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RESEARCH ARTICLE

Assessment of Diversity, Population Structure and Gene Flow in Indigenous and Exotic Accessions of Adzuki Bean (*Vigna angularis*)

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Abstract

Adzuki bean is recognized for its high carbohydrate content and protein composition, featuring a well-balanced amino acid profile, particularly abundant in lysine. This compensates for the typically deficient protein levels found in commonly consumed cereals. Morphological diversity analysis categorized 96 adzuki accessions into three distinct clusters, with the greatest intra-cluster distance observed between cluster I and cluster II. Principal Component Analysis indicated that PC1 accounted for 55.90% of the total variation, primarily attributed to traits such as primary branches per plant, plant height, 1000-seed weight, clusters per plant, seed yield per plant and seeds per pod. Seventy SSR primers were utilized to assess molecular diversity. Among these, 11 primer pairs exhibited polymorphism, with a mean PIC value of 0.241. The average number of effective alleles (Ne) and Shannon's Information Index (SII) were calculated as 1.5051 and 0.4560 per locus, respectively. Clustering analysis using the NTSYS-pc package identified three clusters comprising both indigenous and exotic accessions. Population STRUCTURE revealed the highest ΔK mean value at K=2, dividing the 96 germplasm accessions into two distinct gene pools, consisting of pure lines and admixtures. AMOVA conducted between the two populations unveiled that 79% of the variation exists within populations, while 21% exists among populations. The pure lines and polymorphic SSR markers identified in this study hold promise for the development and evaluation of core collections and the breeding of superior varieties of Adzuki bean, along with harnessing genetic diversity in Adzuki bean to enhance breeding endeavours toward superior varieties and hybrids.

Keywords: Adzuki bean, Genetic resources, Molecular diversity, Population structure, SSR analysis

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Introduction

Adzuki bean, scientifically known as *Vigna angularis* var. *angularis* (2n=2x=22), is a noteworthy crop due to its extreme resilience and capacity to adapt to a wide range of cultivation conditions in both temperate and subtropical regions. It is commonly cultivated in East Asia and is known for its small size, reddish-brown colour, and sweet flavour. It is believed that the weedy adzuki bean, or *Vigna angularis* var. *nipponensis*, originated in the Himalayan and northeastern regions of Asia, where the adzuki bean was first domesticated (Wang *et al*., 2009). This species holds significance within the Asian *Vigna* genus, categorized under the *Ceratotropis* subgenus within the papilionoid subfamily of the Fabaceae family. Adzuki bean, a versatile culinary ingredient, thrives in nutrient-deficient soils and serves as a valuable rotation crop, fixing nitrogen at a rate of 50-500 kg/ha.

Kitano-Okada *et al*., (2012) termed it the 'weight loss bean,' as it is rich in proteins, carbohydrates, antioxidants and essential vitamins while being low in calories and fat. With antioxidant, antihypertensive, anti-inflammatory and anti-

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diabetic properties, it aids in maintaining excellent health and well-being. Adzuki bean's low glycemic index helps regulate blood glucose levels, and its balanced amino acid profile complements cereals' protein deficiency. Additionally, it is a rich source of fibre and essential trace elements such as iron, calcium and phosphorus. Adzuki bean has the potential to have a significant impact in the fight against hunger, for better health and wellbeing and for climate action, particularly in the backdrop of the Sustainable Development Goals (SDGs). Furthermore, its short growth cycle and compact genome make it a potential model species for non-oilseed legumes. China, Japan and Korea house significant adzuki bean germplasm collections (accessions, landraces and cultivars), with China boasting the largest repository (5500 accessions) at the Institute of Crop Germplasm Resources (CAAS), Beijing; Japan's Tokachi Agricultural Experiment Station in Hokkaido-ken holds 3600 accessions (Wang *et al*., 2019), while ICAR-National Bureau of Plant Genetic Resources (NBPGR), India, maintains 203 accessions.

Before improving any variety through breeding strategies, it is important to have a basic comprehension of genetic diversity. Many methodologies are available for the morphological characterization of yield-contributing and biochemical traits (Wang *et al*., 2001), and numerous investigations have utilized amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR) and other molecular markers to examine the genetic diversity of adzuki bean (Akkaya *et al*., 1992; Xu *et* al., 2000; Isemura *et al*., 2001 and Xu *et al*., 2008).

DNA marker analysis reveals pronounced genetic variations among distinct forms of Adzuki bean; however, cultivated germplasm exhibits limited diversity, underscoring the potential significance of wild variants as a vital genetic reservoir for breeding programs (Brown *et al*., 1996). The use of conventional breeding techniques, such as introduction and selection strategies, has made a decent contribution to the improvement of Adzuki bean. Integration of advanced breeding methods and biotechnological approaches into adzuki bean improvement has been hindered by a paucity of genomic data.

The greatest genetic diversity in adzuki bean has been reported in germplasm originating from China, Japan and South Korea, with substantial genetic differentiation observed among the accessions. This suggests prolonged independent domestication processes involving selection for traits such as seed dormancy, pod dehiscence, plant size, early maturity, yield potential and colouration. Several studies have indicated that China has the highest genetic diversity (Yee *et al*., 1999 and Wang *et al*., 2001), whereas other studies have provided insights into the genetic structure of the Japanese wild/weedy/cultivated population (Xu *et al*., 2000). Isemura *et al*., (2001) and Zong *et al*., (2003) found that cultivated adzuki beans in Bhutan and Nepal are genetically distinct from those in other parts of the world.

Researchers from all around the world have looked into the adzuki bean's morphological variation, as well as its molecular characterization. The molecular diversity of accessions from India, however, has not been investigated so far. In light of the foregoing, this study set out to use random simple sequence repeat (SSR) markers to examine the genetic diversity, population structure and gene flow present in indigenous and exotic germplasm accessions sourced from the ICAR-NBPGR, Regional Station, Himachal Pradesh, India.

Materials and Methods

A total of ninety-six (Table 1) adzuki bean genotypes collected from ICAR-NBPGR, Regional Station, Himachal Pradesh, India, were planted in an alpha-lattice design during *Kharif* 2020 at the Department of Genetics and Plant Breeding, CSKHPKV, Palampur, Himachal Pradesh, to assess their morphological and molecular diversity.

Table 1: List of germplasm lines of adzuki bean used for analysis

S.No.	Genotype	S.No.	Genotype	S.No.	Genotype	S.No.	Genotype
1	HPU-51(c)	2	EC-290251	17	EC-240246	18	IC-251353
3	EC-187896	4	IC-341948	19	EC-340275	20	IC-341953
5	EC-340272	6	EC-340258	21	EC-340245	22	EC-87071
7	EC-15648	8	EC-340286	23	IC-485385	24	IC-341949
9	EC-120460	10	IC-341957	25	EC-340257	26	EC-340265
11	EC-340247	12	Local Totru (c)	27	EC-000248	28	IC-108856
13	IC-485886	14	EC-340274	29	EC-000254	30	EC-340240
15	EC-59489	16	EC-340281	31	EC-57159	32	EC-340254

