Available online freely at www.isisn.org

Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network

RESEARCH ARTICLE BIOSCIENCE RESEARCH, 2022 19(4): 2209-2218. **OPEN ACCESS**

Determination of genetic association level with ISSR markers for the development of Canned and Dry Pea Lines

Mustafa Yorgancilar*, Emine Atalay, Ercan Ceyhan and Rahim ADA

Selcuk University, Faculty of Agriculture, Department of Field Crops, Konya, **Turkiye**

***Correspondence:** myorg@selcuk.edu.tr **Received 09-11-2022, Revised: 29-12-2022, Accepted: 30-12-2022 e-Published: 31-12-2022**

Variation between 18 genotypes selected from segregated F_2 population from diallel crosses between 3 commercial edible pea cultivars (Progress, Rondo, and Ultrillo) and 5 pea lines (PS3055, PS3057, PS3073, PS4009 and PS4028) including their parents were investigated using ISSR markers. The DNAs of 26 genotypes selected from the F_2 rank lines and containing parents were isolated. These genotypes were subjected to PCR with ISSR molecular markers determined as a result of preliminary studies. PCR amplification based on ISSR primers showed that the pea genotypes had variation despite sharing the same parents. Some of the genotypes were located in different groups in the dendrogram despite showing similar characteristics which means continuing divergence and gen interaction. As a result of the investigated agronomical characteristics presented a sufficient genetic variation in the population. These data were evaluated together with the ongoing classical selection, and the selection of promising lines for the development of canned and dried peas continued.

Keywords: Pea, breeding, ISSR marker, genetic relationship, variation

INTRODUCTION

With a total global cultivation area of 9.721.945 ha for dry and green consumption and a production of 34.509.067 tons, peas have a significant position in terms of production among edible legumes (FAO, 2020). Its consumed as green, dry and canned food source. Grain size is among one of the most important quality criteria in dry and canned consumption. This is one of the most important quality features in the trade of peas and has indispensable importance. As with all plants, the classification of peas is made according to grain size and is priced accordingly in the market. Grain size is an important yield and adaptation feature, which is partly controlled by the environment and partly genetically, always determined by consumer preferences (Ceyhan and Mülayim, 2003). Today's breeding studies are generally in this direction.

Morphological and genetic identification of plant resources in plant breeding studies allows faster and more effective use of gene resources. Morphological similarity of plant material may cause errors in the selection based on phenotype. It misleads people about whether the genotypes used are different or the same. In this case, the characterization of plant materials with molecular DNA markers has provided significant benefits (Yorgancilar et al. 2009; Filiz and Ibrahim, 2011; Bolouri et al. 2019).

Genetic markers have been defined as chromosomes or regions of chromosomes that can be traced from parents to offspring. It is used intensively to detect intra- and inter-species genetic variation in most cultivated plant, and to distinguish between species or genotypes within a species (Yalım, 2005). DNA marker techniques such as RAPD (Random Amplified Polymorphic DNA), ISSR (Inter Simple Sequence Repeat), SSR (Simple Sequence Repeat), and AFLP (Amplified Fragment Length Polymorphism) are commonly used to determine genetic diversity and polymorphism (Yıldırım and Kandemir, 2001; Ahmad et al. 2012; Bhagyawant et al. 2015; Yorgancılar et al. 2015). These techniques are fast and easy widely used for diversity analysis in peas as in many plant species (Laucou et al. 1998; Burstin et al. 2001; Loridon et al. 2005; Choudhury et al. 2007; Nasiri et al. 2009; Chandrawati et al. 2017). Among these, the ISSR technique is popular due to its benefits, including the ease with which it can be used on all types of plant species and the absence of the need for sequencing (Ng and Tan 2015; Yousefi et al. 2015). Determination of the genotypic relationship level is accepted as one of the appropriate tools for parent plant selection in plant hybridization programs (Bolouri et al. 2019). Kulaeva et al. (2017) reported that pea is the oldest model object of plant genetics and one of the most agriculturally important legumes in the world (Labeeb et al. 2022). The narrow gene pool is especially critical in selecting pea genotypes for cultivation (Baranger et al. 2004). DNA markers are used especially in the recognition of genetic material in pea, and these techniques were recommended in breeding studies (Kapila et al. 2012; Gixhari et al. 2014; Adhikari et al. 2018; Thakur et al. 2018; Sharma et al. 2020).

In this study, genetic diversity of 26 genotypes obtained from selected 18 pea genotypes in F_2 generation of crosses made according to whole diallel analysis method between 3 commercial edible pea cultivars (Progress, Rondo, and Ultrillo) and 5 pea lines (PS3055, PS3057, PS3073, PS4009, and PS4028) was observed.

It was aimed to determine the promising pea lines by determining the relationship level with ISSR molecular markers and evaluating the F_2 lines obtained after hybridization together with the selection based on seed yield, seed size, and quality criteria, and the selection supported by ISSR markers.

MATERIALS AND METHODS

In this study, genotypes selected from F_2 lines and their parents of hybrids made according to the full diallel analysis method between 3 commercial edible pea cultivars (Progress, Rondo, and Ultrillo) and 5 pea lines (PS3055, PS3057, PS3073, PS4009, and PS4028) were used as material. Some morphological characteristics of the pea genotypes used in this study are given in Table 1.

DNA Isolation and Determination of DNA Concentration

The DNAs required to determine the relationship with ISSR molecular markers were obtained from the young leaves of 20-day-old seedlings by the 2xCTAB method (Doyle, 1990; Atalay and Babaoglu 2012). DNA isolation has been done in all genotypes without encountering a problem. It was determined from the 1% agarose gel electrophoresis that DNAs were not damaged and were suitable for further processing.

After isolation, the concentrations of DNA were read at 260 and 280 nm wavelengths in the spectrophotometer. Absorption values (A260) measured at 260 nm were used to determine the DNA concentration in micrograms (µg). The amount of protein at 280 nm wavelength was determined and the A260/A280 value was used to determine the purity of the DNA. Considering the spectrophotometric values of the DNAs used in the study, it is seen that the concentrations and purity are in the desired ratios. DNAs were diluted to a working concentration of 40 ng μL^{-1}, and DNA dilutions were loaded to 1% agarose gel and concentration uniformity was observed. There was no problem in DNA dilution either, it was observed that all of them could be adjusted to equal concentrations and were suitable for ISSR studies.

