ISPEC Tarım Bilimleri Dergisi, 9(3): 677-690, 2025
ISPEC Journal of Agricultural Sciences, 9(3): 677-690, 2025
DOI: https://doi.org/10.5281/zenodo.15783344

Genomic Insights into Cold-Tolerant Pea Genotypes: A Molecular Diversity Study Using ISSR Markers

Emine ATALAY [1*], Mustafa YORGANCILAR [1], Ercan CEYHAN [1]
[1] Selcuk University, Faculty of Agriculture, Department of Field Crops, Konya
*Corresponding author: eatalay@selcuk.edu.tr

Abstract
Pea (*Pisum sativum* L.) is a valuable legume species used in human and animal nutrition due to its rich protein, carbohydrate, vitamin, and mineral content. However, its adaptation to dry and warm climates is limited, necessitating early spring sowing to ensure successful growth and yield. Therefore, the development of cold-tolerant cultivars has become a key objective in pea breeding programs, particularly to enhance adaptability and stability in temperate regions. Molecular marker-based analyses provide a complementary approach to traditional selection methods, offering insights into genetic diversity and parental relationships that are critical for effective hybridization strategies. In the present study, genetic relationships among 32 pea genotypes, including advanced lines, hybrids, and registered varieties, were assessed using 19 Inter Simple Sequence Repeat (ISSR) markers. A total of 88 scorable bands were obtained, with polymorphism levels ranging from 20% to 100%. The mean polymorphism rate across markers was 67%, while the average resolving power ranged from 0.09 to 0.88. Genetic similarity coefficients among genotypes ranged from 0.60 to 0.93. Most hybrids were found to cluster more closely with their cold-tolerant parental lines, suggesting successful introgression of desired traits. The clustering analysis further confirmed the presence of sufficient genetic variability to support ongoing selection and recombination within the breeding program. Overall, the findings highlight the utility of ISSR markers as a reliable tool for assessing genetic diversity and informing the selection of elite lines in pea breeding. The integration of molecular data with morphological and phenological evaluations can significantly accelerate the development of high-yielding, cold-tolerant pea cultivars adapted to early spring sowing conditions.

Research Article

Article History
Received :13.02.2025
Accepted :22.03.2025

Keywords
ISSR
Pisum sativum L.
polymorphism
resolving power

1. Introduction

Pea (*Pisum sativum* L.), one of the eleven leguminous crops recognized by the Food and Agriculture Organization (FAO), serves as an essential source of nutrition for both humans and animals due to its rich content of complex carbohydrates, proteins, vitamins, and minerals (Ton et al., 2018). It is consumed in various forms, including dried seeds, fresh green peas, and edible pods, and holds considerable economic value, particularly in the processing industry for canned and fresh produce (Ceyhan and Avcı, 2015). As a temperate climate crop, pea is highly sensitive to environmental stressors, with yield potential being significantly influenced by temperature and humidity levels. Its limited tolerance to drought and heat necessitates sowing in early spring to ensure optimal growth and productivity. However, prolonged exposure to low temperatures and high relative humidity during early growth stages can delay maturation and adversely affect yield performance (Alan and Geren, 2012). Consequently, the development of cold-tolerant genotypes capable of thriving under early spring conditions has become a central objective in modern pea breeding programs (Raveneau et al., 2011). In this context, assessing genetic distances among genotypes is a valuable strategy for identifying suitable parents in hybridization schemes (Bolouri et al., 2019). Molecular markers, particularly Inter Simple Sequence Repeat (ISSR) markers, have gained prominence in genetic diversity studies due to their ease of use, cost-efficiency, and applicability across diverse plant species without requiring prior sequence information (Yousefi et al., 2015). Compared to other marker systems such as RAPD, SSR, and AFLP, ISSR markers offer higher reproducibility, simplified protocols, and minimal technical infrastructure, making them especially advantageous for resource-limited breeding programs (Arul and Selvakumar, 2019). ISSR markers are also characterized by their genome-wide distribution and high level of polymorphism, features that enhance their resolution in detecting genetic variation, particularly in self-pollinating species such as pea. These advantages are especially critical considering the narrow genetic base observed in many commercially cultivated pea varieties, which limits the effectiveness of conventional selection methods and complicates the differentiation of newly developed cultivars (Singh et al., 2018). This genetic bottleneck presents a significant challenge for breeding programs seeking to enhance early spring adaptation and stress resilience. Thus, integrating molecular marker analyses with traditional phenotypic evaluations has become essential for broadening the genetic base and improving the efficiency of selection in pea breeding (Kole et al., 2015). The primary objective of the present study was to evaluate the genetic diversity among selected pea genotypes developed within a breeding program focused on cold tolerance. The study specifically aimed to assess the genetic relationships among hybrids, advanced lines, and registered varieties using ISSR markers, with the overarching goal of supporting the development of cold-tolerant, high-yielding pea cultivars.

2. Material and Methods

The materials were selected from the collection of a pea breeding program conducted by Prof. Dr. Ercan CEYHAN, which aims to identify cold-tolerant lines using techniques reported by Fiebelkorn (2013). Thirty-two genotypes (6 lines, 22 hybrids, and 4 registered varieties) that were tested and selected after the F_2 generation were scanned with ISSR markers to determine their genetic relationships (Table 1). Şahin, Granger and Melrose were tolerant genotypes, while Ultrillo was a sensitive genotype; the selected lines were closer to the tolerant genotypes (Ceyhan et al., 2019).

