Assessment of Field Pea (*Pisum sativum* var. *arvense*) Genotypes for Morphological Traits, Quality Traits and Molecular Characterization using SSR Marker

Satyendra[1], Vijay Sharma[1], Mukul Kumar[1], Anuj Mishra[2], Kamaluddin[1],
Priya Sharma[1], Shalesh Kumar Singh[1], Nitin Kumar[1], Pawan Kumar Prajapati[1]

ABSTRACT

Background: Field pea is an important cool season grain legume crop. This study was conducted on field pea to explore phenotypic and molecular diversity among field pea genotypes, catering to current and future crop improvement.
Methods: In this investigation forty-six genotypes of field pea were evaluated in randomized block design to assess phenotypic diversity for morphological and quality traits, alongside molecular characterization using simple sequence repeat (SSR) markers.
Result: Variability analysis displayed substantial variability for all traits among test genotypes. Cluster analysis grouped the all genotypes into three distinct multi-genotypic clusters with clusters II and III having highest inter-cluster distance. Molecular characterization revealed the detection of 43 repeatable alleles by 14 polymorphic SSR markers, spanning a size range of 150 bp (AA122) to 970 bp (AA504), with a maximum of 4 fragments intensified by AD147, D21, AA504, AA122 and A9. Utilizing Jaccard dissimilarity coefficients and UNJ methods, all forty-six genotypes were grouped into three clusters. This study on field pea facilitates the identification of diverse parent selections capable of producing desirable segregants in future breeding programs.

Key words: Field pea, Molecular diversity, Phenotypic diversity, SSR marker, Variability.

INTRODUCTION

Field pea (*Pisum sativum* var. *arvense*) is a versatile cool-season grain legume crop belonging to the *Fabaceae* family and *Papilionaceous* sub-family, characterized by a chromosome count of 2n = 14. Globally, it is cultivated in diverse climatic conditions ranging from semi-arid to temperate regions (Olle *et al.*, 2020; Parihar *et al.*, 2024). Renowned for its nutritional richness, pea seeds boast a digestible protein content ranging between 21% and 25%, abundant in lysine, tryptophan and other essential amino acids, underscoring its significance as a high-value crop (Gregory *et al.*, 2016). Additionally, field peas contain notable content of cysteine and methionine amino acids alongside serving as a significant source of both reducing and non-reducing sugars. Moreover, field peas harbor secondary metabolites in the form of phenols, though non-nutritive, they contribute to the overall biochemical composition. The concentration of phenols within the pea's seed coat varies according to the seed variety (Fahim *et al.*, 2016). As per the visionary projections outlined in the ICAR-Indian Institute of Pulses Research's Vision document, India's burgeoning population is anticipated to reach 1.68 billion by 2030, with an estimated pulse demand of 32 million tonnes, necessitating an annual growth rate of 4.2%. Consequently, there exists an imperative for development of high-yielding pea varieties with superior seed quality, catering to multifaceted requirements in the foreseeable future.

Understanding genetic variability is paramount for breeders aiming to enhance complex characteristics that

[1]Department of Genetics and Plant Breeding, Banda University of Agriculture and Technology, Banda-210 001, Uttar Pradesh, India.
[2]Department of Genetics and Plant Breeding, Chandra Shekhar Azad University of Agriculture and Technology, Kanpur-208 002, Uttar Pradesh, India.

Corresponding Author: Vijay Sharma, Department of Genetics and Plant Breeding, Banda University of Agriculture and Technology, Banda-210 001, Uttar Pradesh, India.
Email: vijay.buat@gmail.com
ORCIDs: 0000-0002-6223-2857

exhibit minimal response to direct selection (Pratap *et al.*, 2024a). The estimation of morphological data alone may not fully unveil genetic diversity due to constraints like limited polymorphism and environmental influences. Augmenting morphological markers with molecular markers proves to be a more effective means of accurately estimating genetic diversity. Genotypic characterization facilitated by markers such as simple sequence repeats aids in elucidating genetic relatedness among plant species. SSR markers are preferred for their reproducibility, multi-allelic nature, genome-wide distribution, co-dominant

inheritance and PCR detectability, making them ideal for comprehensive genome characterization. This study aims to explore genetic variability and molecular diversity among field pea genotypes to catering the current and future crop improvement endeavors.