DNA Amplifications with ISSR Molecular Marker Technique

A total of 20 ISSR primers were tried, but 13 ISSR primers with positive results were used (Table 2). Primers were previously used in Selçuk University in the studies carried out in the Field Crops Biotechnology Laboratory of the Faculty of Agriculture, and the primers with high polymorphism rate were selected, which were used in different plant groups (corn, bean, lupine, wheat, barley). All PCR studies were carried out under the same conditions, only the amplification temperature and times were optimized for each PCR primer individually depending on the Tm temperatures of the used primers.

After the reactions were optimized by preliminary experiments, 1 µL of DNA (40 ng μL^{-1}) and 24 µL of reaction mix [2.5 µL 10x PCR buffer solution (SolisBioDyne), 2.5 µL 25 mM Mg^{+2} (SolisBioDyne), 0.4 µL 10 mM dNTP) (Thermo), 0.3 µL 500 U Taq DNA Polymerase (SolisBioDyne), 0.5 µL 10 pmol. μL^{-1} primer and 17.8 µL double distilled water] were performed on PCR thermocycler (Techne, Longgene A300, VWR) programmed to perform 40 cycles in total as Touchdown PCR, taking into account the binding (Tm) temperatures of the primers. The first phase started after 3 min of pre-denaturation at 94°C for 1 min at 94°C, 1 min at a temperature 5-6°C above the standard bonding temperatures (Tm) of the primer (this temperature decreased by 0.5°C in each cycle and the first phase eventually dropped to standard Tm) and 12 cycles set at 72°C for 2 min, the second stage at 94°C for 1 min, at Tm 1°C below standard binding temperature for 1 min and at 72°C. It was completed in 30 cycles of 2 minutes. When the cycles were completed, the PCR reaction was continued for 10 more minutes at 72°C, and at the end of this period, the process was terminated.

PCR products were run in 1% agarose (Sigma-Aldrich) gel electrophoresis and visualized with the Vilber Lourmat (France) imaging system. Consistent bands produced as a result of PCR amplifications were taken into account in scoring.

Analysis of PCR Amplification Data

Determination of polymorphism rates of primers

Polymorphism rates of ISSR primers were found by dividing the number of polymorphic bands/alleles obtained from the primers by the total number of bands / alleles and multiplying by 100.

Polymorphism Rate (%) = Number of polymorphic alleles/Total number of alleles x 100

Table 1: Some morphological properties of pea genotypes used in this study.

No	Code	Genotype name	Flower colour	Grain shape	Hilum colour	Grain shape	Cotiledon colour	Seed pot cracking
1	1	RondoxPS3057	White	Large	White	Wrinkled	Green	Absent
2	2	UltrilloxPS3057	White	Large	White	Wrinkled	Dark Green	Absent
3	4	PS3055xRondo	White	Large	White	Wrinkled	Green	Absent
4	8	PS3055xPS4009	White	Large	White	Wrinkled	Light Green	Existent
5	11	PS3057xPS3055	White	Medium-Large	White	Wrinkled	Light Green	Existent
6	12	ProgressxPS3057	White	Small	White	Round	Light Green	Absent
7	14	PS3057	White	Medium	White	Round	Light Green	Absent
8	16	PS3073	Pink	Medium-Large	Black	Round	Light Brown	Absent
9	17	Rondo	White	Medium	White	Wrinkled	Yellow-Green	Existent
10	18	PS3055	White	Medium	White	Round	Yellow	Absent
11	19	Progress	White	Large	White	Wrinkled	Light Green	Absent
12	21	Ultrillo	White	Large	White	Wrinkled	Yellow-Green	Existent
13	22	UltrilloxPS4028	White	Medium-Large	White	Wrinkled	Light Green	Absent
14	23	PS3073xUltrillo	Pink	Large	Black	Wrinkled	Brown-Green	Absent
15	25	PS4028xPS4009	White	Large	White	Wrinkled	Green	Existent
16	27	ProgressxPS3055	Pink	Large	Black	Wrinkled	Brown-Red	Absent
17	29	PS3073xPS3057	Pink	Medium-Large	Black	Round	Brown	Absent
18	30	RondoxPS3073	White	Small	White	Round	Green	Absent
19	33	RondoxPS4009	White	Large	Black	Wrinkled	Dark Green	Absent
20	34	ProgressxPS3073	Pink	Medium-Large	Black	Round	Brown	Absent
21	36	UltrilloxRondo	White	Small	White	Wrinkled	Light Green	Existent
22	37	PS4009	White	Small	White	Round	Light Green	Absent
23	38	PS4028	White	Medium	White	Wrinkled	Dark Green	Absent
24	41	PS4009xRondo	White	Large	Black	Wrinkled	Dark Green	Absent
25	42	PS3057xRondo	White	Large	White	Wrinkled	Green	Absent
26	43	ProgressxRondo	White	Large	White	Wrinkled	Yellow	Existent

Table 2: Characteristics of ISSR primers used in the study.

Charact	Sequence (5'-3')	GC ratio (%)	Tm (°C)
F3	5'-AGAGAGAGAGAGAGAGCG-3'	55.6	56.0
F4	5'-AGAGAGAGAGAGAGAGTG-3'	50.0	53.7
F6	5'-CCACCACCACCACCA-3'	66.7	53.3
F8	5'-GCCGCCGCCGCCGCC-3'	100.0	67.0
M1	5'-AGCAGCAGCAGCAGCAGCG-3'	68.4	63.1
M2	5'-ACCACCACCACCACCACCG-3'	68.4	63.1
M3	5'- AGCAGCAGCAGCAGCAGCC-3'	68.4	63.1
M5	5'- GAGAGAGAGAGAGAGAGAC-3'	52.6	56.7
M7	5'- AGAGAGAGAGAGAGAGAGC- 3'	52.6	56.7
M9	5'- ACACACACACACACACCG-3'	55.6	56.0
M15	5'-CACACACACACACACAAG-3'	50.0	53.7
M16	5'-CACACACACACACACAGC-3'	55.6	56.0
M17	5'-CAGCACACACACACACACA-3'	52.6	56.7

Determination of polymorphism information content (PIC) of primers

The polymorphism information content (PIC) of the ISSR primers used in the study was determined with the help of the following formula according to Smith et al. (1997). Alleles were scored as present (1) and absent (0) in genotypes, and then their frequencies were calculated separately. Pi in the formula is the frequency of the "i" allele.