Table 1. Pea genotypes used in the study and some morphological properties of genotypes

Numeric code	Genotype name	Flower color	Cotiledone color	Hilum color	Grain size	Grain shape	Seed pot cracking
1	PS3048 x Granger1	White	Yellow	White	Small	Round	Absend
2	PS3048 x Ultrillo	White	Yellow	White	Medium	Round	Absend
3	Şahin x Ultrillo1	White	Yellow	White	Medium	Round	Absend
4	Şahin x Ultrillo2	White	Yellow	White	Medium	Round	Absend
5	PS3037 x Granger1	White	Green	White	Small	Round	Absend
6	Şahin x Ultrillo3	White	Yellowish Green	White	Medium	Round	Absend
7	PS3029 x Utrillo1	White	Green	White	Medium	Round	Absend
8	PS3053 x Ultrillo	White	Green	White	Medium	Round	Absend
9	Şahin x Ultrillo4	White	Yellow	White	Medium	Round	Absend
10	PS3029B x Melrose	White	Green	White	Small	Round	Absend
11	PS3029	White	Green	White	Small	Round	Absend
12	PS3053B X Ultrillo	White	Yellowish Green	White	Medium	Round	Absend
13	PS3037 X Ultrillo1	White	Green	White	Medium	Round	Absend
14	PS3029B	White	Light Green	White	Small	Round	Absend
15	PS3037 X Melrose1	White	Green	White	Small	Round	Absend
16	PS3053 x Granger	White	Yellow	White	Small	Round	Absend
17	PS3048 x Granger2	White	Yellow	White	Small	Round	Absend
18	PS3053B x Melrose	White	Green	White	Small	Round	Absend
19	PS3053 x Melrose	White	Yellow	White	Small	Round	Absend
20	PS3029 x Ultrillo2	White	Green	White	Small	Round	Absend
21	PS3037 x Granger2	White	Green	White	Small	Round	Absend
22	PS3037 x Melrose2	White	Green	White	Small	Round	Absend
23	Şahin x Ultrillo5	White	Green	White	Medium	Round	Absend
24	PS3053B	White	Light Green	White	Small	Round	Absend
25	PS3053	White	Yellow	White	Small	Round	Absend
26	Şahin	Pink	Light Brown	Black	Medium	Round	Absend
27	PS3048	White	Yellow	White	Small	Round	Absend
28	Granger	Pink	Brown Spotted Green	Black	Small	Round	Absend
29	Melrose	Pink	Brown Spotted Green	Black	Small	Round	Absend
30	Ultrillo	White	Yellowish Green	White	Large	Wrinkled	Available
31	PS3037	White	Green	White	Small	Round	Absend
32	PS3037 x Ultrillo2	White	Yellowish Green	White	Medium	Round	Absend

Ten individuals, representing each genotype, were used, and the youngest leaves of 20-day-old seedlings were isolated for DNA. DNA isolation was conducted as a bulk by taking an equal amount of the leaf samples from each plant (~200 mg in total). Modified 2x CTAB method was used (Atalay and Babaoğlu, 2012). A spectrophotometer was used to determine the concentrations and purities of the DNA samples. The PCR amplification products were run for 2-3 hours under 80 V electric current on a 1.5% (w/v) agarose gel containing ethidium bromide. A gel documentation and imaging system captured the image of the agarose gel. Twenty-five ISSR primers, previously shown to produce highly polymorphic bands in our studies, were used for the molecular characterization of the pea genotypes. The 19 primers that yielded positive results were used in the further experiments (Table 2).

Table 2. ISSR primers used in the study

ISSR	Sequence (5'-3')	GC ratio (%)	Temperature melting (Tm) (°C)	Reaction (°C)
M2	ACC ACC ACC ACC ACC ACC G	68.4	63.1	62
M3	AGC AGC AGC AGC AGC AGC C	68.4	63.1	62
M5	GAG AGA GAG AGA GAG AGA C	52.6	56.7	56
M6	GTC ACC ACC ACC ACC ACC AC	65.2	67.8	67
M7	AGA GAG AGA GAG AGA GAG C	52.6	56.7	56
M8	ACA CAC ACA CAC ACA CAC G	52.6	56.7	56
M10	ACA CAC ACA CAC ACA CCY*	52.8	54.8	53
M11	CAC CAC CAC CAC CAC	66.7	53.3	53
M13	CAC ACA CAC ACA RG*	53.6	44.8	42
M14	CAC ACA CAC ACA RY*	50	43.4	42
M15	CAC ACA CAC ACA CAC AAG	50	53.7	53
M16	CAC ACA CAC ACA CAC AGC	55.6	56	56
M17	CAG CAC ACA CAC ACA CAC A	52.6	56.7	56
M18	CGT CAC ACA CAC ACA CAC A	52.6	56.7	56
F1	GAG CAA CAA CAA CAA CAA	38.9	49.1	51
F2	CTC GTG TGT GTG TGT GTG T	52.6	56.7	58.5
F4	AGA GAG AGA GAG AGA GTG	50	53.7	53
F5	AGA GAG AGA GAG AGA G	50	49.2	51
F6	CCA CCA CCA CCA CCA	66.7	53.3	55.5
F8	GCC GCC GCC GCC GCC	100	67	67