MATERIALS AND METHODS

The current investigation was conducted at research farm of Banda University of Agriculture and Technology in Banda, India. The research incorporated a diverse selection of 46 field pea genotypes including two standard control varieties, namely IPFD 10-12 and Ambika, were used for comparison purposes. All the genotypes were planted in a randomized block design with three replicas during winter season of 2021-22. Each experimental plot comprised of two rows, extending 4 meters in length, with row-to-row distances set at 30 cm and plant-to-plant spacing at 15 cm. Data collection involved the observation of 5 randomly selected plants within each genotype, focusing on 10 morphological traits of field pea inclusive of days to 50% flowering and maturity at plot level along with 5 nutritional traits. To conduct various statistical analyses, the averages of data from the selected plants within each plot were utilized for different traits. The observed traits included days to 50% flowering (DF), days to maturity (DM), plant height (PH) in centimeters, total number of pods per plant (TNP), effective pods per plant (EPP), seeds per pod (SP), pod length (PL) in centimeters, 100-seed weight (SW) in grams, biological yield per plant (BY) expressed in grams and seed yield per plant (SY). These observations were made at different growth stages, following the methodologies (Pratap *et al.*, 2024b; Sharma *et al.*, 2023, Yadav *et al.*,

2023). The protein content in seeds was assessed using Kjeldahl method. The protein % is equals to the Nitrogen % ×6.25. Total sugar (TS) content was assessed by using method defined by Dubois *et al.*, (1956). Reducing sugar (RS) content was estimated by using the method outlined by Nelson (1944) with slight adjustments. The proportion of non-reducing Sugar (NRS) was calculated by subtracting RS content from TS content and then multiplying the result by 0.95, as described by Somogyi (1952). The total phenol content of all selected plant extracts was estimated using Folin-Ciocalteu reagent using gallic acid as standard based on oxidation-reduction reaction.

DNA extraction was carried out from leaves of forty-six genotypes using CTAB (Cetyl Tri-methyl Ammonium Bromide) method, following procedure outlined by Doyle and Doyle (1990) with certain adaptations. The Nanodrop spectrophotometer model ND1000 was employed to evaluate the concentration and purity of DNA. Molecular analysis was conducted using a set of 20 SSR markers (Table 1) sourced from the public domain. PCR amplification was carried out using the Agilent Sure Cycler 8800 Thermocycler. The amplified PCR products were resolved on a 1.5% agarose gel and DNA patterns were observed using a gel documentation system under UV illumination.

Statistical analysis utilized the average replication values of each genotype across diverse traits. Observations recorded for each trait underwent analysis of variance following method outlined by Panse and Sukhatme (1957). Statistical analysis of variability parameters was done using R Studio package Variability (version 2023.03.1-446). Similarity estimates were assessed using the unweighted

Table 1: Sequences details of Markers used to assess genetic diversity in Field Pea genotypes.

Marker name	Sequence 5' to 3'	Annealing temperature (°C)
AD147	AGCCCAAGTTTCTTCTGAATCC	57
D21	TATTCTCCTCCAAAATTTCCTT	53
AD148	GAAACATCATTGTGTCTTCTTG	53
AA504	TGAGTGCAGTTGCAATTTCG	56
AA205	TACGCAATCATAGAGTTTGGAA	53
AA175	TTGAAGGAACACAATCAGCGAC	58
AA174	GGAGGGATGATTCTAACAAGGT	58
AA355	AGAAAAATTCTAGCATGATCTG	53
AA122	GGGTCTGCATAAGTAGAAGCCA	59
A9	GTGCAGAAGCATTTGTTCAGAT	56
AA285	TCGCCTAATCTAGATGAGAATA	54
AC58	TCCGCAATTTGGTAACACTG	55
AA399	CCATTGGTATATGAAAGATCGCT	56
AD60	CTGAAGCACTTTTGACAACTAC	56
AD51	ATGAAGTAGGCATAGCGAAGAT	55
AD56	GAAACATTGGTTGAAGAGCGA	56
AD171	TGAGGGCAGACATACCCATAGC	60
AB23	TCAGCCTTTATCCTCCGAACTA	58
AD60	CTGAAGCACTTTTGACAACTAC	56
AB91	CGAGACGACAACGGTAGTGAAA	60

Neighbour Joining (UNJ) method. Cluster analyses was performed using DARWin software (version 6.0.21), while PowerMarker software version 3.25 was employed to determine Polymorphic Information Content (PIC) values, major allelic frequency and gene diversity estimates.

RESULTS AND DISCUSSION

The scope of improvement of field pea crop is dependent on the extent of variability within its gene pool. However morphological diversity is not a reliable measure because it may be influenced due to prevailing environmental factors. This study focused on molecular diversity using different SSR molecular markers. Utilizing molecular markers in conjunction with morphological markers offers a precise way of estimating genetic diversity. Genotypic characterization enables the identification of genetic relatedness among plant species. Employing SSR markers for assessing genetic diversity proves to be an optimal approach as they are highly reproducible, possess multiple alleles, are dispersed throughout genome, exhibit co-dominant inheritance and are easily detectable *via* PCR, rendering them ideal for whole genome characterization.