$PIC = 1 - \Sigma Pi^2$

Determination of the heterozygosity value (H) of the primers

The heterozygosis rate of the SSR primers used in the study was calculated using the basic equation used in population genetics (Nei 1972). Here p, "i". The number of times the allele exists, and q is "i". Represents the number of absences of the allele.

$H = 1-p^2-q^2$

The Resolving power of the primers (RP)

The separation power of the primers was calculated with the help of the formula developed by Prevost and Wilkinson (1999). The p in the formula is the ratio of the "i" allele in the 26 genotypes.

$RP = \Sigma\ Ib \quad Ib = 1 - [2 \times (0.5 - p)]$

Generating Genetic Relationship Coefficients and Dendrogram

The score able bands obtained as a result of PCR amplifications with ISSR primers were evaluated, and the products amplified by the primers were scored as 1/0 based on presence/absence of bands. From these scoring values, a genetic similarity matrix was created according to SM (Simple Matching) coefficient by using NTSYS-2.1 pc program and genetic relationship dendrogram in which genotypic variation was exhibited was obtained according to UPGMA method (Rohlf, 1993). Bootstrapping analysis of the genotypes was performed using the WINBOOT Package Program (Yap and Nelson, 1996).

RESULTS

A total of 87 scoreable bands were obtained from the 13 ISSR primers used. Of these scoreable bands, 83 were polymorphic and 4 were monomorphic. While F3 and M16 primers were the primers that produced the most (10) polymorphic bands, the F6 primer was determined as the primer that formed the least (3) polymorphic bands. The rate of polymorphism in the 13 primers varied between 75% and 100%, the lowest polymorphism was observed in F6 primer (75%), while the highest polymorphism (100%) was observed in F3, F4, F8, M3, M5, M7, M9, M16 and M17 primers. The mean polymorphism was calculated as 95% (Table 3).

It was determined that a total of 953 alleles were obtained with 13 primers in 26 genotypes with PCR amplifications, and 849 of these alleles were polymorphic (Table 4). The polymorphic allele rate was calculated as 89%. While most alleles were obtained from the M15 primer (107), the primer with the highest number of polymorphic alleles was the F3 primer with a value of 94.

The data of the products obtained in PCR amplifications with ISSR primers were evaluated and the resolving power (RP), heterozygosity ratio (H), and polymorphism information content (PIC) values were determined separately for each primer. The average resolving power (RP) values of the primers were found in the range of 0.28-0.77, and the total resolving power was calculated as 5.85. The highest value was obtained from primer M5 (0.77), and the lowest value was obtained from primer M7 (0.28). The mean RP value was determined as 0.45, and the majority of the primers were found to have RP values close to the mean value. This situation can be interpreted as the primers used in the study being of equal quality in terms of resolving power.

The primers used in the study showed heterozygosity in the range of 0.21-0.46. The highest value was obtained from primer M5 (0.46), and the lowest value was obtained from primer M17 (0.20). Considering the mean heterozygosity value of the polymorphic information contents (PIC) of the 13 ISSR primers used ranged from 0.41 to 0.96. While the mean PIC was determined as 0.75, the highest value was obtained from the F6 primers (0.96) and the lowest value was obtained from the M17 primer (0.41). When evaluated in general, it is seen that the PIC values of the primers used in the study are 0.31, it can be interpreted that heterozygosity is low in pea genotypes or that they are similar in terms of the examined primer regions.

The distribution of observed allele numbers of the products obtained in PCR amplifications with ISSR primers in terms of genotypes and primers is also given in Table 5. Considering the average number of alleles in the pea genotypes used as material, the least allele is in the PS3055xRondo genotype (2 pieces), the most alleles (6 pieces) are RondoxPS4009, UltrilloxRondo, PS4009, PS4009xRondo, PS3057xRondo and ProgressxRondo were observed in 6 genotypes. It was determined that the Rondo cross-genotypes used in the study were similar in terms of allele numbers and the allele numbers were higher than the other genotypes. Considering that it is a combination of hybrids and contains common parents, it can be concluded that the genetic information is similar in the genotypes examined and that the primers can give similar or close values (Table 6).

The similarity coefficients of the genotypes showed an observation between 0.49-0.89. When the closest and farthest genotypes were examined according to the similarity coefficients, it was seen that genotypes 3055 and 3073 are far from the majority of the others. It was determined that 3055 was significantly different from other genotypes with an average similarity coefficient of 0.46 and 3073 an average of 0.55 (Table 7).

Table 3: Band (number) and polymorphism rates (%) obtained with ISSR primers used.

ISSR Primers	Sequence (5'-3')	Scorable Band Order (number)	Polymorphic Bands Order (number)	Polymorphism (%)
F3	$(AG)_8CG$	10	10	100
F4	$(AG)_8TG$	7	7	100
F6	$(CCA)_5$	4	3	75
F8	$(GCC)_5$	5	5	100
M1	$(AGC)_6G$	8	7	88
M2	$(ACC)_6G$	6	5	83
M3	$(AGC)_6C$	8	8	100
M5	$(GA)_9C$	5	5	100
M7	$(AG)_9C$	6	6	100
M9	$(AC)_8CG$	6	6	100
M15	$(CA)_8AG$	7	6	86
M16	$(CA)_8GC$	10	10	100
M17	$CAG(CA)_8$	5	5	100
Total		87	83	-
Mean		6.69	6.38	95