*R: G/A; Y: C/T

PCR conditions were optimized through preliminary experiments. After optimization, a 20 µL PCR reaction mixture was prepared by combining 2 µL of DNA (20 ng μL^{-1}) with 18 µL of reaction components [Bioneer AccuPower HotStart PCR Premix, 1 µL of primer (10 pmol μL^{-1}), and 17 µL of deionized distilled water]. The PCR amplification was performed using a Techne thermal cycler, programmed for 35 cycles based on the melting temperature (Tm) of the primers. Following an initial denaturation at 95 °C for 3 minutes, the PCR reaction was performed for 35 cycles consisting of denaturation at 95 °C for 1 minute, annealing at a temperature ±2 °C from the standard melting temperature (Tm) of the primer for 1 minute (Table 2), and extension at 72 °C for 2 minutes. After completion of the cycles, the PCR reaction was continued with a final extension at 72 °C for 10 minutes and then the operation was ended at 4 °C. The scorable bands that were produced as a result of PCR amplifications and that were obtained after agarose gel electrophoresis were considered. The polymorphism rates of the primers were determined by dividing the number of polymorphic bands or alleles received from the primers by the total number of bands or alleles and then multiplying by 100. The polymorphism information contents (PIC) of the primers were determined using $PIC= 1-\Sigma pi^2$ formula (Smith et al., 1997). The alleles were scored in genotypes as there is (1) and none (0), and then the frequencies were calculated separately. The “pi” in the formula is the frequency of the “i” allele. The heterozygosity value of the primers (H) was calculated by making use of the fundamental equation $H = 1-p^2-q^2$ used in population genetics (Nei, 1972). Here, “p” refers to the number of existences of the “i” allele, and “q” is the number of the absence of the “i” allele. The Resolving power of the primers (RP) was calculated with the $RP = \Sigma Ib$ formula. The “Ib” value was determined according to the equation $Ib = 1 - [2 \times (0.5- p)]$. The “p” in the formula refers to the rate of the “i” allele in 32 genotypes (Prevost and Wilkinson, 1999). The scoring bands obtained using ISSR primers in PCR were evaluated, and they were scored as 1 or 0 based on the presence or absence of the primers. The genetic relation matrix was created from these scoring values using the NTSYS-2.1 PC program, based on the Simple Matching (SM) coefficient. The genetic relation dendrogram was then obtained using

the UPGMA Method (Rohlf, 2004). The resampling analyses of the genotypes (bootstrapping) were made with 5000 repetitions by using the WINBOOT Package Program (Immanuel and Nelson, 1996).

3. Result and Discussion

3.1. Evaluation of PCR amplification products

A total of 88 scorable bands, of which 56 were polymorphic, were obtained from 19 ISSR primers (Figure 1, Figure 2).

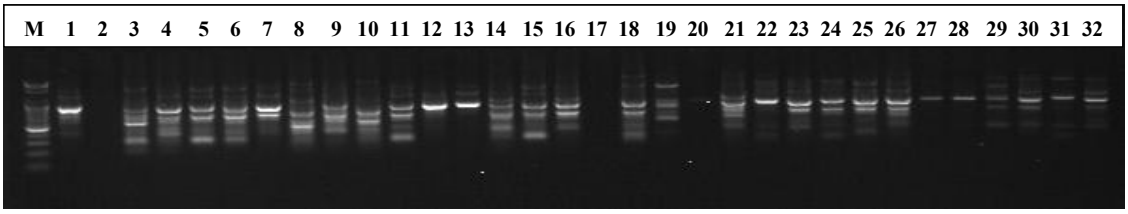


Figure 1. Agarose gel image of PCR products obtained with the M3 primer

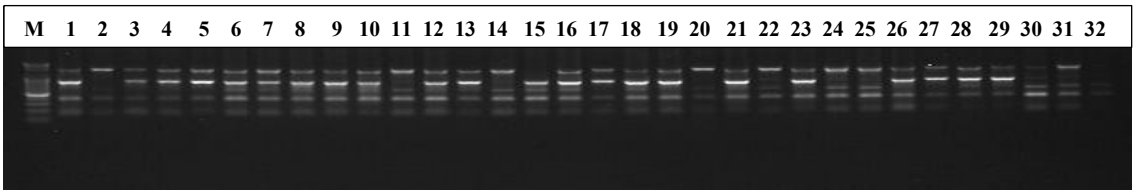


Figure 2. Agarose gel image of PCR products obtained with the M5 primer

The polymorphism values were higher than 50% in 13 of the 19 primers with a mean polymorphism rate of 67% (Table 3). Afzal et al. (2018) emphasized that primers can be valuable in terms of polymorphic information content even if they do not show high polymorphism. The primers used in the present study exhibited adequate polymorphism and could be utilized in further breeding studies.

Table 3. Band order obtained by ISSR primers and polymorphism rates

ISSR	Sequence (5'-3')	Scorable band order (number)	Polymorphic bands order (number)	Polymorphism (%)
M2	(ACC)6 G	2	2	100
M3	(AGC)6 C	3	3	100
M5	(GA)9 C	5	3	60
M7	(AG)9 C	5	5	100
M8	(AC)9 G	3	3	100
M10	(AC)8 CY	4	3	75
M11	(CAC)5	5	1	20
M13	(CA)6 RG	7	5	71
M14	(CA)6 RY	6	2	33
M15	(CA)8 AG	6	5	83
M16	(CA)8 GC	4	4	100
M17	CAG (CA)8	5	4	80
M18	CGT (CA)8	6	4	67
F1	GAG (CAA)5	6	2	33
F2	CTC(GT)8	5	3	60
F4	(AG)8TG	3	3	100
F5	(AG)8	5	1	20
F6	(CCA)5	5	2	40
F8	$(GCC)_5$	3	1	33
Total		88	56	
Mean		5	3	67