S.No.	Genotype	S.No.	Genotype	S.No.	Genotype	S.No.	Genotype
33	EC-000249	34	IC-469175	65	IC-108855	66	IC-89957
35	EC-340283	36	EC-290652	67	EC-340251	68	EC-340278
37	IC-341958	38	EC-340285	69	IC-341947	70	EC-340287
39	EC-000251	40	EC-340284	71	IC-341952	72	IC-341956
41	EC-340282	42	EC-340253	73	EC-340246	74	EC-87896
43	EC-000377	44	IC-100072	75	IC-469172	76	EC-120466
45	EC-15256	46	EC-000276	77	IC-241041	78	EC-120480
47	EC-24102	48	EC-340276	79	IC-341944	80	IC-469174
49	IC-24105	50	IC-485396	81	EC-340277	82	EC-18257
51	EC-240251	52	EC-18959	83	EC-30256	84	IC-341963
53	EC-34264	54	IC-341946	85	IC-469173	86	IC-341945
55	IC-341941	56	EC-18256	87	IC-16761	88	EC-08707
57	IC-485388	58	IC-485382	89	EC-24523	90	EC-108080
59	IC-140848	60	EC-87895	91	EC-340288	92	EC-340261
61	EC-87815	62	IC-341937	93	EC-30270	94	EC-30253
63	IC-341954	64	EC-340256	95	EC-340263	96	EC-340250

Table 2: List of qualitative and quantitative traits studied

S. No.	Qualitative trait	S. No.	Quantitative trait
1	Early plant vigour	1	Days to 50% flowering
2	Plant habit	2	Days to 80% maturity
3	Leaf colour	3	Plant height(cm), primary branches per plant
4	Leaf surface	4	Clusters per plant
5	Leaflet shape	5	Pods per cluster
6	Flower colour	6	Pods per plant
7	Stem colour	7	Seeds per pod
8	Stem surface	8	1000-seed weight (g)
9	Pod angle	9	Seed yield per plant (g)
10	Pod surface		
11	Seed coat colour		

Morphological characterization

Quantitative data (Table 2) recorded on the adzuki bean accessions were analyzed using TNAUSTAT software to estimate descriptive statistics (mean and range), phenotypic coefficient of variation (PCV), genotypic coefficient of variation (GCV), broad-sense heritability (h^2bs) and expected genetic advance as a per cent of mean (Table 3). Analysis of variance (ANOVA) was performed to determine the significance of differences among genotypes. Qualitative traits (Table 2) were analyzed based on frequency distribution.

Estimation of morphological diversity

A measure of group distance based on multiple traits was given by Mahalanobis (1936). With x_1, x_2, x_3,.... x_p as the multiple measurements available on each individual and d_1, d_2, d_3 .....d_p as $x_1^{-1} - x_1^{-2}$, $x_2^{-1} - x_2^{-2}$ .....$x_p^{-1} - x_p^{-2}$, respectively, being the difference in the means of two populations, Mahalanobis D^2-analysis is defined as follows: $pD^2 = b_1d_1 + b_2d_2 + \ldots\ldots\ldots\ldots b_pd_p$. In terms of variances and covariances, the D^2 value is obtained as follows: $pD^2 = W^{ij}(x_i^{-1} - x_i^{-2})(x_j^{-1} - x_j^{-2})$, where W_{ij} is the inverse of the estimated variance-covariance matrix. The test of significance was done as suggested by Rao (1952). Using D^2 values, different genotypes were grouped into various clusters following Tocher's method as suggested by Rao (1952).

Grouping of genotypes into various clusters

Average intra-cluster distance $(D^2) = \Sigma D_i^2/n$

Average inter-cluster distance $(D^2) = \Sigma D_{ij}^2/n_i \ldots n_j$

where, ΣD_i^2 = sum of all distances between all possible

combinations (n) of the genotypes included in the cluster. ΣD_{ij}^2 = sum of all distances between all possible combinations ($n_i.n_j$) of the genotypes between the clusters

n_i = number of genotypes in i^{th} cluster s

n_j = number of genotypes in j^{th} cluster

Table 3: List of adzuki bean SSR primer sequences used in the present study

S. No.	Primers	Forward sequence	Reverse sequence
1	VES0001	TGTTTCCACAGCAACACCAT	GTTGTAAAGGGCGTCATGCT
2	VES0002	GGATTTATTGACTTCAAAATGCAC	GATGGGGCCTGTTAAGATGA
3	VES0003	GGGATCATGATATTGCATTGG	AATGTCAGCCAGCATGAGAA
4	VES0004	ATTATAACTCGTGCGTGCCC	TCTGAAGGAACACGCTGATG
5	VES0005	AGGATGGAAATGGCTAGGCT	ATCAAATCACATGAAGGCCC
6	VES0006	GTGTTCCATTCCCATTCCAC	ATTGGAGGGCCGTTAAGAGT
7	VES0008	CCTGCTCGGAATGAGAGTTC	CCCACATGGACTTTGTTTTT
8	VES0009	TCTCTCACCCCCTTAACCCT	TCGCAGAATTCGGAGAACTT
9	VES0010	AAAATCAGAAGCTCCCCTCC	TGGACTTTGCTGCTGCTAGA
10	VES0013	CGAAACCTTTTCAGCACCTC	CACCCTTTCGTCCACTCAAT
11	VES0014	CACACATCCAGTGCCATTTC	AAATCCTGGAGGCCCTCTT
12	VES0015	AATAGGCCATGCTGTCCTTG	AGTAATCCAGGCGGTGATTG
13	VES0016	CACCAAAGTGGTATCGTGGA	AAAGAAAAAGGGGAGGGGAT
14	VES0017	ACGAAAGAGTCCGCTTGAAA	GATGGAAGGGGTTGTTGCTA
15	VES0018	CATTCCAATCCCAAATGGAC	TGCTTGAAATAGGTGGGGAG
16	VES0019	GGCTCACACTCATTCTTCCC	TCACACGAGCTGTCTATGCC
17	VES0020	AAGCAAGCCTTCCATGCTAA	TGCTGATACAAAGGCTGCTG
18	VES0021	GAGTCACATGCAAAAGGTCG	GGAACACTGTGTGCAAGATCA
19	VES0022	CTGTTCCCGGACATTGTTCT	GCTCAGCGATCAAATTCCTC
20	VES0023	CATGGCGAAGAAGAAGGAAG	TTGTGAAACACCATCCATGC
21	VES0024	GCTCCAGAGAGAGGAGCAAA	CCAATTGCCCTTGATCATTC
22	VES0025	CCCACTCTTGGAATGGAAAA	TTGGTGAGCAATGTTGTGGT
23	VES0026	TCCCTTACTGTTAGGCCATCC	AGGTCCACAACTTCCACCAA
24	VES0027	TCTTTGGCGAGTTGATGATG	CCAGGGTGGTGAATGACTTT
25	VES0028	CCTGAGCAACATCGGAATCT	TCCCTTCCTTTAACCCCAAC
26	VES0029	TGCTACACAAAACCAAGGCA	AAACGTAGCTACCACCACCG
27	VES0030	GCCACAAGCTCAAAGACCTC	ATTCAGGGATGGTGACAAGC
28	VES0031	ACCTTTCGGCATTTTCCTCT	AAGAAGAAGGGGTTCGCATT
29	VES0032	ACAGTGGTGGAGTGGACCTC	TCACGTGTCTCCAGTTTTGC
30	VES0033	GGAGGGGAAGAAGGAGAATG	CCCACTCATTCAACAGGCTT
31	VES0034	AGATGAGCAACCCATGAAGG	TTATCATCGACCATGCCAGA
32	VES0035	GCCCCAACCATAACCCTAAT	GGGATCGTAGGAGGTGGAAT
33	VES0036	TCCCTTGTTTTTCGATCTGG	AAATTCGGGCAGAATCACAG
34	VES0037	ACCAATCACACCCACATCAA	ATCATCAACTCCTGGGAACG
35	VES0038	TGCTTGCCTTTTTCTTTTCC	GAGCAGAGGAGATTTTTGGG
36	VES0039	AACTTACCCACCTGCAAACG	GAGGAGGGTGAACTGGAACA
37	VES0040	GGAACAGTGTCAGAGAGCCC	TTCCCAAAGATCATTTTCGC
38	VES0041	TGGAGATCAACAGACAGACCA	GATCAGAAGGTAGCAAGCCG
39	VES0043	CTTGGGATGCTTTGTTTGGT	CATCAGCTAGTGAAAGCCCC
40	VES0044	CCTCATTCTTACATCCTTAAACACTTG	CCAGAAGAATCACCAGCCAT
41	VES0045	TCCCAACCGTCAAGTTTACC	TTCATCTTGACAATGCGGAG