Table 4: Calculated values obtained from PCR amplifications.*

ISSR Primers	Total Allel Number	Polymorfic Allel Number	Resolving Powder (RP)	Heterozygous Rate (H)	Polymorphic Information Content (PIC)
F3	94	94	0.42	0.30	0.79
F4	50	50	0.31	0.24	0.85
F6	40	14	0.36	0.28	0.96
F8	59	59	0.60	0.40	0.75
M1	75	49	0.34	0.28	0.87
M2	95	69	0.54	0.38	0.66
M3	79	79	0.57	0.38	0.81
M5	68	68	0.77	0.46	0.71
M7	76	76	0.28	0.21	0.62
M9	45	45	0.58	0.38	0.90
M15	107	81	0.32	0.24	0.60
M16	75	75	0.45	0.31	0.87
M17	90	90	0.31	0.20	0.41
Total	953	849	5.85	4.06	9.8
Mean	73.30	65.30	0.45	0.31	0.75

*Values are calculated considering all alleles obtained from a primer. RP, H and PIC values were determined as average by dividing the obtained value by the number of polymorphic alleles

Tablo 5: Genotypes and primary distribution of allele numbers observed in pea.*

	Genotypes*																								
Primers	1	2	4	8	1	1	1	1	1	1	1	2	2	2	2	2	2	3	3	3	3	3	3	4	4	4
F4	2	2	1	3	2	3	1	2	2	3	1	3	3	3	1	1	1	1	3	2	2	1	1	1	3	2
F6	1	1	1	1	1	2	2	3	1	2	2	1	1	1	1	4	2	2	2	1	1	2	1	1	2	1
F8	1	1	3	3	3	2	3	3	2	1	2	2	3	3	2	2	3	2	3	3	2	2	2	2	2	2
F3	4	6	2	4	4	3	5	5	3	4	3	3	4	5	3	4	5	4	3	2	4	3	4	2	3	2
M1	2	2	1	2	5	4	2	2	3	4	4	6	5	6	3	2	3	2	2	2	3	2	2	2	2	2
M2	5	3	1	3	4	5	6	4	3	5	4	3	3	3	3	4	4	4	4	2	3	5	4	4	2	4
M3	3	3	1	4	4	5	1	6	5	3	2	2	3	5	4	0	4	1	4	2	5	3	2	3	3	1
M5	2	2	3	3	3	2	3	1	2	1	2	2	3	2	4	3	4	3	4	3	4	3	2	2	3	2
M7	2	2	3	3	3	3	4	4	2	3	3	4	2	2	5	3	3	3	4	4	3	3	2	3	2	1
M9	1	1	1	2	1	4	1	1	1	5	1	2	3	4	1	1	1	3	0	3	1	1	2	2	1	1
M15	4	4	4	4	4	6	1	3	5	3	5	5	4	3	5	5	3	6	3	3	3	6	4	4	5	5
M16	5	5	0	3	3	4	1	2	5	3	1	3	2	1	3	1	4	3	3	2	4	3	4	4	3	3
M17	3	3	4	3	3	3	4	3	3	4	4	4	3	3	4	4	4	4	4	4	3	3	3	4	3	3
Total Allel	36	37	29	46	51	58	48	55	54	59	53	61	61	64	64	61	70	68	72	67	74	74	71	75	76	72
Mean Allel	3	3	2	4	4	4	4	4	4	5	4	5	5	5	5	5	5	5	6	5	6	6	5	6	6	6

* The numerical codes of the genotypes are given in Table 1.

Table 6: The closest and farthest genotypes according to the similarity coefficients.*

Genotypes	Furthest	Nearest	Genotypes	Furthest	Nearest
1	16 (0.58)	38 (0.85)	23	27 (0.51)	22 (0.77)
2	18 (0.60)	1 (0.80)	25	18 (0.51)	21 (0.79) - 36
4	18 (0.53)	19 (0.78) - 43 (0.78)	27	23 (0.51)	14 (0.78)
8	18 (0.53)	22 (0.83)	29	18 (0.49)	33 (0.79)
11	27 (0.56)	8 (0.77) - 22 (0.77)	30	16 (0.57) - 18	19 (0.77)
12	16 (0.54)	11 (0.73)	33	18 (0.51)	34 (0.83)
14	18 (0.54)	19 (0.79)	34	18 (0.51)	41 (0.79)
16	21 (0.52)	33 (0.68) - 34 (0.68) - 37	36	18 (0.49)	25 (0.75)
17	27 (0.54)	42 (0.79)	37	18 (0.51)	27 (0.77)
18	29 (0.49) - 36	23 (0.64)	38	18 (0.58)	1 (0.85)
19	18 (0.56)	14 (0.79) - 21 (0.79)	41	18 (0.54)	1 (0.84)
21	16 (0.52)	25 (0.79)	42	18 (0.52)	43 (0.89)
22	16 (0.53)	8 (0.83)	43	16 (0.56)	42 (0.89)

*The numerical codes of the genotypes are given in Table 1.

Table 7: Genetic similarity coefficients of pea genotypes determined according to the SM coefficient of ISSR data.*

