTTGGTTACGATCGAGCAT	58, 56
11	PSBLOX13	CTGCTATGCTATGTTTCACATC	CTTGCTTGCAACTTAGTAACAG	57, 57

To further explain the population structure and genetic variation of pea populations, we carried out Principal Component Analysis (PCA) based on genomic compositions. PCA results suggested two main clusters separation but with some minor exceptions. Generally, 7 varieties and 11 populations of forage peas separated each other into two main groups. Ulubatlı(10) and

Özkaynak(11) varieties are exceptionally located in the center. In addition, Yerlisu(1), Ortadüzü(15), and Kars(18) populations were also located among 7 varieties contrary to expectations (Figure 4, 5).

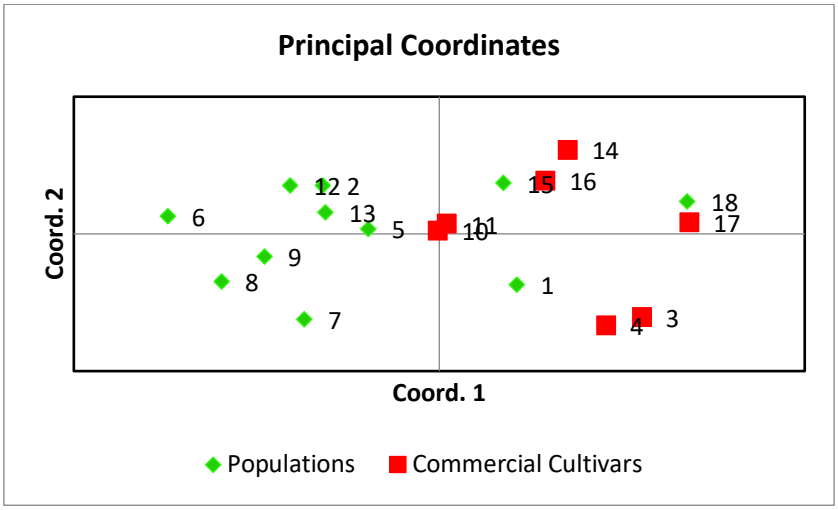

Figure 4. Clusters of eighteen pea populations based on the first two principal components obtained from an analysis of 11 SSR markers. (Red colors show commercial cultivars of forage pea; 3: *Taşkent*, 4: *Töre*, 10: *Ulubatlı*, 11: *Özkaynak*, 14: *Kirazlı*, 16: *Ürünlü*, 17: Livioletta and 11 populations of pea (*Pisum Sativum* L.); 1:Yerlisu, 2: *Bayburt*, 5: Arıbahçe, 6: Altınbulak, 7: Altınbulak, 8: Umudum, 9: Kumluyazı, 12: Yusufeli, 13: Mülkköy, 15: Ortadüzü, 18: *Kars*.)

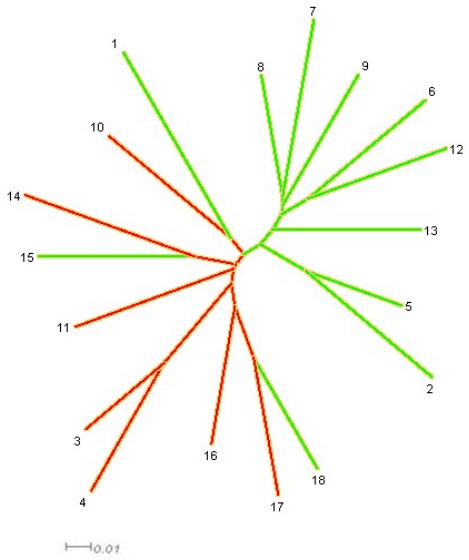

Figure 5. Neighbor-joining dendrogram of 18 populations of forage peas.