S. No.	Primers	Forward sequence	Reverse sequence
42	VES0046	CCGAACCCTATCTTGTGTGG	GTGATCGATGAGGGAAGGAA
43	VES0047	CACGAACGCAATTTAAAGCA	TCGTGTTCGACAAGCTCAAC
44	VES0048	AGATTCTTCTGCGGGCTCTT	GCCGACAAGGTCAAGGAATA
45	VES0050	TCAATCCCGACACAAAATGA	GCTAAATTGGTTGCACGGTT
46	VES0051	GCGAGAACCTTCTTACCCCT	TCCTGCAAGAGACTGTGGTG
47	CEDG127	GGTTAGCATCTGAGCTTCTTCGTC	CTCCTCACTTGGTCTGAAACTC
48	CEDG018	AGCGTGTTTGTGGTGATAGC	ACACAGGAACGAACAAACCC
49	CEDG150	GAAGGGAATGAAAATGAAACCC	GTTCAATCCATTCAGTCTCC
50	CEDG214	CACTCACTGCAAAGAGCAAC	CTACCTATCTGAGGGACAC
51	CEDAAG002	GCAGCAACGCACAGTTTCATGG	GCAAAACTTTTCACCGGTACGACC
52	CEDG204	CCTTGGTTGGAGCAGCAGC	CACAGACACCCTCGCGATG
53	CEDG0	AGGATTGTGGTTGGTGCATG	ACTATTTCCAACCTGCTGGG
54	CEDG043	GCAGAATTTTAGCCACCGAG	AAAGGATGCGAGAGTGTAGC
55	CEDG021	ATCAACTGAGGAGCATCATCGA	CAACATTTCAACCTTGGGACAG
56	CEDG084	CCCGATGAACGCTAATGCTG	CGCCAAAGGAAACGCAGAAC
57	CEDG015	TCAGCAATCACTCATGTGGG	TGGGACAAACCTCATGGTTG
58	CEDG026	CCCCGAAATTCCCCTACAC	AACACCCGCCTCTTTCTCC
59	CEDG073	AGGCGAGGTTTCGTTTCAAG	GCCCATATTTTTACGCCCAC
60	CEDG008	CGAGCAGAACACTGATCATG	CCTCTTAGAGGTCATTGCTC
61	CEDG286	CACCTTCTTAATCTCTTCACC	GGGTTTCTCTTAATTCATTGAGTC
62	CEDG294	GATGACCAAGGTAACGTG	GGACAGATCCAAAACGTG
63	CEDG232	GGTCCATTGAGACGGATCGAG	TCCCACCTCAGCGGAATCC
64	CEDG071	CACTTCCATGATGACTCACC	CACCCTTCTTTATCCTCTTCG
65	CEDG253	ATAAGTAGAAATTGGTTCAAATG	GGTTCGTTAAAGTAACTTTTAAT
66	CEDG090	TCAGCAACCTTGCATTGCAG	TTTCCCGTCACTCTTCTAGG
67	CEDG044	CCAGGCATCCATGATGACC	GAAGTTGTTGGTAATGGTTGCCTC
68	CEDG141	CGGAAGAAGAACGCAGAGTG	GCATCAACAAGGACTTCTGC
69	CEDG178	AACCCAACCAACCCTTGTGGTAAG	GCTGGAATCATAATACCGCCTTGT
70	CEDG118	GTCCTTGTTTTCCTCTCCATGG	CATCAGCTGTTCAACACCCTGTG
	CEDG154		

Cluster means

Cluster means of adzuki bean genotypes falling under different clusters in individuals as well as combined over environments were also calculated.

Contribution of individual character towards divergence

In all combinations, each character was ranked on the basis of $di = Y_{ij} - Y_{ik}$ values. The contribution of individual character to the divergence has been worked out in terms of 'n' number of times it appeared first.

Principal component analysis

The main goal of PCA is to reduce the dimensionality in a set of correlated attributes into a smaller set of uncorrelated attributes that explain the majority of variation in the original attributes and was calculated using the statistical software StatistiXL version 1.10.

Extraction of plant genomic DNA

Genomic DNA was isolated from young leaf tissue (0.5-1 g) of each accession using the CTAB method Murray and Thompson (1980). Leaf tissues were rinsed in deionized water, dried on tissue paper discs, then pulverized to fine powder in liquid nitrogen in autoclaved, pre-cooled pestles and mortars. Ground tissue was transferred into 2 ml eppendorf tubes with 800 µl extraction buffer (2% CTAB, 100 mM Tris, 20 mM EDTA, 1.4 mM NaCl, 1% PVP, pH 8.0) in a water bath at 60^{0}C and firmly stirred. With intermittent mixing, the mixture was incubated at 60^{0}C for 1 hour. The tubes were gently mixed with an equal amount of chloroform: isoamyl

alcohol (24:1). The mixture was centrifuged at 10,000 rpm for 10 minutes at 4^{0}C. The aqueous phase was transferred to a new tube, and 500 µl of pre-chilled isopropanol was added. DNA was precipitated by centrifuging the tubes at 10,000 rpm for 10 minutes after gently mixing them and incubating at -20^{0}C for 1 hour. The DNA pellets were washed twice with 1 ml of 70% cold ethanol after removing the supernatant. The pellet was dried for 3-4 hours in sterile air in a laminar airflow cabinet. The dried DNA pellet was diluted in 1 ml TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0).

Purification of DNA

To purify DNA, 1 µl of RNase (10 mg/ml) and 100 µl of phenol: chloroform: isoamyl alcohol (25:24:1) were added to tubes and gently mixed. The mixture was centrifuged at 10,000 rpm for 10 minutes at 4^{0}C. The aqueous phase was moved to a new tube, and 200 µl of pre-chilled isopropanol was added. After gently mixing the tubes, they were incubated at -20^{0}C for 30 min. Centrifugation at 10,000 rpm for 10 minutes to precipitate DNA. The particle was washed twice with 70 µl cold ethanol after draining the supernatant. The pellet was subjected to a laminar airflow cabinet's sterilized air stream in order to dry it. The dried DNA pellet was dissolved in 80 µl TAE buffer. DNA quantity and quality were assessed by electrophoresis on a 0.8% agarose gel (HIMEDIA).

PCR amplification of DNA

For the amplification of genomic DNA, a reaction mixture of 12.5 µl volume was prepared using 7.15 µl of sterilized distilled water, 1.0 µl template DNA (25 ng/µl), 0.5 µl of forward and 0.5 µl of reverse primer (5 µM), 1.0 µl $MgCl_2$ (25 mM), 1.25 µl 10 X PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 1.0 µl dNTP mix (0.2 mM each of dATP, dGTP, dCTP and dTTP) and 0.1 µl *Taq* polymerase (5 U/µl). The amplifications were carried out in Veriti Applied Biosystems using the protocol given in Table 4.