	1	2	4	8	11	12	14	16	17	18	19	21	22	23	25	27	29	30	33	34	36	37	38	41	42	43
1	1																									
2	0.80	1																								
4	0.78	0.78	1																							
8	0.75	0.68	0.75	1																						
11	0.60	0.60	0.72	0.77	1																					
12	0.73	0.67	0.63	0.73	0.73	1																				
14	0.69	0.64	0.74	0.64	0.69	0.58	1																			
16	0.58	0.65	0.58	0.56	0.57	0.54	0.64	1																		
17	0.75	0.70	0.70	0.73	0.74	0.64	0.59	0.56	1																	
18	0.69	0.69	0.53	0.63	0.67	0.54	0.64	0.63	0.56	1																
19	0.68	0.65	0.78	0.68	0.64	0.59	0.70	0.63	0.65	0.56	1															
21	0.63	0.63	0.69	0.69	0.75	0.69	0.69	0.53	0.67	0.59	0.70	1														
22	0.70	0.70	0.73	0.83	0.77	0.69	0.63	0.53	0.73	0.60	0.63	0.67	1													
23	0.67	0.69	0.67	0.67	0.75	0.70	0.56	0.59	0.73	0.64	0.59	0.69	0.77	1												
25	0.70	0.63	0.75	0.70	0.74	0.63	0.64	0.53	0.75	0.51	0.73	0.70	0.65	0.63	1											
27	0.67	0.63	0.77	0.64	0.56	0.65	0.78	0.59	0.54	0.54	0.69	0.58	0.59	0.51	0.67	1										
29	0.73	0.67	0.69	0.69	0.69	0.63	0.69	0.63	0.74	0.60	0.69	0.65	0.64	0.65	0.74	0.68	1									
30	0.63	0.67	0.69	0.67	0.65	0.68	0.75	0.57	0.59	0.57	0.77	0.70	0.67	0.58	0.67	0.70	0.63	1								
33	0.78	0.70	0.70	0.68	0.67	0.63	0.69	0.68	0.63	0.51	0.68	0.63	0.69	0.59	0.75	0.63	0.78	0.63	1							
34	0.68	0.70	0.75	0.65	0.63	0.63	0.67	0.68	0.56	0.51	0.65	0.59	0.69	0.63	0.65	0.67	0.73	0.63	0.83	1						
36	0.74	0.69	0.73	0.74	0.75	0.65	0.69	0.54	0.73	0.40	0.67	0.73	0.67	0.65	0.70	0.63	0.70	0.65	0.77	0.73	1					
37	0.73	0.73	0.69	0.63	0.59	0.64	0.74	0.68	0.58	0.51	0.73	0.59	0.58	0.53	0.63	0.77	0.74	0.73	0.70	0.73	0.74	1				
38	0.95	0.75	0.73	0.78	0.67	0.69	0.77	0.63	0.75	0.58	0.75	0.73	0.73	0.64	0.68	0.73	0.64	0.73	0.65	0.69	0.73	0.74	1			
41	0.84	0.70	0.71	0.67	0.65	0.69	0.70	0.63	0.74	0.54	0.69	0.69	0.70	0.65	0.74	0.68	0.69	0.65	0.77	0.70	0.73	0.74	0.93	1		
42	0.77	0.74	0.77	0.64	0.65	0.63	0.70	0.59	0.70	0.53	0.69	0.69	0.69	0.65	0.69	0.68	0.63	0.68	0.67	0.67	0.70	0.73	0.79	0.83	1	
43	0.78	0.73	0.78	0.65	0.67	0.67	0.74	0.56	0.78	0.58	0.70	0.64	0.68	0.64	0.68	0.69	0.62	0.69	0.65	0.65	0.72	0.68	0.83	0.79	0.89	1

*The numerical codes of the genotypes are given in Table

3057xRondo and ProgressxRondo genotypes were the two genotypes that showed the closest similarity with a value of 0.89. These two genotypes gave similar results from the allelic condition.

Genetic association dendrogram was obtained according to UPGMA method using NTSYS-2.1 pc program from the data of PCR products obtained with ISSR primers (Figure 1).

When the genetic relationship dendrogram given in Figure 1 was examined, it was seen that the pea genotypes were gathered in 2 main groups. While the genotype PS3055 took place alone in the first basic group, all other genotypes were included in the second basic group. The 2nd main group showed a distribution divided into 2 groups again. In this 2nd basic group, the outermost genotype was genotype 3073 and showed a separation of 0.59 from the others.

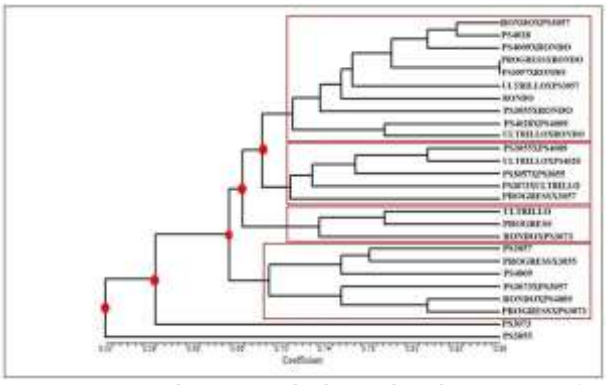

Figure 1: Genetic association dendrogram of pea genotypes.

The other 23 genotypes used showed clustering in the other branch of the 2nd group. The similarity of the genotypes in this cluster varies between approximately 0.55-0.89.3rd degree branching occurred on 2 branches

again and PS3057, Progress x PS3055, PS4009, PS3073xPS3057, RondoxPS4009, ProgressxPS3073 genotypes were separated from other genotypes. At the 4th degree of branching, Progress and Ultrillo cultivars and RondoxPS3057 genotype were separated from the others.

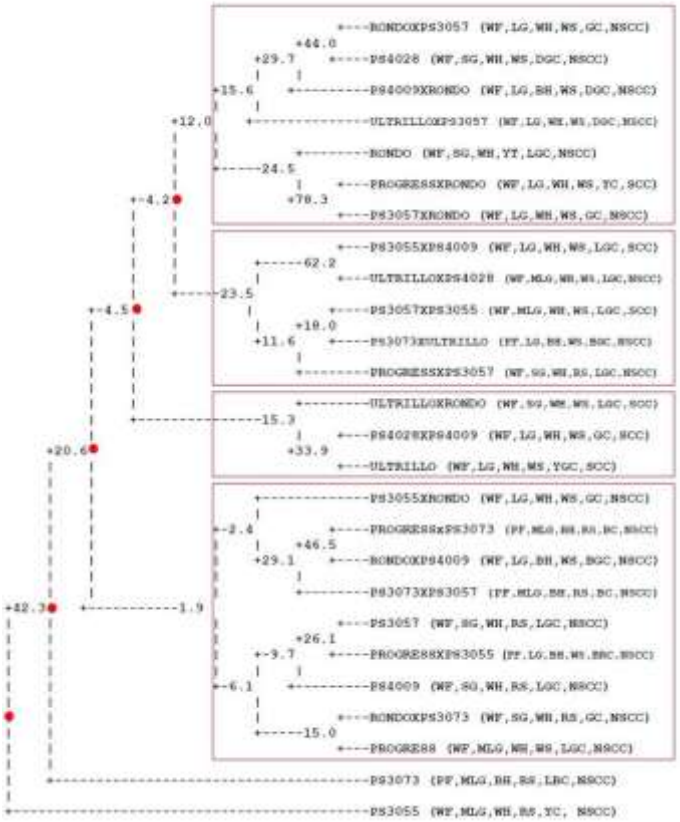

Figure 2:Dendrogram obtained according to the result of retroactive resampling analysis; (**WF**: White flower, **PF**: Pink flower, **LG**: Large grain, **SG**: Small grain, **MLG**: Medium large grain, **WH**: White hilum, **BH**: Black hilum, **WS**: Wrinkled seed, **RS**: Round seed, **GC**: Green cotyledon, **DGC**: Dark green cotyledon, **LGC**: Light green cotyledon, **YGC**: Yellow green cotyledon, **YC**: Yellow cotyledon, **BRC**: Brown red cotyledon, **BC**: Brown cotyledon, **YC**: Yellow cotyledon, **LBC**: Light brown cotyledon, **BGC**: Brown green cotyledon, **SCC**: There is seed coat cracking, **NSCC**: No seed coat cracking).