Red colors show commercial cultivars of forage pea;3: *Taşkent*, 4: *Töre*, 10: *Ulubatlı*, 11: *Özkaynak*, 14: *Kirazlı*, 16: *Ürünlü*, 17: Livioletta and Green colors show 11 populations of pea:1: Yerlisu, 2: *Bayburt*, 5: Arıbahçe, 6: Altınbulak, 7: Altınbulak, 8: Umudum, 9: Kumluyazı, 12: Yusufeli, 13: Mülkköy, 15: Ortadüzü, 18: *Kars*.

As another approach, genetic distance matrices were determined and Neighbor-joining (NJ) dendrograms were created to investigate genomic separations of pea populations. With the genetic association dendrogram created using NJ from the genetic similarity matrix, it was seen that 18 forage pea populations were generally divided into two main groups with small differences. Ulubatlı (10) was located close to the central part of the dendrogram, while Livioletta (17) and *Kars* (18) populations were among the varieties as different groups (Figure 5). When the PCA and NJ analysis results are evaluated in general, while both 7 and Kars (18) populations are close to varieties, Ulubatlı (10) varieties are located in completely different regions in terms of their genetic structures. The closest genetic relationship was obtained between the two varieties Taşkent (3) and Töre (4) varieties, and the most distant ones were Yerlisu (1) and Altınbulak (6) populations among all forage pea varieties groups (Table 3). The main reason why some genotypes were exceptionally found in different regions in PCA and NJ analysis is that these genotypes used in the study have genetic diversities based on distance. These findings line up with those of the studies below. The UPGMA dendrogram as used SSRs showed two main groups for 11 varieties of peas [24]. Tahir et al. [28], found that pea genotypes were separated into three major clusters using PCA but with small exceptions again Rana et al. [1] constructed the NJ tree using the Dice coefficient and SSR information and separated pea accessions into 3 groups. The genetic similarity among the 19 peas was graphically represented by a UPGMA dendrogram using 5 SSR markers. The dendrogram showed two main groups [4].

Genetic diversity within the species allows the development of varieties with desired agronomic characteristics. In this respect, it is important to know the genetic diversity and genetic affinity of local varieties as well as commercial varieties. Because local varieties can play an important role in eliminating the deficiencies of commercial varieties. DNA-based molecular markers are mainly used to evaluate genetic diversity [17, 18]. Many marker techniques were developed for the molecular characterization of pea varieties and populations [17, 19, 20] but SSR markers have been often preferred due to their high level of polymorphism and reliability for pea genetic diversity research [1, 18, 21, 22, 23, 24, 25, 26, 27, 28, 29].

Table 3. Genetic Distance Matrix (GD) of forage pea populations calculated for all loci in GenAlEx 6.1 program.

Pop	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	0																	
2	25	0																
3	22	31	0															
4	28	29	**14**	0														
5	24	15	22	26	0													
6	**33**	22	29	31	19	0												
7	23	28	23	27	19	22	0											
8	22	19	24	24	18	15	17	0										
9	22	27	28	28	20	19	19	16	0									
10	21	26	25	25	21	26	26	21	21	0								
11	26	23	22	24	20	19	23	20	24	19	0							
12	28	23	28	30	22	17	27	18	18	23	24	0						
13	24	21	24	28	20	19	23	20	18	17	24	18	0					
14	25	24	27	31	23	28	30	29	29	22	21	27	25	0				
15	28	25	22	28	24	25	27	26	24	23	22	22	18	19	0			
16	27	22	25	23	19	26	28	25	23	22	19	19	23	20	25	0		
17	22	29	22	26	22	31	29	28	28	23	24	28	26	25	24	19	0	
18	25	28	19	25	23	30	30	29	25	28	23	23	25	22	21	18	15	0

In this study, the overall number of alleles ranged from 6 to 11 with an average of 6. Major allele frequency ranged from 0,50 to 0,94 with an average of 0,77, gene diversity states heterozygous, gene diversity ranged from 0,10 to 0,50 with an average of 0,34, while PIC values ranged from 0,10 to 0,38 with an average of 0,28 (Table 4).