Among the total of 2079 alleles amplified through PCR using 19 ISSR primers, 1058 were identified as polymorphic, corresponding to a mean polymorphism rate of 50.88% (Table 4). This level of polymorphism indicates a moderate degree of genetic diversity among the evaluated pea genotypes. While the observed polymorphism rate varied among primers, it was sufficient to distinguish genotypes with varying levels of genetic similarity. Such polymorphism is essential for ensuring genetic variability in early-generation materials, particularly in self-pollinated species like *Pisum sativum* L., where the genetic base is often limited. When evaluated on a primer basis, the total number of alleles identified across all genotypes ranged from 56 to 69 (Table 5). On average, each primer amplified between 3 and 4 alleles per genotype. This finding is consistent with previous studies. For instance, Nisar et al. (2017) reported an average of 4.6 alleles per ISSR primer in cultivated pea genotypes, while Nasiri et al. (2010) observed an average of 5.9 alleles in wild pea accessions using SSR markers. The relatively similar allele counts obtained in the present study may be attributed to the fact that most hybrids and advanced lines used share common parental backgrounds, thereby limiting the diversity of alleles. Nevertheless, the number of polymorphic loci detected was sufficient to discriminate among the genotypes and to establish genetic relationships. The mean Resolving Power (RP) value of the primers was calculated as 0.50, with most primers exhibiting relatively uniform RP values. The RP metric, which reflects a primer's ability to resolve genetic variation among individuals, further supports the informativeness of the selected ISSR primers. According to Singh et al. (2018), primers with RP values of 0.5 or higher are considered adequate for diversity analysis in pea. The comparable RP values across primers in this study indicate that all selected primers contributed evenly to the resolution of genetic differences, without any single primer dominating the dataset. Heterozygosity values obtained using the primers ranged from 0.09 to 0.49. These relatively low levels of heterozygosity are typical of autogamous species, such as the pea, which primarily undergo self-pollination. The low heterozygosity observed may also be a reflection of the conserved genomic regions targeted by the ISSR primers. Singh et al. (2018) and Tahir et al. (2018) previously reported that the ongoing use of narrow breeding pools and the inherent selfing nature of *Pisum sativum* have significantly contributed to reduced genetic diversity and heterozygosity. Consequently, the identification and incorporation of diverse parental lines remain crucial to expanding the genetic base of cultivated pea. In summary, while the genetic diversity observed in this study is moderate, the level of polymorphism, allele number, resolving power, and heterozygosity collectively demonstrate the effectiveness of ISSR markers for characterizing genotypic variation in pea. These findings provide a valuable molecular foundation for future selection and crossing decisions within cold-tolerant breeding programs. The average Polymorphic Information Content (PIC) value obtained for the ISSR primers in this study was 0.55, indicating that the markers used possess a high level of informativeness. According to the classification proposed by Alizadeh et al. (2016), primers with a PIC value greater than 0.50 are considered highly informative, those ranging between 0.25 and 0.50 are moderately informative, and values below 0.25 are deemed to have low informativeness. Based on this scale, the majority of the ISSR primers employed in this study fall into the "highly informative" category, thereby confirming their suitability for detecting genetic variation among the analyzed pea genotypes. This is particularly important in species like *Pisum sativum* L., where limited genetic variability and self-pollination often obscure subtle differences between closely related lines. High PIC values not only indicate a primer's ability to detect polymorphic loci but also enhance the resolution power of the marker system in distinguishing among genotypes with similar phenotypic traits or common ancestry.

Table 4. Calculated values obtained from PCR amplifications*

ISSR	Total allel number	Polymorphic allel number	Resolving powder (RP)	Heterozygous rate (H)	Polymorphic information content (PIC)
M2	55	55	0.28	0.23	0.26
M3	66	66	0.58	0.37	0.50
M5	147	83	0.27	0.20	0.24
M7	62	62	0.50	0.32	0.77
M8	61	61	0.73	0.46	0.59
M10	98	66	0.63	0.35	0.49
M11	146	18	0.88	0.49	0.68
M13	139	78	0.56	0.34	0.69
M14	162	34	0.25	0.22	0.58
M15	87	55	0.46	0.32	0.82
M16	66	66	0.75	0.45	0.71
M17	105	73	0.58	0.39	0.62
M18	134	70	0.47	0.32	0.61
F1	182	54	0.31	0.23	0.27
F2	154	90	0.13	0.11	0.12
F4	65	65	0.44	0.31	0.48
F5	140	12	0.75	0.47	0.86
F6	127	31	0.09	0.09	0.56
F8	83	19	0.81	0.48	0.65
Total	2079	1058	9.5	6.2	11.5
Mean	109.42	55.68	0.50	0.32	0.55

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

Furthermore, the genetic similarity coefficients calculated among the 32 pea genotypes revealed relatively narrow ranges, with values clustering closely together. This pattern suggests a generally low degree of genetic divergence among the genotypes under investigation. Such a finding may be attributed to multiple factors. First, the use of genetically related parental lines in the breeding program likely reduced the overall variability, particularly if specific elite lines were repeatedly used in crosses for cold tolerance. Second, the narrow genetic base observed may reflect the consequence of prolonged selection pressure during the development of adapted lines, which can result in the fixation of similar alleles across genotypes. Third, the genomic regions targeted by the ISSR primers may include conserved loci with limited mutation rates, thereby restricting the observed polymorphism and resulting in high similarity coefficients. Taken together, the combination of high PIC values and closely related genetic similarity coefficients highlights both the strength and the limitation of the molecular approach used. While ISSR markers proved effective in detecting polymorphism and distinguishing between genotypes to a certain extent, the narrow genetic variability underscores the importance of introducing novel allelic sources and expanding the breeding gene pool. Future studies could benefit from complementing ISSR data with high-throughput marker systems such as SNP arrays or genotyping-by-sequencing (GBS) to uncover deeper levels of genetic diversity within elite and wild pea germplasm.

Table 5. Distribution of allel numbers determined in pea genotypes by genotypes and primers*