The 5th-degree branching showed 2 large clusters in itself. It was determined that there were 10 genotypes (RondoxPS3057, PS4028, PS4009xRondo, ProgressxRondo, PS3057xRondo, UltrilloxPS3057, Rondo, PS3055xRondo, PS4028xPS4009, UltrilloxRondo) in one of the branches and Rondo hybrids formed a group together. PS3057xRondo and ProgressxRondo genotypes were the two genotypes with the closest similarity (0.89). In the other branch, 5 genotypes (PS3055xPS4009, UltrilloxPS4028, PS3057xPS3055, PS3073xUltrillo, and ProgressxPS3057) were included.

Although the separation power of the primers used was high, the similarity between the pea genotypes showed close values. For this reason, there are intertwined branches in the dendrogram. However, genotypes could still be distinguished from each other by the primers used.

It was suggested that the close values in the similarity coefficients might be due to the genotypes being hybrids with common parents. For this reason, bootstrapping analysis of clusters in the dendrogram was performed with 5000 replications using the WINBOOT package program developed by Yap and Nelson (1996) and the genotypic relationship was interpreted by considering some morphological features (Figure 3).

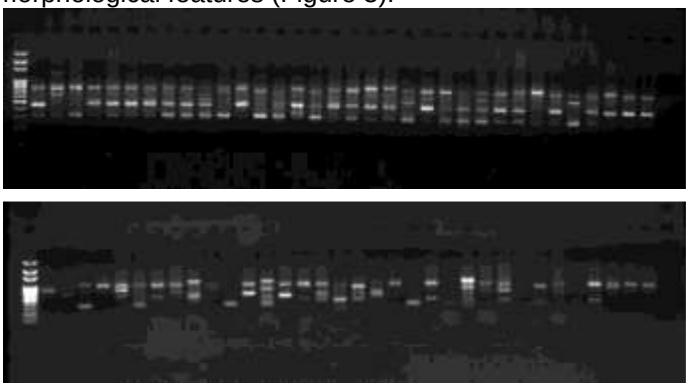

Figure 3: Agarose gel image of PCR products obtained with F3 and M9 primer.

It was seen that the dendrogram obtained according to the result of the retroactive resampling analysis also overlapped with the dendrogram obtained by the UPGMA method. Genotypes were clustered into 5 main branches.

Among the examined genotypes, the 2 most distant genotypes were PS3055 and PS3073. PS3055 differed from other pea genotypes with its yellow cotyledon colour, and took place in the outermost group in the dendrogram, and the PS3073 genotype also differed with pink flower colour. It was determined that the genotypes on the first branch in the 3rd-degree branching had pink flower colour and brown cotyledons. All remaining genotypes were located on the other branch. In the 4th-degree branching, clustering occurred on 2 branches again, and UltrilloxRondo, PS4028xPS4009 and Ultrillo genotypes, which had pod cracking features at this level, were separated from the others. On the 5th-degree branches, 2 large clusters were again formed. It was determined that genotypes with light green cotyledons were dominant in one cluster, and green and dark green cotyledons were found in the other cluster.

DISCUSSION

The pea genotypes studied appear to have variation despite having co-parents. Although some genotypes have similar features, they are located in different arms in the dendrogram, suggesting that the expansion is still ongoing and they may have differed due to gene interactions.

Baloch et al. (2015) reported that the 12 iPBS-retrotransposon primers generated yielded 106 scorable well resolved clear bands, and 81 of these were found to be

polymorphic DNA fragment (76.4%), with an average mean of 6.75 polymorphic fragments for each primer. Polymorphism information content (PIC) varied from 0.33 to 0.84 with a calculated mean value average of 0.61. It was obvious that field pea landraces from the same geographical region were frequently placed in various groups in the neighbour-joining analysis, pointing out that grouping on the basis of genetic parameters was not closely related to the geographical origin. In this study, we scored 87 allelic bands, out of which 83 were polymorphic While AMOVA (Analysis of Molecular Variance) has been found to be accurate even with a small number of bands (30-50 bands), it has been reported that more than this number of bands is usually required for STRUCTURE analysis (Nelson and Anderson, 2013). This supports that a sufficient number of polymorphic bands were produced in our study. The rate of polymorphism in the 13 primers used varied between 75% and 100%. It was determined that a total of 953 alleles were obtained with 13 primers in 26 genotypes with PCR amplifications, and 849 of these alleles were polymorphic. The average resolving power (RP) values of the primers were found in the range of 0.28-0.77, and the total resolving power was calculated as 5.85. As observed from results the primers used in the study were of equal quality in terms of resolving power. The primers used in the study showed heterozygosity in the range of 0.21-0.46. PIC of the primers used ranged from 0.41 to 0.96. The similarity coefficients of the genotypes showed an observation between 0.49-0.89. Al-Musawi et al. (2020) have found that the genetic distance values ranged from 0.11738 to 0.30065 between pea genotypes. Molecular markers serve as an effective tool in recognizing genotypes for their use in future breeding program (Arul and Selvakumar, 2019). Babayeva et al. (2018) stated that the primers used in the research should show high polymorphism and have the ability to generate multi-locus data from the genome examined. Afzal et al. (2018) emphasized that primers can be valuable in terms of polymorphic information content, even if they do not show high polymorphism. Pea is a autogamous plant, therefore many researchers have reported the narrowing in its genetic pool (Kole et al. 2015; Singh et al. 2018; Tahir et al. 2018). El-Fatah and Nafea (2020) used ISSR and SRAP markers to study the molecular diversity among pea genotypes. They reported that both ISSR and SRAP markers were able to amplify unique bands specific to a particular genotype. The primers used in our study showed sufficient polymorphism in pea genotypes. In this context, it can be concluded that these primers can be easily used in marker-assisted breeding programs of peas.