Table 4: PCR conditions used for amplification of *Vigna angularis* genomic DNA

Primer Type	Steps	Temperature and time	Cycles
SSR	Initial denaturation	94^{0}C for 5 minutes	1
	Denaturation	94^{0}C for 30 seconds	
	Annealing	57^{0}C for 30 seconds	40
	Extension	72^{0}C for 1 minute	
	Final extension	72^{0}C for 5 minutes	
	Storage	4^{0}C for ∞	1

The amplification products were electrophoresed in a 3% agarose gel (HIMEDIA) and stained with ethidium bromide (0.5 µg/ml). The gels were visualized and photographed under UV light using the Gel-Documentation Unit.

Analysis of SSR profiles

Binary data matrices were created, wherein '1' and '0' represented the presence and absence of particular molecular weights, respectively, for each primer. Jaccard's coefficient was utilized to construct a similarity matrix ($J_{ij} = C_{ij}/(n_i + n_j - c_{ij})$), where '$C_{ij}$' denotes the number of positive matches between two genotypes, while n_i and n_j represent the total number of bands in genotypes i and j, respectively. Genetic distances (GD) were calculated as GD = 1- [$C_{ij}/(n_i + n_j - C_{ij})$]. These data were then utilized to generate a dendrogram through the unweighted pair group method with arithmetic averages (UPGMA) in the SAHN program of the NTSYS-pc package. The genetic diversity parameters, including observed heterozygosity (H_o), expected heterozygosity (H_e), average heterozygosity (Av. He), effective number of alleles (N_e), number of alleles (N_a), and Shannon's Information index (I) per locus, were computed using version 1.32 of the POPGEN computer software, as described by Yeh and Boyle (1997). Additionally, the Polymorphic Information Content (PIC) was determined using the formula PIC = 1 - Σ(pi)^2, where k represents the total number of alleles and pi is the frequency of the i^{th} allele in the set of genotypes investigated. Effective multiplication ratio (EMR) was calculated as EMR = n × β as described by Prevost and Wilkinson (1999**).** Marker index was calculated by MI = PIC × EMR as described by Powell *et al.,* (1996).

Clustering analysis

Binary data were used to calculate a genetic dissimilarity matrix using the Jaccard dissimilarity index (d_{ij}) between pairs of accessions (units). Jaccard's similarity index (Jaccard, 1901) was calculated with data provided by the observation of the presence (1) or absence (0) of bands between pairs of accessions using the formula $D_{(ij)}$ = a/(a+b+c) where a is the number of fragments shared by accessions i.

STRUCTURE version 2.3.4 Pritchard *et al*., (2000) was used to infer the genetic structure so as to obtain an estimate of the likely number of population genetic clusters (K). The number of clusters of the population (K) was identified by performing 6 iterations and setting the value of k from 1 to 25, with a burn-in period of

100,000 and 100,000 Markov Chain Monte Carlo (MCMC) repeats after burn-in. The maximal value of LnP (D) and the posterior probability of data as per Evanno *et al*., (2005), were obtained using STRUCTURE HARVESTER Earl and vonHoldt (2012).

Analysis of molecular variance (AMOVA)

The Analysis of Molecular Variance procedure in GenALEx 6.5 Peakall and Smouse, (2006) was used to estimate the components of variance on the basis of a pairwise individual-by-individual genetic distance matrix (Euclidean distance matrix) generated for binary data.

Principal coordinate analysis (PCoA)

The Patterns of genetic relationships contained in the matrix were visualized by Principal Coordinate Analysis (PCoA) in GenALEx 6.5 Peakall and Smouse (2006).

Results

Morphological characterization

We observed considerable variability among the 96 adzuki bean genotypes for all quantitative traits studied (Table 5). Days to 50% flowering ranged from 54.27 to 82.77 days, while days to 80% maturity varied from 100.17 to 125.67 days.

The highest phenotypic coefficient of variation (PCV) and genotypic coefficient of variation (GCV) were recorded for pods per cluster (30.01 and 26.47%), followed by primary branches per plant (29.10 and 25.30%), indicating substantial genetic variability for these traits. High heritability, coupled with high genetic advance as a per cent of the mean, was observed for clusters per plant (97.80%, 38.79%) and 1000-seed weight (98.75%, 37.37%).

Twelve qualitative traits were evaluated through visual observations, revealing considerable phenotypic variation among the 96 adzuki bean accessions. Most accessions (77.80%) exhibited good early plant vigour, while 15.70% showed very good vigour. All accessions possessed an indeterminate growth habit. Green leaf colour was predominant (52.30%), followed by yellowish green (38.60%), and 69.30% of the accessions had pubescent leaves. Entire leaflet shape was observed in 63.40% of the germplasm, whereas 34.00% had lobed leaflets. Green stem pigmentation was recorded in 94.80% of the accessions, and 90.20% exhibited pubescent stems. Yellow flowers predominated (92.80%), with the remaining accessions bearing light yellow flowers. All accessions had an erect pod angle, and 96.10% possessed glabrous pods. Seed coat colour showed marked variation, with red being the most common (80.40%), followed by other colours (17.60%) and maroon (2.00%), indicating substantial morphological diversity within the germplasm.

Morphological diversity using Mahalanobis D^2 statistics

In the present investigation, with non-hierarchical Euclidean cluster analysis, 96 genotypes of adzuki bean were grouped into three major clusters (Fig. 1). The maximum number of genotypes were placed in cluster I

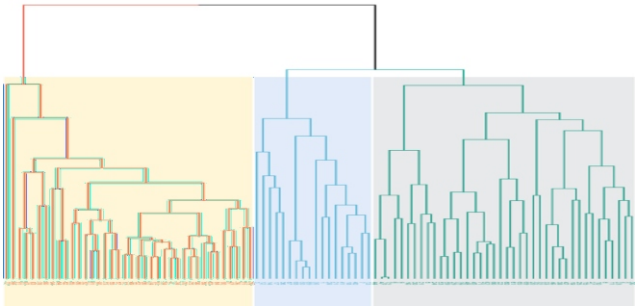


Fig. 1: Phenogram of adzuki bean accessions generated using Mahalanobis D^2-analysis.

Table 5: Descriptive statistics for ten morphological traits studied in the current investigation

S. No.	Traits	Mean	Range	PCV (%)	GCV (%)	Heritability h^2bs (%)	Expected GA (% of mean)
1.	Days to 50% flowering (days)	68.36	54.27- 82.77	7.54	7.29	93.38	14.51
2.	Days to 80% maturity (days)	109.28	100.17-125.67	4.02	3.54	77.61	6.43
3.	Plant height (cm)	85.16	46.82-102.54	9.57	4.76	24.78	4.89
4.	Primary branches per plant	3.72	1.59-6.59	29.10	25.30	75.58	45.31
5.	Clusters per plant	15.97	7.46-22.06	19.25	19.04	97.80	38.79
6.	Pods per cluster	3.21	1.0-6.1	30.01	26.47	77.78	48.08
7.	Pods per plant	36.40	14.41-58.31	19.64	18.16	85.54	34.61
8.	Seeds per pod	7.74	4.43-11.38	15.01	14.10	88.27	27.29
9.	1000-seed weight	90.90	58.14-162.54	18.37	18.26	98.75	37.37
10.	Seed yield/plant (g)	12.66	7.91-16.51	13.43	12.64	88.51	24.49