	Genotypes																													
Primers	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
M2	1	1	1	2	2	1	2	2	2	2	2	1	2	2	2	2	2	1	2	2	0	2	2	2	2	2	1	2	2	2	2	2
M3	2	0	3	2	3	3	2	3	3	2	3	1	1	3	3	2	0	3	3	0	2	2	2	3	3	2	1	1	2	2	2	2
M5	4	4	4	4	4	5	5	5	5	5	5	5	5	5	4	5	4	5	4	4	4	5	4	5	5	4	4	5	5	5	5	5
M7	3	1	2	2	2	1	2	2	3	2	2	3	1	2	1	1	2	2	3	2	2	2	3	2	1	3	2	1	2	2	2	1
M8	0	2	2	2	2	3	3	1	1	3	2	1	2	2	2	1	3	1	1	2	2	2	2	2	2	0	1	2	3	3	3	3
M10	3	3	3	3	3	4	3	3	3	2	3	4	3	3	3	3	4	3	3	3	3	3	2	3	3	3	3	3	4	3	2	4
M11	5	4	4	5	4	5	4	5	4	5	4	5	5	4	5	5	5	5	5	4	5	5	4	4	4	4	5	5	5	5	4	4
M13	4	6	5	2	5	3	4	5	5	3	5	5	4	5	5	4	4	4	5	4	4	5	5	4	6	5	4	4	4	3	5	3
M14	5	5	6	5	6	5	5	5	6	5	5	5	5	5	5	5	5	5	5	5	5	5	5	4	5	5	5	5	6	5	5	4
M15	4	2	4	3	2	3	3	2	3	2	3	4	3	2	3	3	2	5	5	2	2	3	3	2	2	3	1	2	2	3	2	2
M16	1	0	2	2	1	2	2	1	2	2	2	3	2	2	3	3	0	4	1	1	3	3	4	4	3	4	3	2	0	0	3	1
M17	4	3	3	3	2	3	3	3	2	4	3	4	4	3	4	4	4	3	3	3	3	4	3	3	3	3	4	4	4	3	3	3
M18	4	4	4	4	3	4	4	4	3	5	4	5	5	4	5	5	3	5	3	5	4	4	3	4	4	3	4	5	5	5	5	5
F1	6	5	6	5	6	5	5	5	6	6	6	6	5	6	5	5	6	6	6	6	6	6	6	6	6	6	6	5	6	6	6	5
F2	5	5	5	4	5	5	5	5	5	5	5	5	4	5	5	5	3	5	5	4	5	5	5	5	5	5	5	4	5	5	5	5
F4	2	2	2	3	2	2	2	2	1	2	2	2	2	2	2	2	2	2	3	1	2	2	2	2	2	2	2	2	3	2	2	2
F5	5	4	5	4	5	4	4	4	5	4	4	4	4	4	4	4	4	4	5	5	5	4	5	4	4	5	5	4	5	4	5	4
F6	4	3	4	4	4	4	4	4	4	4	4	4	4	4	4	4	5	4	4	3	4	4	4	4	4	4	4	4	4	4	4	4
F8	2	2	3	3	2	3	3	3	3	3	2	2	2	3	3	3	2	2	3	3	3	3	3	3	3	3	2	3	2	2	2	2
Total Allel	64	56	68	62	63	65	65	64	66	66	66	69	63	66	68	66	60	69	69	59	64	69	67	66	67	66	62	63	69	64	67	61
Mean Allel	3	3	4	3	3	3	3	3	3	3	3	4	3	3	4	3	3	4	4	3	3	4	4	3	4	3	3	3	4	3	4	3

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

3.2. Similarity matrix and genetic relationship dendogram

The genetic relationships among the 32 pea genotypes were visualized using a UPGMA (Unweighted Pair Group Method with Arithmetic Mean) dendrogram constructed from ISSR marker data (Figure 3). According to the dendrogram, the initial divergence among genotypes began at a similarity coefficient of 0.60, suggesting a moderate level of genetic differentiation within the germplasm pool. This initial bifurcation separated the genotypes into two primary clusters. The first major cluster consisted of 10 genotypes, while the second major cluster comprised the remaining 22 genotypes, indicating asymmetrical distribution of genetic variation. As the similarity threshold decreased further, a more pronounced divergence among genotypes became evident at the 0.65 level, where the genotypes were further stratified into three distinct main groups. Within these major groupings, genotypic similarity coefficients ranged from 0.66 to 0.93, indicating the presence of both closely related and moderately divergent genotypes within the population. This level of resolution reflects the utility of ISSR markers in differentiating even closely related hybrids and lines. Each main group exhibited internal sub-clustering, suggesting that while genotypes shared a high degree of overall similarity, there existed finer-scale genetic variation likely attributable to differences in parental lineage, recombination events, or specific genomic regions captured by the ISSR primers. The relatively high similarity coefficients among many genotypes, especially those sharing one or both parents, confirm the genetic proximity among breeding lines developed under similar selection pressures for cold tolerance. Nevertheless, the observed stratification also points to sufficient genetic variability for further selection and recombination in future breeding cycles. The hierarchical branching pattern observed in the dendrogram supports the hypothesis that although certain genotypes cluster tightly due to shared ancestry, the accumulation of subtle genetic differences—possibly due to recombination or selection—contributes to the observed grouping dynamics. These results underscore the effectiveness of ISSR-based clustering in revealing the underlying genetic structure of pea breeding populations and provide valuable information for selecting genetically diverse parental combinations in future hybridization efforts.