CONCLUSION

It is understood that the pea genotypes examined in PCR amplifications with ISSR primers had variations despite having common parents. Although some genotypes have similar features, the fact that they are located in different branches in the dendrogram had shown us that the expansion is still ongoing and the interactions of gene continue.

When evaluated in terms of genetic groupings and morphological features in the dendrogram, 9 of the 18 lines in the PCR analysis were among the 16 lines selected by classical methods (Ceyhan et al. 2018). This showed that the choices made by classical methods should be supported by molecular markers. It has been revealed that there will be no narrowing and loss in the gene pool, thanks to the association of the existing variation in the lines obtained as a result of crossing in breeding studies with the lines to be selected with molecular markers.

The enhancement of breeding programs by the use of molecular techniques and their application is a useful methodology prospect for the improvement of pea. In comparison, traditional strategies pose the limitation of genetic erosion, while relying totally on molecular and genomic approaches will not be a dependable option. Hence, the simultaneous use of both traditional and molecular breeding especially marker-assisted selection would be the recommended approach for pea breeding (Sharma et al. 2020).

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEDGEMENT

This study was produced from the project named “Development of the Pea Lines that are Big Grained and High Yielded for Canned and Dry Grains which are Favorable for Central Anatolian Conditions” (Project no: 114O276) supported by TUBITAK

AUTHOR CONTRIBUTIONS

MY, EA, EC, RA designed, performed and reported the experiments. EC and RA contributed to the hybridization and cultivation of the plant, MY and EA contributed to laboratory experiments, MY contributed to the writing and publication the manuscript. All authors have read review and approved the final version.

REFERENCES

Adhikari D, Khanna V, Singh K, 2018. Crossability and genetic diversity studies in pea (*Pisum sativum*). Curr. Investig. Agric. Curr. Res, 3: 443-452.

Afzal M, Alghamdi SS, Migdadi HM, Khan MA, Farooq M, 2018. Morphological and Molecular genetic diversity analysis of chickpea genotypes. Int. J. Agric. Biol, 20: 1062-1070.

Ahmad S, Singh M, Lamb-Palmer ND, Lefsrud M, Singh J, 2012. Assessment of genetic diversity in 35 *Pisum sativum* accessions using microsatellite markers. Canadian Journal of Plant Science, 92(6): 1075-1081.

Al-Musawi BH, Al-Bdairi NAH, AL-Tamimi AJT, 2020. Assessment of genetic polymorphism in cultivated pea (*Pisum sativum* L.) genotypes using ISSR marker. Paper presented at the AIP Conference Proceedings, AIP Publishing LLC, Vol. 2290, No. 1, p. 020006.

Arul S, Selvakumar R, 2019. Genetic diversity and application of DNA markers in garden pea-review. Acta Scientific Agriculture, 3(2): 153-161.

Atalay E, Babaoglu M, 2012. Determination of genetic relationship in Turkish chickpea (*Cicer arietinum* L.) genotypes using SSR molecular markers and capillary electrophoresis. The Journal of Animal and Plant Sciences, 22(2): 369-375.

Babayeva S, Akparov Z, Amirov L, Shikhaliyeva K, Hasanova S, Rustamov K, Mirzayev R, Izzatullayeva V, Mirzaliyeva I, Mammadov A, 2018. Genetic relationship among introduced lentil germplasm using agronomic traits and ISSR markers. Genetika, 50(2): 575-590.

Baloch FS, Alsaleh A, de Miera LES, Hatipoğlu R, Çiftçi V, Karaköy T, Yıldız M, Özkan H, 2015. DNA based iPBS-retrotransposon markers for investigating the population structure of pea (*Pisum sativum*) germplasm from Turkey. Biochemical Systematics and Ecology, 61: 244-252.

Baranger A, Aubert G, Arnau G, Lainé AL, Deniot G, Potier J, Weinachter C, Lejeune-Henaut I, Lallemand J, Burstin J, 2004. Genetic diversity within *Pisum sativum* using protein-and PCR-based markers. Theoretical and Applied Genetics, 108(7): 1309-1321.

Bhagyawant SS, Gupta N, Gautam A, Chaturvedi S, Shrivastava N, 2015. Molecular diversity assessment in chickpea through RAPD and ISSR markers. *World* Journal of Agricultural Research, 3(6): 192-197.

Bolouri P, Hossein Pour A, Jannati G, Haliloglu K, 2019. Genetic diversity of Pea (*Pisum arvense* L.) genotypes according to the tissue culture traits. Atatürk Üniversitesi Ziraat Fakültesi Dergisi, 50(1): 11-17.

Burstin J, Deniot G, Potier J, Weinachter C, Aubert G, Barranger A, 2001. Microsatellite polymorphism in *Pisum sativum*. Plant Breeding, 120(4): 311-317.

Ceyhan E, Mülayim M, 2003. Relationships between Grain Yield and Some Agricultural Characters of Pea in F1 and F2 Generations. SU Journal of Agricultural Faculty, 17(31): 68-73.

Ceyhan E, Harmankaya M, Yorgancılar M, Atalay E, Ada R, 2018. Development of the Pea Lines that are Big Grained and High Yielded for Canned and Dry Grains which are Favorable for Central Anatolian Conditions Project no: 114O276. TUBITAK: Scientific and Technological Research Institution of Turkiye (In Turkish, Abstract in English).

Chandrawati SN, Kumar R, Kumar S, Singh P, Yadav V, Ranade S, Yadav HK, 2017. Genetic diversity, population structure and association analysis in linseed (*Linum usitatissimum* L.). Physiology and molecular biology of plants, 23(1): 207-219.

Choudhury P, Tanveer H, Dixit G, 2007. Identification and detection of genetic relatedness among important varieties of pea (*Pisum sativum* L.) grown in India. Genetica, 130(2): 183-191.

Doyle J, 1990. A rapid total DNA preparation procedure for fresh plant tissue. Focus, 12: 13-15.