Variabilit analysis

The estimates of mean value, range, magnitude of phenotypic coefficient of variability (PCV), genotypic coefficient of variance (GCV), heritability in broad sense (h^2b), expected genetic advance (GA) and genetic advance as percent of mean (GAM) for each trait under analysis is provided in Table 2. Among the studied traits, an extensive array of PCV and GCV was found. The results reflect that PCV values were greater than GCV for all traits, proving a substantial influence of the environment on trait expression. Notably, the difference between PCV and GCV estimates was most pronounced for SP, followed by SW, suggesting a notable environmental influence on these traits. PCV value ranged from 4.63% (DM) to 49.82% (NRS), whereas the GCV ranged from 2.71% (DM) to 49.62% (NRS). The high estimates of PCV (>20%) was observed for NRS, SY, PH, EPP, TNP, BY, TS, RS and TS. Moderate estimate of PCV (between 10 to 20%) were recorded for SP, SW, PL and P, while DF and DM showed low PCV (<10%). Similarly, higher GCV estimates were observed for PH, NPP, EPP, BY, SY, NRS, RS, TS and TP, signifying potential for improvement through selection. Thus, these traits could serve as valuable selection parameters. The magnitude of h^2b varied from 34.38% (DM) to 99.21% (NRS). Higher estimates of h^2b (>80%) were observed for PH, TNP, EPP, BY, SY, NRS, RS, TS and TP suggest that selection for these traits could be relatively easy, because there would be a close association between genotype and phenotype, with the environment exerting relatively partial influence on phenotype. The lowest estimates of h^2b (<40%) was observed for DM (34.38%), while moderate estimates of h^2b were recorded for the remaining traits. The GA was estimated at 5% selection intensity and was transformed into GAM for comparison between the traits. Estimates of GA varied from 0.76 for PL to 90.95 for TP. Magnitude of GA for SY was 14.31. The estimates of GAM ranged from 3.28 (DM) to 101.82 (NRS). Low estimates of GAM (<10%) at 5% selection intensity were noted for DM. While it was moderate (11-20%) for SW, PL, DM and P. In current study, high values of GAM (>20%) were observed for EPP, TNP, PH, SP, BY, SY, NRS, RS, TS and TP. The combination of high heritability and high GAM for traits such as PH, TNP, EPP, BY, SY, NRS, RS, TS and TP, suggests that they are predominantly controlled by additive gene effects and selection may be potentially effective for improvement of these traits. Assessing heritability along with genetic advance aids in expecting gains achievable through selection. These findings are in consistence with studies conducted previously by Tiwari *et al.* (2001); Pratap *et al.*, (2024b) and Jagadeesh *et al.* (2023). The genetic variability observed within the evaluated germplasm serves as the

Table 2: Estimates of mean and genetic parameters of field pea genotypes.

Traits	Mean	GCV (%)	PCV (%)	h²b (%)	GA	GAM
DF	76.6	6.32	8.14	60.42	7.76	10.13
DM	120.16	2.71	4.63	34.38	3.94	3.28
PH	94.66	33.8	35.31	91.64	63.12	66.67
TNP	40.54	29.73	32.21	85.21	22.92	56.54
EPP	36.64	30.03	33.37	81.01	20.4	55.69
SP	4.09	13.97	17.8	61.57	0.92	22.58
PL	5.67	8.48	10.92	60.21	0.76	13.55
SW	14.8	12.18	15.6	61.02	2.9	19.61
B Y	59.69	28.59	31.11	84.48	32.32	54.15
S Y	18.99	39.06	41.72	87.67	14.31	75.34
P	25.53	10.27	12.08	72.2	4.59	17.97
NRS	2.92	49.62	49.82	99.21	2.94	101.82
RS	9.64	24.75	25.13	97.01	4.84	50.22
TS	12.73	20.36	20.62	97.47	5.27	41.41
TP	148.84	29.91	30.17	98.95	90.95	61.1

foundation for crop breeding program enabling selection of superior genotypes. Consequently, the greater the variability within the breeding material for a given trait, the higher the potential for enhancement through selection.