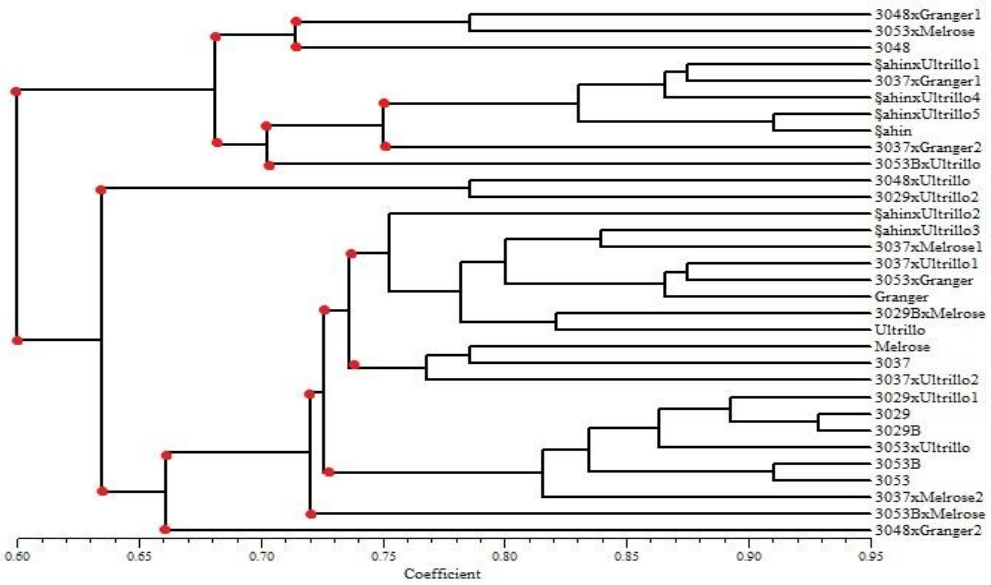


Figure 3. Genetic relationship dendogram of pea genotypes

In the UPGMA dendrogram constructed from ISSR marker data, the first major cluster comprised 20 genotypes and was subsequently divided into two well-defined subgroups. Within this cluster, PS3048 × Granger2 was the first genotype to diverge from the others at a similarity threshold of 0.66, suggesting moderate genetic distinctiveness—likely influenced by recombination effects or residual heterozygosity stemming from early-generation hybridization. The remaining genotypes in this subgroup continued to diverge at varying levels of similarity, reflecting subtle differences in their genetic makeup. A noteworthy observation within this cluster was the separation of PS3053B × Melrose at a similarity level of 0.72, forming a branch that later bifurcated into two genetically similar but distinguishable genotypes: PS3053 and PS3053B, which showed a high similarity coefficient of 0.91. Similarly, PS3029 and PS3029B, two genotypes with shared parentage and highly similar morphological characteristics—such as white flowers, green cotyledons, and round, small seeds—exhibited a close genetic relationship, separating at a similarity level of 0.93. These findings highlight a strong correlation between morphological uniformity and genetic proximity, particularly among genotypes that are likely near-isogenic derivatives or sister lines within the same breeding cohort. The second main cluster included 10 genotypes and displayed further sub-structuring at a similarity level of 0.68. The first sub-branch within this group included PS3053B × Ultrillo, which separated at 0.70, followed by Şahin × Ultrillo1, PS3037 × Granger1, Şahin × Ultrillo4, Şahin × Ultrillo5, and Şahin itself. These genotypes not only shared genetic similarity but also displayed similar morphological and agronomic features, such as medium seed size, yellow or yellowish-green cotyledons, and round seed shapes, which suggests that the cold-tolerant genotype Şahin contributed significantly to the phenotypic profile of these hybrids. The pair Şahin × Ultrillo5 and Şahin were particularly close, with a separation level of 0.91, confirming both their genetic proximity and phenotypic resemblance, particularly in terms of cold tolerance and seed morphology. Another distinct sub-branch of this group consisted of PS3048 × Granger1 and PS3053 × Melrose, with both genotypes separating from PS3048 at similarity thresholds of 0.79 and 0.71, respectively. Interestingly, although these genotypes share a common parent, their placement in separate sub-clusters may reflect differences in morphological traits, such as

cotyledon color or seed cracking characteristics, or the influence of non-additive gene interactions that contribute to phenotypic divergence despite their shared ancestry. The third main group was the most distinct and comprised only two genotypes: PS3048 × Ultrillo and PS3053 × Melrose, which diverged at a similarity level of 0.78. Despite limited sample size in this cluster, the relatively early separation of these genotypes implies a higher degree of genetic divergence, potentially resulting from the contribution of genetically distant parental combinations or differential selection history. Results from bootstrap resampling analysis further reinforced the presence of meaningful variation among genotypes. Some genotypes with similar morphological profiles were placed in different branches of the dendrogram, highlighting the complexity of genotype-phenotype associations. This divergence likely stems from gene interactions (e.g., epistasis or dominance effects) influencing agronomic traits such as cold tolerance, seed morphology, and growth habit, which are not always directly reflected by neutral molecular markers. The ongoing segregation of quantitative traits in early-generation materials, particularly in self-pollinated species like pea, underscores the need to integrate both molecular and phenotypic data for accurate genetic evaluation and parent selection in breeding programs. The genetic similarities identified through the UPGMA dendrogram corresponded with several morphological and agronomic traits (Figure 3, Figure 4). For instance, PS3053 and PS3053B genotypes showed 91% genetic similarity and were also highly similar in terms of morphological characteristics, such as small, round seeds with a white hilum. Their derivation from the same parental line suggests that ISSR marker-based clustering reliably reflects phenotypic similarity in closely related genotypes. Similarly, PS3029 and PS3029B, which shared 93% genetic similarity, also exhibited matching flower colors (white), cotyledon colors (green), and round seed shapes. These findings support the idea that sister lines originating from the same breeding pool tend to retain both genetic and morphological coherence. On the other hand, Şahin and ŞahinxUltrillo5 genotypes also displayed a high level of genetic similarity (91%). They showed phenotypic resemblance in traits such as flower color (pink), hilum color (black), and cotyledon shade (light brown to greenish tones). This indicates that the dominant morphological features of the cold-tolerant parent, Şahin, were inherited in the hybrid, which aligns with the molecular similarity detected via ISSR markers. However, some inconsistencies were also observed. For example, PS3048 × Granger1 and PS3048 × Granger2, although derived from the same parental combination, displayed a lower genetic similarity (66%). This suggests that due to recombination, genetic reshuffling, or epistatic interactions occurring in the F2 or later generations, phenotypic similarity may not always correlate directly with molecular similarity, highlighting the complexity of genotype–phenotype relationships in segregating populations. The genetic patterns revealed by ISSR markers showed partial alignment with morphological and agronomic characteristics, particularly in genotypes sharing the same pedigree. However, these associations were not universally consistent, likely due to environmental influences and gene interactions. Therefore, combining molecular marker data with phenotypic evaluations enhances the accuracy and efficiency of selection in pea breeding programs.