El-Fatah A, Nafea DS, 2020. Genetic Relationships and Diversity among Pea (*Pisum sativum* L.) Genotypes Assessed using Agro-Morphological and Molecular Markers. Journal of Agricultural Chemistry and Biotechnology, 11(12): 353-363.

Filiz E, Ibrahim K, 2011. Molecular markers in plant biotechnology. Journal of Gaziosmanpaşa University Faculty of Agriculture, 2011(2).

Food Agriculture Organization, 2020. Crops and livestock products staticial data: FAO, Rome.

Gixhari B, Pavelkova M, Ismaili H, Vrapi H, Jaupi A, Smýkal P, 2014. Genetic diversity of Albanian pea (*Pisum sativum* L.) landraces assessed by morphological traits and molecular markers. Czech Journal of Genetics and Plant Breeding, 50(2): 177-184.

Kapila R, Naryal S, Dhiman K, 2012. Analysis of genetic diversity among garden-and field-pea genotypes of higher Indian Himalayas. Journal of Plant Biochemistry and Biotechnology, 21(2): 286-291.

Kole PR, Sharma MK, Kumar S, Kumar R, 2015. Genetic diversity assessment in Indian cultivated pea (*Pisum sativum* L.) varieties using RAPD markers. Journal of Applied and Natural Science, 7(1): 316-323.

Kulaeva OA, Zhernakov AI, Afonin AM, Boikov SS, Sulima AS, Tikhonovich IA, Zhukov VA, 2017. Pea Marker Database (PMD)–A new online database combining known pea (*Pisum sativum* L.) gene-based markers. Plos One, 12(10): e0186713.

Labeeb M, Badr A, Haroun SA, Mattar MZ, El-Kholy AS, 2022. Ultrastructural and molecular implications of ecofriendly made silver nanoparticles treatments in pea (*Pisum sativum* L.). Journal of Genetic Engineering and Biotechnology, 20(1): 1-14.

Laucou V, Haurogné K, Ellis N, Rameau C, 1998. Genetic mapping in pea. 1. RAPD-based genetic linkage map of *Pisum sativum*. Theoretical and Applied Genetics, 97(5): 905-915.

Loridon K, McPhee K, Morin J, Dubreuil P, Pilet-Nayel ML, Aubert G, Rameau C, Baranger A, Coyne C, Lejeune-Henaut I, 2005. Microsatellite marker polymorphism and mapping in pea (*Pisum sativum* L.). Theoretical and Applied Genetics, 111(6): 1022-1031.

Nasiri J, Haghnazari A, Saba J, 2009. Genetic diversity

among varieties and wild species accessions of pea (*Pisum sativum* L.) based on SSR markers. African Journal of Biotechnology, 8(15).

Nei M, 1972. Genetic distance between populations. The American Naturalist, 106(949): 283-292.

Nelson MF, Anderson NO, 2013. How many marker loci are necessary? Analysis of dominant marker data sets using two popular population genetic algorithms. Ecology and Evolution, 3(10): 3455-3470.

Ng W, Tan S, 2015. Inter-simple sequence repeat (ISSR) markers: are we doing it right. ASM Science Journal, 9(1): 30-39.

Prevost A, Wilkinson M, 1999. A new system of comparing PCR primers applied to ISSR fingerprinting of potato cultivars. Theoretical and Applied Genetics, 98(1): 107-112.

Rohlf F, 1993. Numeric taxonomy and multivariate analysis system. NTSYS-pc.

Sharma A, Sekhon BS, Kumar R, Sharma S, Mahajan R, 2020. Marker-assisted selection in pea breeding, 137-154 Accelerated Plant Breeding, Volume 2, Springer, 137-154 pp.

Singh BK, Sutradhar M, Singh AK, Singh AK, Vyas RP, 2018. Analysis of genetic diversity in twelve cultivars of pea based on morphological and simple sequence repeat markers. Indonesian Journal of Agricultural Science, 19(2): 57-66.

Smith J, Chin E, Shu H, Smith O, Wall S, Senior M, Mitchell S, Kresovich S, Ziegle J, 1997. An evaluation of the utility of SSR loci as molecular markers in maize (*Zea mays* L.): comparisons with data from RFLPs and pedigree. Theoretical and Applied Genetics, 95(1): 163-173.

Tahir NA, Lateef DD, Omer DA, Kareem SH, Ahmad DA, Khal LH, 2018. Genetic diversity and structure analysis of pea grown in Iraq using microsatellite markers. Jordan Journal of Biological Sciences, 11(2): 201-207.

Thakur B, Sharma S, Sharma I, Sharma P, Zargar SM, 2018. Diversity analysis of pea genotypes using RAPD markers. Legume Research-An International Journal, 41(2): 196-201.

Yalım D, 2005. Determination of genetic diversity in barley cultivars grown in Turkiye by ISSR (Inter Simple Sequence Repeats) molecular marker technique. Graduate School of Natural and Applied Sciences, Department of Field Crops, M.Sc., Adana.

Yap I, Nelson R, 1996. Winboot: A program for performing bootstrap analysis of binary data to determine the confidence limits of UPGMA-based dendrograms. Manila, Philippines: International Rice Research Institute.

Yıldırım A, Kandemir N, 2001. Genetic Markers and Analysis Methods. In S Özcan, E Gürel, M Babaoğlu (Eds.), Plant Biotechnology II: Genetic Engineering and Applications, Chapter 23, Plant Genetic Engineering and Applications, SU Press., Konya, ISBN: 975-6652-04-7, pp: 334-363.

Yorgancilar M, Babaoglu M, Hakki EE, Atalay E, 2009. Determination of the relationship among Old World Lupin (*Lupinus* sp.) species using RAPD and ISSR markers. African Journal of Biotechnology, 8(15): 3524-3530.

Yorgancılar M, Yakışır E, Tanur Erkoyuncu M, 2015. Use of Molecular Markers in Plant Breeding. Journal of Bahri Dagdas Crop Research, 4(2): 1-12.

Yousefi V, Najaphy A, Zebarjadi A, Safari H, 2015. Molecular characterization of Thymus species using ISSR markers. JAPS: Journal of Animal and Plant Sciences, 25(4).