Phenotypic diversity analysis

Evaluating the genetic diversity within field pea provides crucial insights necessary for broadening the narrow genetic pool and selecting parental lines to initiate crop improvement programs. In this investigation, dendrograms constructed based on Mahalanobis genetic distance revealed that genotypes can be clustered in 3 groups (Fig 1). Cluster I, II and III comprised of 17, 17 and 12 genotypes, respectively. Highest intra-cluster distance was observed in cluster III followed by cluster II and cluster I whereas highest inter-cluster distance was between cluster II and III followed by I and III and I and II (Table 3). Cluster I had higher mean values for the characters TNP and EPP and lower mean values for DM, P, TS, RS, NRS and TP, cluster II had higher mean values for characters DF, DM, PL and SW and lower mean values for PH, TNP, EPP, SP and BY and Cluster III had higher mean values for characters PH, SP, BY, P, TS, RS, TP and SY and lower mean values for DF, PL and SW (Table 4). The genotypes showed less diversity belonging to same cluster than that of belonging to different clusters. Cluster III had highest diversity among its genotypes and also had highest number of superior characters making this an important cluster from breeding

point of view. As inter-cluster distance indicated cluster II and III were highly diverse to each other and superior cross may be obtained by making crosses between the genotypes of these clusters. Cluster I and II also had enough intra-cluster distances showing ample variability in these groups as well as they have superior characters like DM, TNP, EPP and SW, NRS, respectively which can be used to improve genotypes with these character combinations. Inter-cluster distances between cluster I and II and cluster I and III were also high indicating that they can play important role in making new gene combinations by crossing them which may ultimately lead to get improved population. Comparable conclusions were also described and withdrawn earlier (Tiwari *et al*, 2004; Pratap *et al*., 2024b).

Molecular diversity analysis

Besides phenotypic diversity, SSR markers were employed for genetic diversity assessment in field pea due to their co-dominant nature and high reproducibility, as noted by Negisho *et al*. (2017). This study involved evaluating the genetic diversity of forty-six field pea genotypes utilizing twenty SSR markers. Each primer exhibited easily detectable bands and a distinct banding pattern for analysis. Notably, the primers AD147, D21, AD148, AA504, AA205, AA175, AA174, AA355, AA122, A9, AA285, AC58, AA399 and AD60 produced polymorphic bands in the genotypes, while the remaining six primers generated
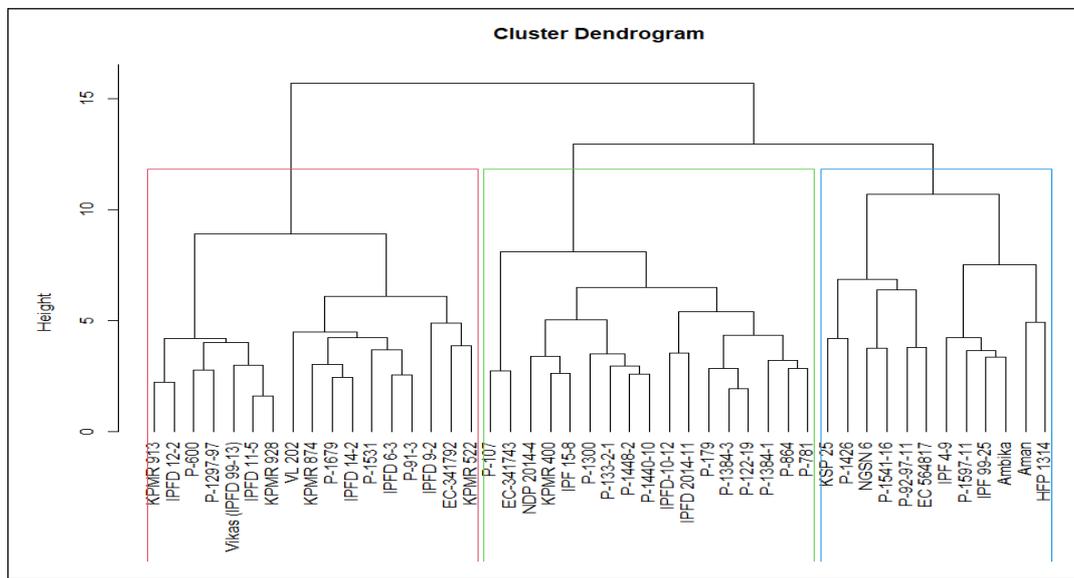

Fig 1: Cluster dendrogram of forty-six genotypes of field pea.

Table 3: Estimates of average distances within and between clusters of field pea.

Cluster number	Number of genotypes	Intra-cluster distance	Inter-cluster distance		
			I	II	III
I	17	4.059	-	5.321	5.863
II	17	4.147	5.321	-	6.200
III	12	5.664	5.863	6.200	-

monomorphic bands (Table 5). The DNA profiles of the forty-six field pea genotypes, depicted using the fourteen SSR markers, are provided in Supplementary Fig S1. To evaluate variability among microsatellite loci, we analyzed parameters such as band size, total number of alleles, major allelic frequency, gene diversity and PIC. The overall fragment length of PCR amplified products extended from 150 bp (AA122) to 970 bp (AA504). Across 14 polymorphic loci, we identified 43 alleles within the forty-six-field pea genotypes ranged from 2 (in AA205, AA285, AC58 and AA399) to 4 (in AD147, D21, AA504, AA122 and A9) and a mean of 3.07 alleles per locus. Major allele frequencies