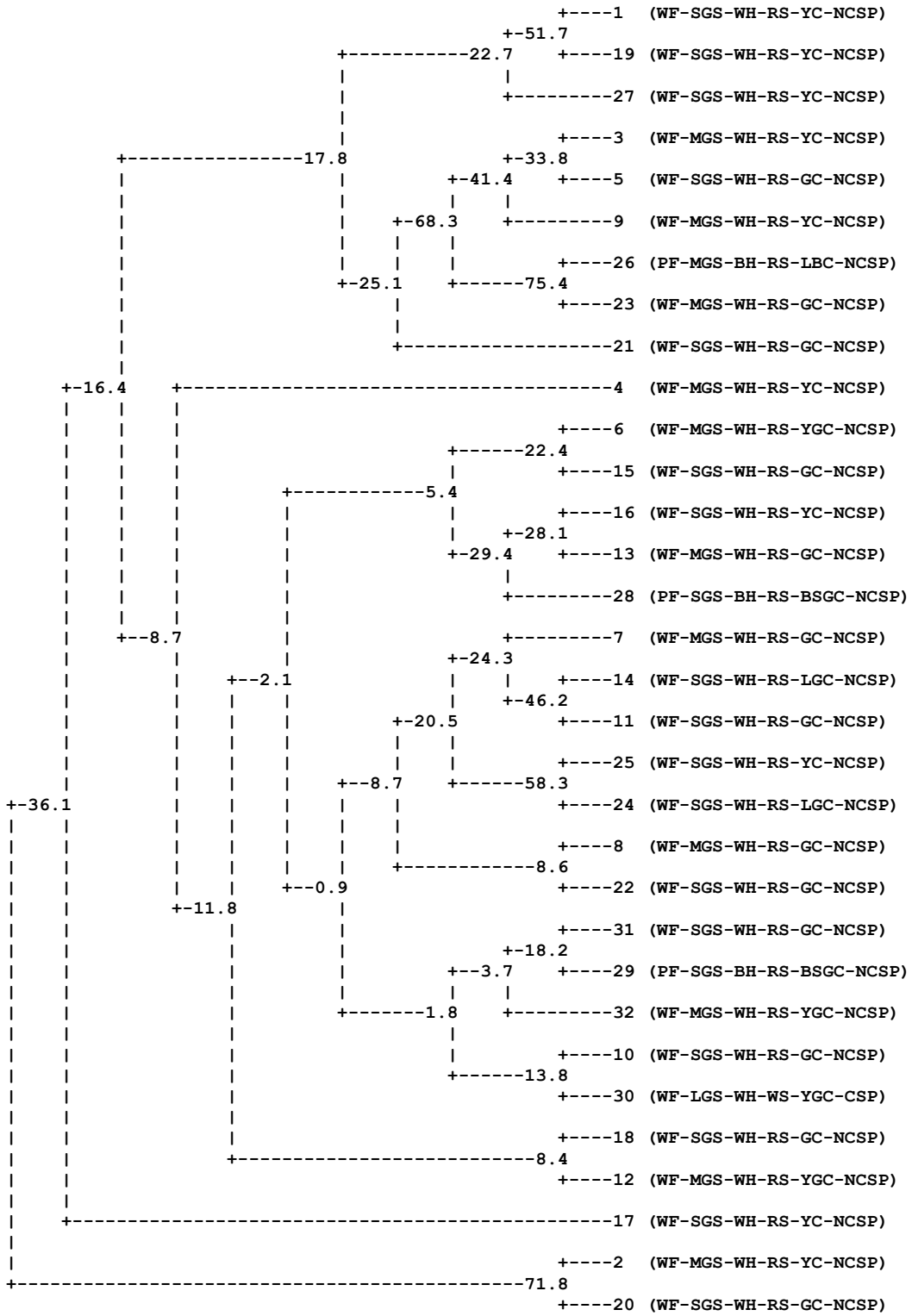


Figure 4. Dendrogram of pea genotypes with bootstrap analysis

(WF: White Flower, PF: Pink Flower, SGS: Small Grain Size, MGS: Medium Grain Size, LGS: Large Grain Size, RS: Round Seed, WS: Wrinkled Seed, WH: White Hilum, BH: Black Hilum, GC: Green Cotyledon, LGC: Light Green Cotyledon, YGC: Yellowish Green Cotyledon, YC: Yellow Cotyledon, LBC: Light Brown Cotyledon, BSGC: Brown Spotted Green Cotyledon, CSP: cracking seed pod, NCSP: non cracking seed pod)

4. Conclusion

This study demonstrated that sufficient genetic diversity exists among selected hybrid pea genotypes derived from cold-tolerant and sensitive parental lines. Using 19 ISSR primers, a total of 88 scorable bands, 56 of which were polymorphic, were successfully amplified, indicating moderate to high levels

of polymorphism and marker informativeness across the genotypes. The observed genetic similarity coefficients (ranging from 0.60 to 0.93) suggest that while some hybrids retain close genomic proximity to their tolerant parents (e.g., Şahin, Granger, Melrose), others display moderate divergence, likely due to ongoing recombination and segregation processes typical of early-generation crosses. The dendrogram-based clustering largely supported the pedigree information, with hybrids clustering near at least one of their parents. However, the placement of several hybrids in distinct sub-clusters, despite their agronomic resemblance to a parent, highlights the complex genetic architecture underlying traits such as cold tolerance and seed morphology. This also emphasizes the necessity of integrating molecular markers with field-based phenotypic selection to capture both visible and cryptic genetic variation. Importantly, ISSR markers proved effective not only in detecting polymorphism among the genotypes but also in differentiating between closely related hybrids—an essential advantage considering the narrow genetic base often encountered in modern pea germplasm. The study reaffirms the potential of marker-assisted selection (MAS) in early-generation breeding, particularly for traits such as cold tolerance that exhibit complex, quantitative inheritance patterns. Among the evaluated hybrids, PS3053 × Ultrillo, PS3037 × Ultrillo1, PS3048 × Granger, and PS3037 × Ultrillo showed promising molecular diversity, along with phenotypic resemblance to tolerant parents. These combinations may serve as elite lines in subsequent generations and should be prioritized in future field trials under cold-prone environmental conditions. In conclusion, the integration of molecular tools such as ISSR with conventional selection criteria offers a valuable approach to accelerate pea improvement programs. Future studies should focus on validating these findings through multilocational trials, integrating genomic selection approaches, and further dissecting the genetic basis of key traits using high-resolution markers. Such integrative strategies will support the development of high-yielding, stress-resilient pea cultivars adapted to the agro-climatic conditions of Central Anatolia and similar temperate regions