Table 6: Grouping of adzuki bean accessions into different clusters based on Mahalanobis D^2 analysis

Clusters	No. of accessions	accessions
Cluster I	40	EC-290251, EC-340272, EC-340286, EC-120460, IC-341957, EC-340247, EC-87815, IC-108855, EC-87071, IC-89957, EC-340258, EC-34264, EC-340257, EC-340240, EC-340275, IC-341949, IC-140848, EC-15256, EC-000276, EC-340276, EC-240246, EC-000254, EC-000251, EC-59489, IC-341953, EC-340285, EC-340253, IC-341937, EC-24102, EC-87895, IC-485385, EC-57159, EC-340254, EC-000248, IC-100072, IC-341952, IC-341956, IC-341947, EC-000249 , EC-340265
Cluster II	18	IC-485886, IC-485396, EC-24523, EC-340278, IC-16761, EC-15648, EC-340284, IC-251353, EC-340282, EC-340245, IC-108856, HPU-51, EC-340283, EC-290652, EC-240246, EC-340274, EC-187896, Local *Totru*
Cluster III	38	IC-341948, IC-341958, EC-240251, IC-341963, IC-469175, IC-485388, IC-485382, EC-18256, IC-469174, EC-340277, EC-18257, EC-30256, EC-18959, EC-340287, EC-340246, EC-30253, IC-24105, IC-469173, EC-340288, EC-340261, EC-30270, IC-341945, EC-08707, EC-108080, EC-000377, EC-340265, EC-120480, IC-341946, IC-341941, EC-340251, EC-120466, EC-340250, IC-241041, IC-341944, EC-340263, IC-341954, IC-469172, EC-87896

(40 genotypes), followed by cluster III (38 genotypes) and cluster II (18 genotypes). Genotypes falling under each cluster are presented in Table 6.

Maximum intra-cluster distance was observed in cluster II (27.20) followed by cluster III (17.87), indicating genetic diversity among the genotypes belonging to these clusters. The minimum intra-cluster distance was observed in cluster I (8.40) (Table 7); thus, the chances of developing good segregants by crossing the genotypes of these clusters show low values for Intra-cluster distances are very low. Therefore, the crosses should be made between the genotypes of clusters separated by large intra-cluster distances.

Table 7: Inter- and intra-cluster distances among the adzuki bean accessions

		Inter cluster distance	Cluster I 8.40	Cluster II 27.20	Cluster III 17.87
Intra cluster distance:		Cluster I	**0.00**	35.83	40.81
		Cluster II		**0.00**	33.81
		Cluster III	**0.00**		

The maximum inter-cluster distance was observed between cluster I and cluster III (40.81), followed by the distance between cluster I and cluster II (35.83), suggesting wide diversity between these clusters. Therefore, genotypes belonging to these clusters may be used in hybridization programme for the improvement of adzuki bean. The least inter-cluster distance was observed between cluster II and cluster III (33.81), indicating a close relationship between the genotypes of these clusters.

Cluster I showed the maximum cluster mean values for primary branches per plant, 1000-seed weight, clusters per plant, seeds per pod and seed yield per plant, along with the lowest values of days to 50% flowering and days to 80% maturity. Cluster III showed the maximum values for pods per plant and pods per cluster, along with the lowest value of plant height (Table 8). Thus, the genotypes falling under these clusters hold great promise as parents for obtaining promising elite lines through hybridization and to create further variability for these traits.

Table 8: Cluster means of three clusters for different traits of adzuki bean accessions

Trait	Custer I	Cluster II	Cluster III
Days to 50% flowering	57.99*	65.38	68.76**
Days to 80% maturity	106.99*	107.90**	107.10
Plant height	96.14**	83.32	82.81*
Primary branches/plant	5.48**	3.35*	3.46
Clusters/plant	19.98**	16.40	16.39*
Pods/cluster	3.50	2.99*	3.58**
Pods/plant	35.91*	40.50	41.49**
Seeds/pod	8.78**	7.62*	7.49
1000-seed weight	98.99**	90.53	74.27*
Seed yield/plant	15.80**	12.60	12.54*

**and *: Maximum and minimum values, respectively.

The relative contribution of individual traits to the genetic divergence among adzuki bean genotypes was presented in Table 9. The maximum contribution towards the genetic divergence was exhibited by pods per cluster (33.42%), followed by primary branches per plant (19.04%), pods per plant (11.98%) and clusters per plant (10.52%). The lowest contribution towards the genetic divergence was exhibited by seeds per pod (0.27%).

Table 9: Relative contribution (%) of individual trait to the genetic divergence among adzuki bean accessions

Trait	No. of times ranked first	Contribution (%)
Days to 50% flowering	5	0.67
Days to 80% maturity	9	1.20
Plant height	50	6.66
Primary branches/plant	143	19.04
Clusters/plant	79	10.52
Pods/cluster	251	33.42
Pods/plant	90	11.98
Seeds/pod	2	0.27
1000-seed weight	54	7.19
Seed yield/plant	68	9.05

Morphological diversity using principal component analysis (PCA)

Principal component analysis (PCA) showed that the first principal component (PC1) was the most important and explained 55.90% of the total variation, which was mainly contributed by primary branches per plant, plant height, 1000-seed weight, clusters per plant, seed yield per plant and seeds per pod. PC2 accounted for 12.95 per cent of the variation and was attributed to primary branches per plant and clusters per plant.

PC3 contributed 8.86 per cent to the total variation through primary branches per plant, days to 50% flowering, pods per cluster, pods per plant, clusters per plant and seeds per pod. Eigenvalues and proportion of accounted variance for each variable have been shown in Table 10.

A Scree plot depicting the per cent contribution of each variable with the help of eigenvalues was made and depicted in Fig. 2. The loading plot drawn for PC1 and PC2 components, including all the traits, also indicated the importance of these traits for explaining the variation among accessions and understanding species relationships and is presented in Fig. 3.

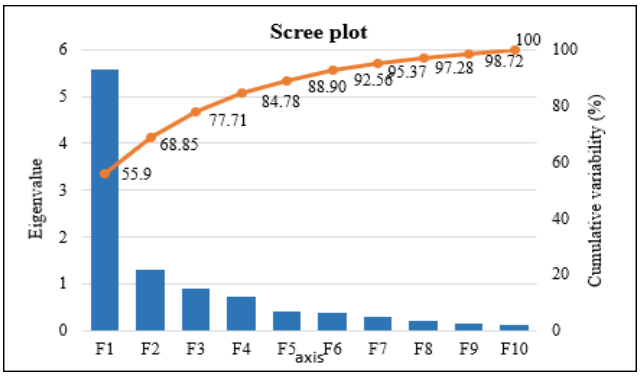


Fig. 2: Scree plot of per cent contribution of each variable in adzuki bean.

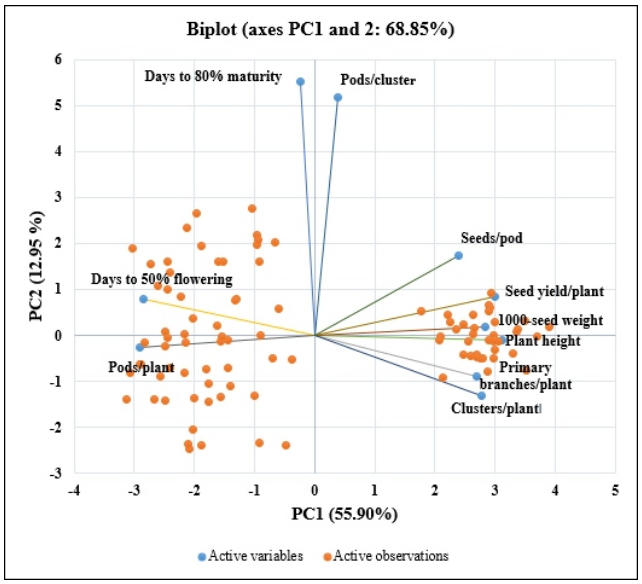


Fig. 3: Principal component analysis biplot of adzuki bean accessions.