varied from 0.35 (in D21) to 0.91 (in AA174), with an overall mean of 0.61. Gene diversity and PIC values ranged from 0.15 to 0.16 (in AA174) to 0.72 and 0.66 (in D21), respectively, with mean values of 0.49 and 0.43. In general, PIC value serves as an indicator of marker effectiveness in linkage analysis during inheritance studies among parental lines and hybrids. Across 14 polymorphic loci, the mean PIC value of 0.43, varied from 0.15 to 0.66, closely aligns with previous studies by Dhutmal *et al.* (2021) and Adhikari *et al.* (2018). It's recognized that a PIC value exceeding 0.5 indicates high locus diversity (Botstein *et al.,* 1980). In our investigation, five primers (AD147, D21, AA355, AA122 and A9) exhibited PIC values e"0.5, indicating their efficacy in genotype identification and robust support for detecting polymorphism at specific SSR loci. SSR polymorphism observed in our study is consistent with earlier outcomes by Gupta *et al.* (2014) and Prajapat *et al.* (2014).

In our study, a distance-based tree was constructed (Fig 2) using the UNJ method and Jaccard's dissimilarity coefficient, which categorized all genotypes into three distinct clusters. Cluster I comprised 15 genotypes, further subdivided into two sub-clusters of 9 and 6 genotypes. Cluster II included 15 genotypes, with sub-clusters of 5 and 10 genotypes. Lastly, Cluster III encompassed 16 genotypes, featuring sub-clusters of 4 and 11 genotypes. The clustering based on SSR markers indicated that genotypes within same cluster exhibit genetic similarity, while those in different clusters are less closely related. Utilizing diverse genotypes from different clusters holds promise for effectively selecting desirable recombinants in field pea breeding programs. Molecular analyses unveiled considerable genetic diversity among evaluated field pea genotypes. Corresponding findings regarding the efficacy of SSR markers in assessing genetic diversity for

Table 4: Mean values of clusters for various traits of field pea.

Characters	Cluster number		
	I	II	III
DF	76.37	79.17**	73.30*
DM	118.61*	122.10**	119.64
PH	100.29	73.72*	116.38**
TNP	49.61**	29.66*	43.13
EPP	44.63**	26.84*	39.22
SP	4.14	3.88*	4.36**
PL	5.71	5.95**	5.23*
SW	15.06	15.40**	13.59*
BY	65.59	42.81*	74.14**
P	23.77*	25.31	28.35**
TS	10.57*	13.81	14.26**
RS	7.60*	10.66	11.13**
NRS	2.83*	3.00**	2.97
TP	124.03*	153.60	177.26**
SY	19.15	14.08*	25.73**

*, ** Indicates lowest and highest values for the traits, respectively.

Table 5: Characteristics of amplification products obtained from fourteen SSR markers.