Table 4. Means along with minimum and maximum ranges of genetic diversity statistics based on 11 SSR primers of 18 individual genotypes of forage pea

Parameters of Genetic Diversity	**Means**	**Minimum values**	**Maximum values**
The Number of Allele	6	3	11
Major Allele Frequency	0,77	0,50	0,94
Gene Diversity	0,34	0,10	0,50
PIC	0,28	0,10	0,38

PIC: Polymorphic Information Content

The 11 SSR markers successfully produced a total of 66 polymorphic bands by a percentage of 89,2% for 18 forage pea populations. Primer “PSAD270” has the lowest number of polymorphic bands (3 bands), while primer “PSGAPA1a” has the highest number of polymorphic bands (11 bands) (Table 5).

Table 5. The Numbers of monomorphic, polymorphic and total bands with rates of Polymorphic bands (%) based on 11 SSR Primers of 18 individual genotypes of forage pea

The Number of SSR Primers	Primers	The Number of Monomorphic Bands	The Number of Polymorphic Bands	The Number of Total Bands	Rates of Polymorphic Bands (%)
1	PSGAPA1a	-	11	11	100
2	PSADH1a	-	9	9	100
3	PSMPSAA278b	1	4	5	80
4	PSMPSAD237	1	4	5	80
5	PSAD270	1	3	4	75
6	PSAA456	1	7	8	87,5
7	PSMPSAA476e	-	6	6	100
8	PSMPSAA473	2	4	6	66,5
9	PSP4OSGa	-	4	4	100
10	AA430902	1	9	10	90
11	PSBLOX13	1	5	6	83
Total		8	66	74	89,2

This result is consistent with some previous studies. Ford et al. [31] reported that the mean number of alleles was close to 6 in a study on 20 *P. sativum* accessions and Baranger et al. [17] determined the mean number of alleles per locus was 5 using 148 pea accessions. Nasiri et al. [9], obtained the mean of alleles per locus was 5.9 in 20 pea varieties and 57 wild pea accessions using 10 SSR markers. Nisar et al. [32], noticed an average of 4.69 alleles per SSR locus in a research investigated with Pakistani pea. Hanci and Gökçe [33] stated that the mean number of alleles was 3.49. In addition, several studies have also reported a wide range (3,4-9,9) in the

average number of alleles per locus in pea [17, 21, 22, 31, 32, 33, 34, 35, 36, 37]. This variability of the mean of allele frequency per locus is based on the type of marker system and the number of genotypes [35].

Various PIC values were reported in different research conducted by pea varieties [9, 21, 36, 38, 39]. In a study conducted by 8 pea varieties, the average PIC value was reported as 0.62 using 188 polymorphic bands [21]. Jain et al. [27] noticed an average PIC value of 0.29 for SSR markers varied from 0.01 to 0.56. Similarly, the PIC value was found as the mean of 0.50 with a range of 0.32-0.63 [32]. In another study conducted by Sharma et al. [18], the minimum PIC value was found to be 0.095 while the maximum PIC value was found to be 0.500 with an average of 0.349. The PIC values are the demonstration of marker effectiveness. Therefore, it varies according to the number of markers and genotypes used in studies.

4. Conclusion

In conclusion, PCA and NJ dendrogram analysis supported the population structure analysis results. Forage pea populations are represented by 2 clusters of 7 varieties and 11 populations. Some small exceptions were observed based on genetic distance. In light of diversity parameters, it is seen that there is moderate genetic diversity between both varieties and populations of forage peas and similar results in similar studies. In addition, values of diversity parameters are varied depending on the number of markers and genotypes used. Therefore working with more markers and genotypes will contribute to the reliability of the study. With this study, it was concluded that there are genetic differences between pea varieties and that landraces can be used in breeding programs.

Ethics in Publishing

There are no ethical issues regarding the publication of this study.

Author Contributions

Concept: B.Y., P.U., Design: B.Y., P.U., Execution: B.Y., P.U., Material supplying: B.Y., Data acquisition: B.Y., P.U., Data analysis/interpretation: B.Y, Writing: B.Y., Critical revision: B.Y., P.U.

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