Table 10: Eigenvectors for the ten components of quantitatively measured traits in 96 adzuki bean accessions

	Eigenvectors									
Variable	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
Eigenvalue	5.59	1.30	0.89	0.71	0.41	0.36	0.28	0.19	0.14	0.13
Variability (%)	55.90	12.95	8.86	7.08	4.12	3.65	2.81	1.91	1.44	1.28
Cumulative %	55.90	68.85	77.71	84.78	88.90	92.56	95.37	97.28	98.72	100
Days to 50% flowering	-0.36	0.1	0.28	0.37	0.24	-0.08	0.20	0.31	0.04	0.67
Days to 80% maturity	-0.03	0.69	-0.58	0.27	0.19	0.11	0.06	-0.18	0.17	-0.02
Plant height	0.39	-0.01	0.03	-0.04	-0.04	0.17	-0.31	0.40	0.74	0.11
Primary branches/plant	0.34	-0.11	0.17	0.21	0.75	-0.37	0.06	-0.06	0.06	-0.30
Clusters/plant	0.35	-0.16	0.19	0.24	**-0.08**	0.53	0.53	-0.41	0.12	0.11
Pods/cluster	**0.05**	0.65	0.59	-0.43	**0.02**	**0.06**	0.16	**0.04**	**-0.01**	-0.15
Pods/plant	-0.36	**-0.03**	0.14	0.44	-0.14	0.21	0.16	0.38	0.12	-0.64

Seeds/pod	0.30	0.22	0.26	0.52	-0.50	-0.43	-0.23	-0.19	**-0.06**	**0.03**
1000-seed weight	0.36	**0.02**	-0.29	-0.11	-0.22	-0.33	0.62	0.47	-0.12	**0.01**
Seed yield/plant	0.37	0.10	**-0.01**	0.18	0.14	0.45	-0.30	0.36	-0.61	**0.05**

Bold values are approximately equal to zero and thus do not contribute to the eigenvectors of PCA.

Diversity analysis at the molecular level

Marker informativeness: In the present study, a total of 70 SSR primers were screened for PCR amplification on 96 adzuki bean genotypes. Out of these, 11 primers were found polymorphic. Polymorphic bands of markers CEDG021 and CEDG294 were shown in Fig. 4a and 4b, respectively. SSR markers produced amplified product sizes ranging between 100-300 bp. Polymorphic information content (PIC) values ranged from 0.0399 to 0.3733, with an average of 0.241 per primer. However, the average number of effective alleles (N_e) varied from 1.043 to 1.986, with a mean value of 1.506. The mean value of Shannon's Information Index (SII) was 0.456, with a range of 0.101 to 0.689. Observed (H_0) and expected heterozygosity (H_e) varied from 0 to 0.235 and 0.041 to 0.499, respectively (Table 11).

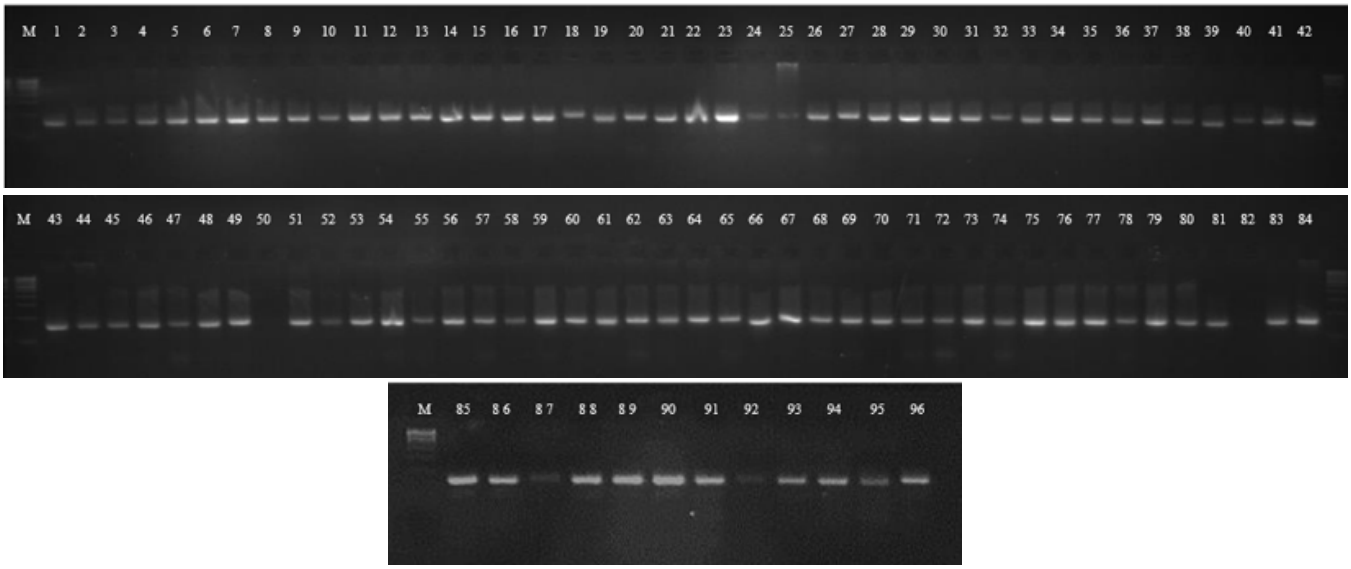


Fig. 4a: SSR polymorphism in 96 accessions revealed by primer *CEDG021*. M, 100 bp DNA ladder.