| Marker name	Band Size	Number of alleles	Major allele frequency	Gene diversity	PIC
AD147	300-370	4	0.39	0.71	0.65
D21	230-270	4	0.35	0.72	0.66
AD148	180-200	3	0.54	0.52	0.40
AA504	350-970	4	0.76	0.40	0.37
AA205	200-220	2	0.67	0.44	0.34
AA175	270-300	3	0.85	0.26	0.24
AA174	190-280	3	0.91	0.16	0.15
AA355	160-200	3	0.37	0.66	0.59
AA122	150-280	4	0.52	0.58	0.50
A9	400-600	4	0.50	0.62	0.55
AA285	200-220	2	0.63	0.47	0.36
AC58	200-230	2	0.74	0.39	0.31
AA399	200-240	2	0.67	0.44	0.34
AD60	150-250	3	0.59	0.56	0.49
Mean		3.07	0.61	0.49	0.43
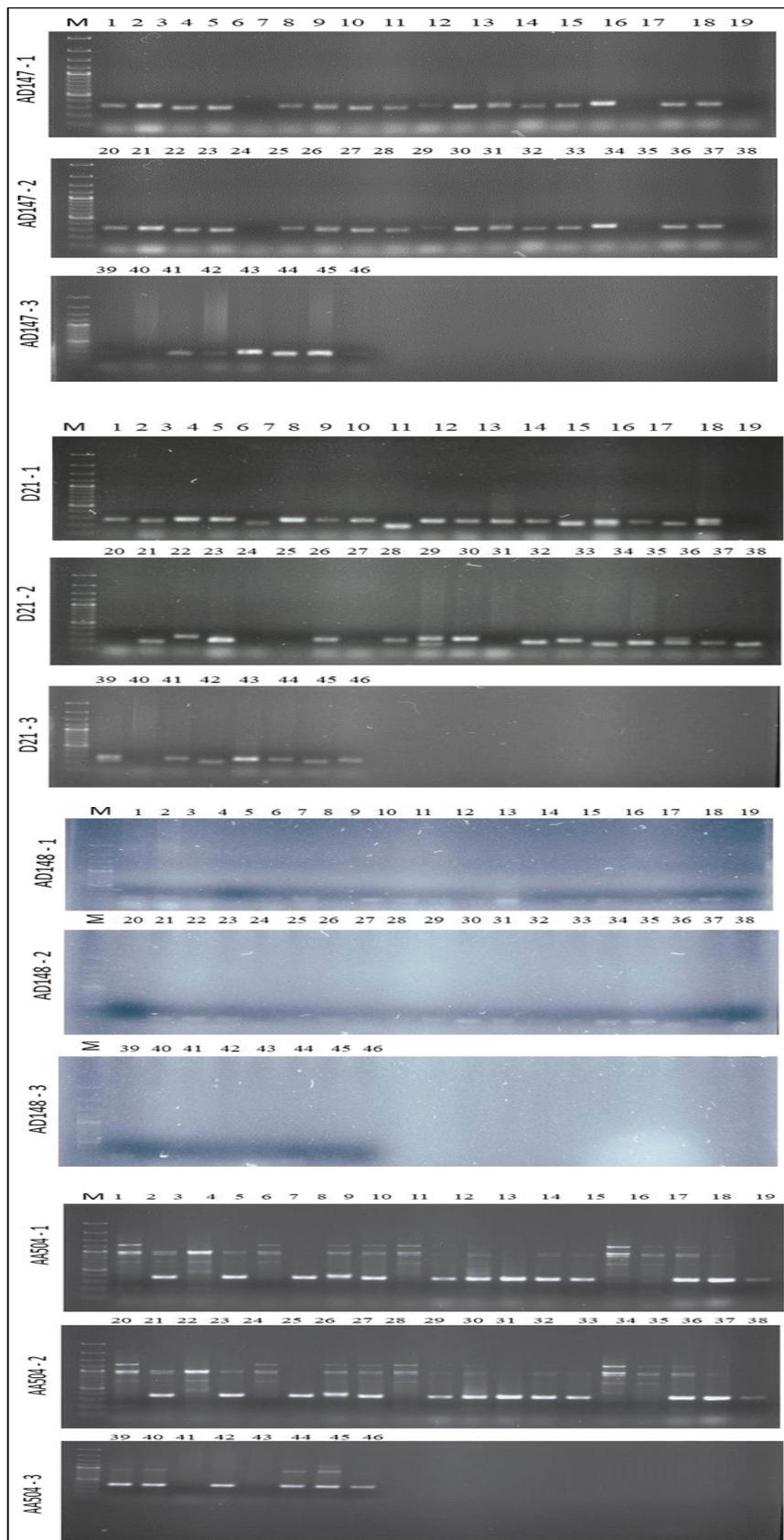
Fig S1: Continue...