Declaration of Author Contributions

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

Declaration of Conflicts of Interest

All authors declare that there is no conflict of interest related to this article.

Acknowledgements

This study was conducted as part of the project entitled "Development of High-Yielding and Cold-Tolerant Pea Lines Adapted to Central Anatolian Conditions." The authors gratefully acknowledge the financial support provided by the Research and Development Program of the Ministry of Agriculture and Forestry of the Republic of Turkey (Project no: TAGEM-15/AR-GE/60).

References

Afzal, M., Alghamdi, S.S., Migdadi, H.M., Khan, M.A., Farooq, M., 2018. Morphological and molecular genetic diversity analyses of chickpea genotypes. *International Journal of Agriculture and Biology*, 20: 1062-1070.

Alan, Ö., Geren, H., 2012. Effects of different sowing dates on the seed yield and some other agronomical characteristics of pea (*Pisum sativum* L.). *Journal of Agriculture Faculty of Ege University*, 49(2): 127-134.

Alizadeh, Z., Ahmadi, D.N., Mohammadi, M., Karimizadeh, R.A., Memari, H.R., Bahmankar, M., 2016. Assessment of molecular variation in pea germplasm using RAPD markers. *Electronic Journal of Biology*, 12(1): 22-27.

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 & Plant Sciences*, 22(2): 369-375.

Bolouri, P., Hossein Pour, A., Jannati, G., Haliloğlu, K., 2019. Genetic diversity of pea (*Pisum arvense* L.) genotypes according to the tissue culture traits. *Atatürk University Journal of Agricultural Faculty,* 50(1): 11-17.

Ceyhan, E., Avci, M., 2015. Determination of some agricultural characters of developed pea (*Pisum sativum* L.) lines. *International Journal of Agricultural and Biosystems Engineering*, 9(12): 1235-1238.

Ceyhan, E., Yorgancılar, M., Ada, R., Kahraman, A., Atalay, E., 2019. Development of high grain yield and cold resistant pea lines suitable for central Anatolian conditions. Ministry of Agriculture and Forestry, Research - Development Support Program, Project no: TAGEM-15/AR-GE/60.

Fiebelkorn, D.M., 2013. Characterization of selected winter hardiness traits in pea (*Pisum sativum* L.). Master thesis, North Dakota State University, North Dakota, USA.

Immanuel, V.Y., Nelson, R.J., 1996. WINBOOT: A program for performing bootstrap analysis of binary data to determine the confidence limits of UPGMA-Based Dendrograms.

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

Nasiri, J., Haghnazari, A., Saba, J., 2010. Genetic diversity among varieties and wild species accessions of pea (*Pisum sativum* L.) based on SSR markers. *African Journal of Biotechnology*, 8: 3405-3417.

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

Nisar, M., Khan, A., Wadood, S.F., Shah, A.A., Hanci, F., 2017. Molecular characterization of edible pea through EST-SSR markers. *Turkish Journal of Botany*, 41(4): 338-346.

Prevost, A., Wilkinson, M.J. 1999. A new system of comparing PCR primers applied to ISSR fingerprinting of potato accessions. *Theoretical and Applied Genetics*, 98: 107-112.

Raveneau, M., Coste, F, Moreau-Valancogne, P., Lejeune-Hénaut, I., Durr, C., 2011. Pea and bean germination and seedling responses to temperature and water potential. *Seed Science Research,* 21(3): 205-213.

Rohlf, J.F., 2004. NTSYS-pc:2.2, Numerical Taxonomy and Multivariate Analysis System.

Singh, B.K., Sutradhar, M., Singh, A.K., Singh, A., Vyas, R.P., 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.S., Wall, S.J., Senior, M.L., 1997. An evaluation of the utility of SSR loci as molecular marker in maize comparisons with data from RFLP and pedigree. *Theoretical and Applied Genetics*, 95: 163-173.

Tahir, N., Lateef, D., Omer, D., Kareem, S., Ahmad, D., Khal, L., 2018. Genetic diversity and structure analysis of pea grown in Iraq using microsatellite markers. *Jordan Journal of Biological Sciences,* 11(2): 201-207.

Ton, A., Karaköy, T., Anlarsal, A.E., Türkeri, M. 2018. Investigation of grain yield and yield components of some field pea (*Pisum sativum* L.) genotypes in Mediterranean climate conditions. *Legume Research*, 41(1): 41-47.

Yousefi, V., Najaphy, A., Zebarjadi, A., Safari, H., 2015. Molecular characterization of Thymus species using ISSR markers. *The Journal of Animal & Plant Sciences*, 25(4): 1087-1094.

To Cite Atalay, E., Yorgancılar, M., Ceyhan, E., 2025. Genomic Insights into Cold-Tolerant Pea Genotypes: A Molecular Diversity Study Using ISSR Markers. *ISPEC Journal of Agricultural Sciences*, 9(3): 677-690.
DOI: https://doi.org/10.5281/zenodo.15783344.