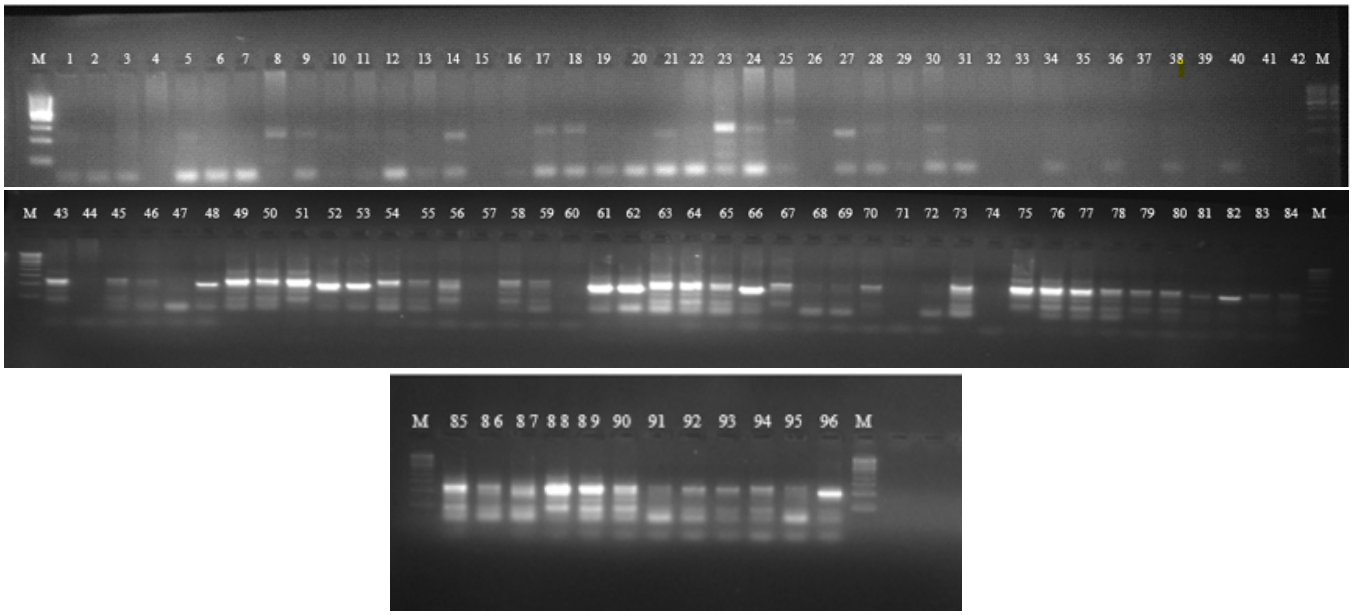


Fig. 4b: SSR polymorphism in 96 accessions revealed by primer *CEDG294*. M, 100 bp DNA ladder.

Table 11: Number of scorable and polymorphic SSR bands along with their fragment size generated by 11 primers

S. No.	Primers	N_e	SII	PIC value	Ho	He	N_g	PB (%)	Fragment size (bp)	EMR	MI
1	CEDG214	1.087	0.173	0.077	0.000	0.080	0.079	100	200-300	0.958	0.074
2	CEDG021	1.043	0.101	0.039	0.025	0.041	0.041	100	100-200	0.979	0.039
3	CEDAAG002	1.156	0.261	0.126	0.063	0.136	0.135	100	100-200	0.927	0.117
4	CEDG294	1.465	0.498	0.267	0.115	0.319	0.318	100	200-300	0.802	0.214
5	VES0039	1.986	0.689	0.373	0.235	0.499	0.497	100	200-300	0.542	0.202
6	CEDG090	1.932	0.676	0.366	0.154	0.485	0.482	100	100-200	0.406	0.149
7	VES0047	1.156	0.261	0.126	0.081	0.136	0.135	100	200-300	0.927	0.117
8	VES0024	1.843	0.650	0.353	0.185	0.459	0.458	100	200-300	0.646	0.228
9	VES0046	1.546	0.538	0.291	0.172	0.355	0.353	100	200-300	0.771	0.224
10	CEDG026	1.519	0.525	0.283	0.057	0.344	0.342	100	100-200	0.781	0.221
11	VES0021	1.822	0.644	0.349	0.012	0.454	0.451	100	200-300	0.656	0.229
	Mean	1.505	0.456	0.240	0.099	0.300	0.299				

N_e, Number of effective alleles; SII, Shannon's Information Index; H_e, Expected heterozygosity; H_o, Observed heterozygosity; N_g, Nei's genetic diversity (Average expected heterozygosity); P_B, Polymorphic bands; EMR, Effective multiplex ratio; MI, Marker index.

Cluster I comprised the maximum number of accessions, i.e., 40 accessions; 18 accessions were grouped in cluster II, and 38 accessions were grouped in cluster III. Accessions belonging to cluster I and cluster II are more closely related than those in cluster III containing distantly related accessions (Table 12, Fig. 5).

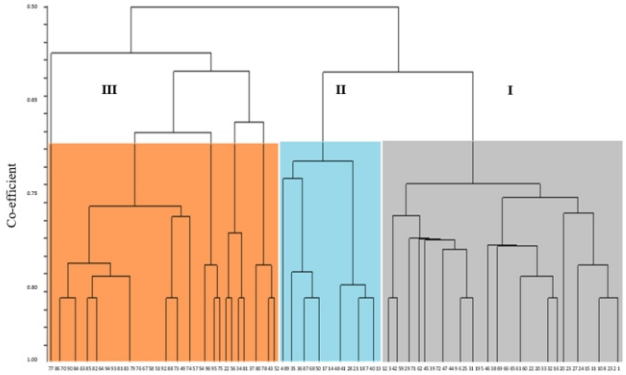


Fig. 5: Dendrogram depicting genetic relationships among the adzuki bean accessions constructed by NTSYS-pc (version 2.0).

Table 12: Grouping of adzuki bean accessions into different clusters based on SSR data

Clusters	No. of accessions	accessions
Cluster I	40	HPU-51, EC-290251, IC-485385, EC-340286, IC-341957, EC-340247, EC-59489, IC-341949, EC-000248, EC-34264, EC-340265, EC-340281, EC-340254, EC-000249, IC-341953, EC-87071, EC-87895, EC-87815, IC-108855, IC-89957, IC-341947, EC-340240, EC-340285, EC-000276, EC-340272, EC-340275, EC-57159, EC-340257, EC-340258, EC-120460, IC-100072, EC-24102, IC-341956, EC-000251, EC-15256, IC-341937, IC-341952, EC-000254, IC-140848, EC-340253
Cluster II	18	EC-187896, Local *Totru*, IC-485886, EC-340284, EC-15648, IC-251353, EC-340245, IC-108856, EC-340282, EC-340276, EC-340274, EC-240246, IC-485396, EC-340278, IC-16761, EC-290652, EC-340283, EC-24523
Cluster III	38	IC-341948, EC-18959, EC-000377, EC-120480, IC-469174, IC-341958, EC-340277, IC-469175, EC-18256, IC-341941, IC-469172, EC-340263, EC-340250, IC-341946, IC-485388, EC-87896, IC-24105, EC-340246, EC-08707, EC-340261, EC-240251, IC-485382, EC-340251, EC-120466, IC-341944, EC-30256, EC-340288, EC-30270, EC-30253, EC-340256, EC-18257, IC-469173, IC-341954, IC-341963, EC-108080, EC-340287, IC-341945, IC-241041

Population structure

Detailed knowledge of genetic relationships among individuals in an association panel is a key factor in avoiding spurious associations. Population structure (Q matrix), estimated using STRUCTURE (Pritchard *et al*., 2000; Falush *et al*., 2003) and expressed as membership probabilities, is one way to correct spurious associations due to genetic relatedness. It is often difficult to estimate the true number of populations (k). Generally, k is taken to be the value with the highest estimated LnP (D) value returned by STRUCTURE. However, in real situations, few data sets fit precisely to the STRUCTURE model and the LnP (D) value keeps increasing when k reaches the true number**.** Evanno *et al*., (2005) suggested an ad hoc method, Delta K, the second-order rate of change of the likelihood function with respect to K, which performed well in indicating the real number.

For the estimation of the number of populations and the best-fit value of K, STRUCTURE harvester was used for the given range, i.e., K=1 to K=10, using a panel of 11 SSR markers. Results concluded that the highest value of K delta mean, 47.33, was shown at K=2 (Fig. 6a).

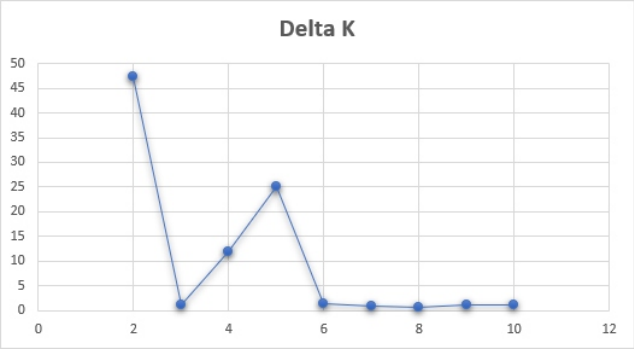


Fig. 6a: Population structure analysis using LnP(D) derived delta K for determining optimum number of subpopulations.