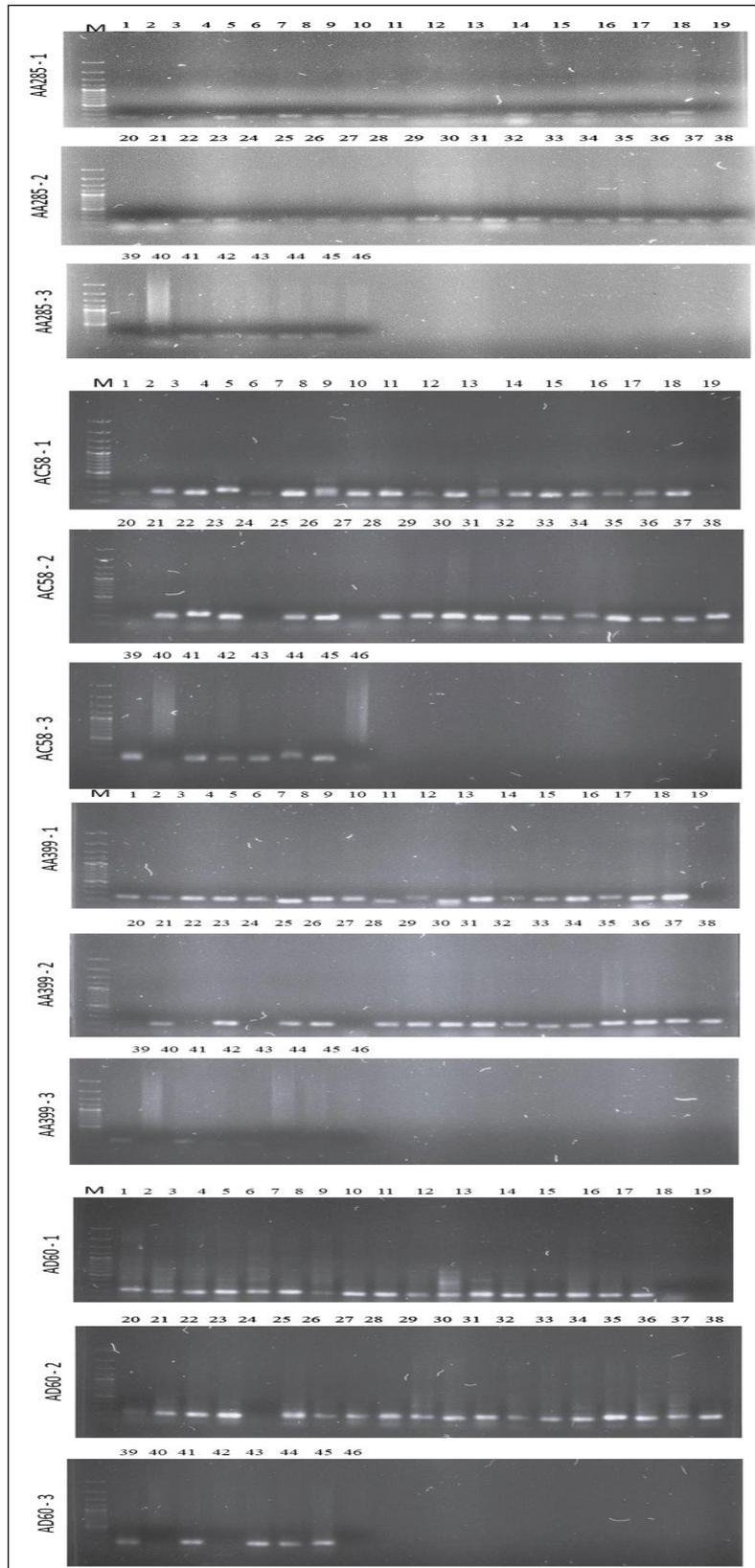**Fig S1: Continue...**
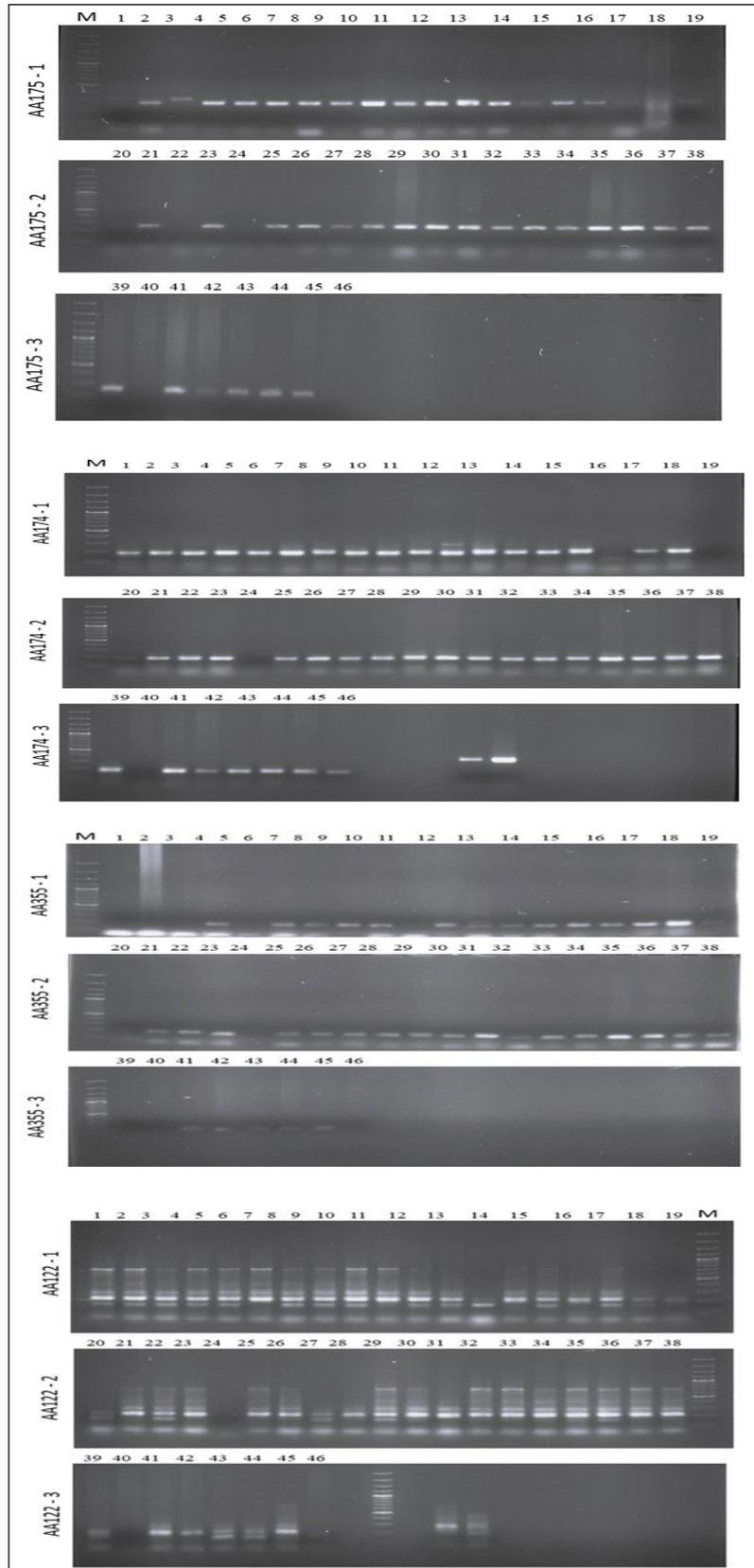
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Fig S1: Banding pattern of 46 field pea genotypes with 14 SSR marker on agarose gel. Here, M is 100bp DNA ladder.
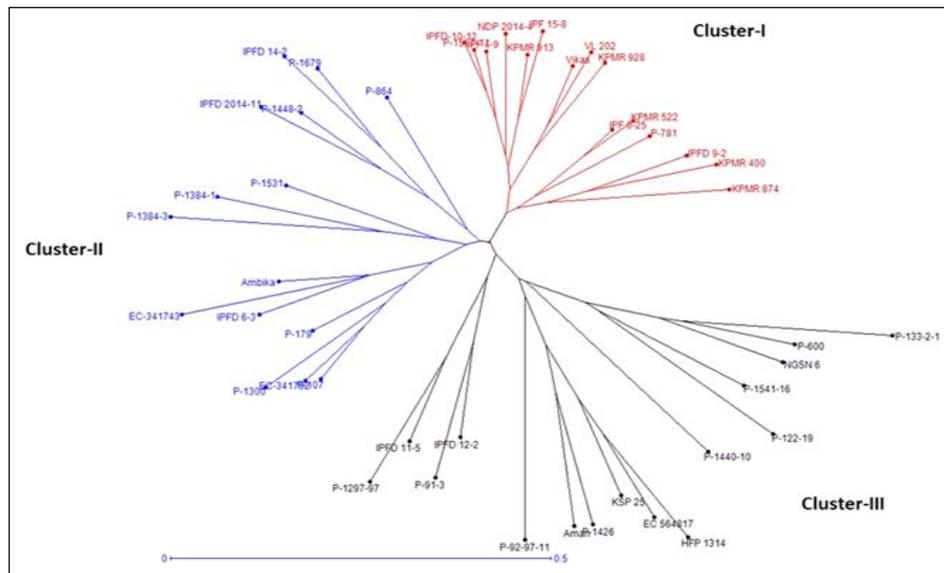
Fig 2: UNJ clustering of 46 field pea genotypes obtained from 14 SSR markers.

yield and its related traits were also stated by Negisho *et al.* (2017) and Osman *et al.* (2021).

CONCLUSION

The primary aim of this investigation was to assess phenotypic diversity based on morphological and quality traits, alongside molecular diversity among field pea genotypes. Variance analysis revealed significant variability among test genotypes for morphological and quality traits. Molecular analysis using 14 polymorphic SSR markers detected a total of 43 repeatable alleles, varied from 150 bp (AA122) to 970 bp (AA504), with AD147, D21, AA504, AA122 and A9 amplifying the maximum of 4 fragments. Both phenotypic and molecular diversity studies was in agreement that all genotypes under study can be divided in 3 clusters however in both studies genotypes were different in different cluster indicating significance of both studies. Cluster III was highly diverse with higher number of superior characters and also showed high divergence with cluster II indicating that it should be utilized to improve the yield by making superior and diverse combination of yield contributing characters. Genotype IPFD 10-12 for 100-seed weight and pod length; HFP 1314 for seed yield per plant, biological yield per plant, seeds per pod and reducing sugar; Aman for seed yield per plant and seeds per pod; EC 564817 for biological yield per plant and protein content was found promising. These SSR markers used in the study highlighted significant genetic variability among the studied genotypes.

ACKNOWLEDGEMENT

Disclaimers

Conflict of interest

The authors declare that no conflict of interest.

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