Therefore, Structure analysis was conducted for K=2, which largely clustered 96 adzuki bean accessions into two gene pools. The allelic variation patterns depicted in the bar diagram (Fig. 6b) suggest the existence of substantial admixture, indicating accessions with mixed parentage derived from distinct gene pools. The number of accessions falling under these two clusters is represented in Table 13. Cluster I contains 52 accessions having 32 pure lines and 20 admixtures, while Cluster II contains 44 accessions having 21 pure lines and 23 admixtures. Accessions in the two different gene pools represented a purity of 61.54% and 47.72% for Cluster-I and Cluster-II, respectively.

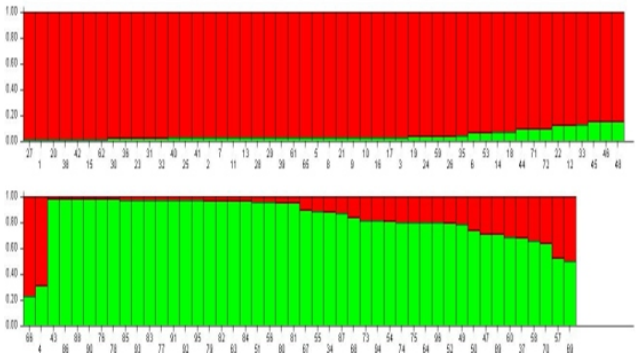


Fig. 6b: Population structure of 96 adzuki bean accessions using structure software program (K=2).

Table 13: Grouping of adzuki bean accessions using STRUCTURE software program

Group	No. of accessions	Accessions
Cluster I	52	(Pure lines: 32)
		EC-000248, HPU-51, IC-341953, EC-340285, EC-340253, EC-59489, IC-341937, EC-340240, EC-290652, IC-485385, EC-57159, EC-340254, EC-340284, EC-340257, EC-340282, EC-290251, EC-15648, EC-340247, IC-485886, IC-108856, EC-000254, EC-000251, EC-87815, IC-108855, EC-340272, EC-340286, EC-340245, EC-120460, IC-341957, EC-340281, EC-240246, EC-187896
		(Admixtures: 20)
		EC-340275, IC-341949, IC-140848, EC-340265, EC-340283, EC-340258, EC-34264, EC-340274, IC-251353, IC-100072, IC-341952, IC-341956, EC-87071, *Local Totru*, EC-000249, EC-15256, EC-000276, EC- 340276, IC-89957, IC-341948
Cluster II	44	(Pure lines: 21)
		EC-000377, IC-341945, EC-08707, EC-108080, EC-120466, EC-120480, IC-469173, EC-30270, EC-30256, IC-241041, EC-340288, EC-340261, EC-340263, IC-341944, EC-18257, IC-341954, IC-341963, EC-240251, EC-18256, IC-469174, EC-340277
		(Admixtures: 23)
		EC-340251, IC-341941, IC-469175, IC-16761, EC-340278, EC-340246, EC-30253, IC-341946, EC-87896, IC-469172, EC-340256, EC-340250, EC-18959, IC-24105, IC-485396, EC-24102, EC-24523, EC-87895, IC-341958, IC-485382, EC-340287, IC-485388, IC-341947

Analysis of molecular variance (AMOVA)

To assess molecular variation among adzuki bean accessions, AMOVA was computed for two population groups based on SSR data, as revealed by the population structure. The variation within populations accounted for 79% of the total variation, while variation among population it was only 21%, as shown in Table 14 and Fig. 7. The analysis of molecular variance (AMOVA) reveals a limited degree of variation between populations, juxtaposed with a substantial portion of genetic diversity within populations.

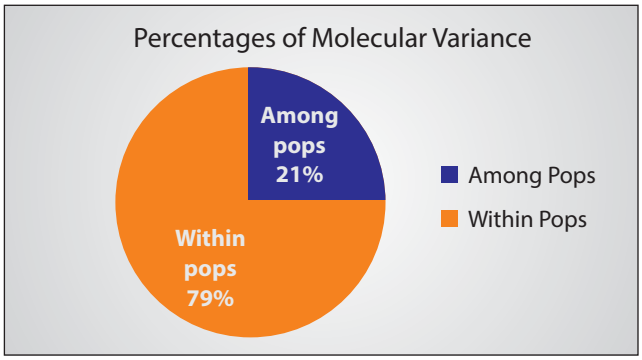


Fig. 7: Percentage of total variation.

Table 14: Analysis of molecular variance (AMOVA)

Marker system	Source	Df	SS	MS	Variation	% of Total Variation	P value
SSR	Among populations	1	19.73	19.73	0.38	21%	0. 001
	Within Population	94	138.25	1.47	1.47	79%	0. 001
	Total	95	157.98		1.85	100%	0. 001

Principal coordinate analysis (PCoA)

Clustering data based on similarity coefficients or variance-covariance values provides greater insights into significant categories; Principal Coordinate Analysis (PCoA) is a multivariate approach that uses these metrics. In contrast, cluster analysis improves resolution, especially for populations that are closely connected to one another. PCoA examines relationships among several quantitative variables by deriving a small number of linear combinations (principal components) that retain as much of the information in the original data as possible.

Principal Coordinate Analysis (PCoA) was used to analyse substructures of adzuki bean accessions. Using SSR markers, the percentage of variation is explained by the first 3 axis. Axis 1 contributes 25.47% variation, axis 2 contributes 17.84% variation, and axis 3 contributes 15.05% variation (Table 15).

Table 15: Percentage of variation explained by the first 3 axis

Axis	1	2	3
%	25.47	17.84	15.05

It is clear that test accessions have significant variation, but the entire germplasm was distributed in a scattered pattern across the plot of the entire collection (Fig. 8).

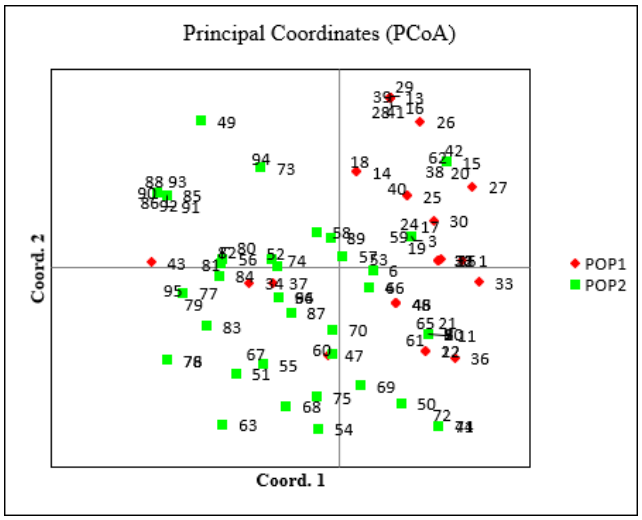


Fig. 8: Principal Coordinate Analysis (PCoA) for analyzing substructures of adzuki bean accessions.

Discussion

Accessions of adzuki bean were grouped into three distinct clusters using Tocher's method, and dendrograms based on Mahalanobis D^2 statistics revealed substantial genetic variability among them. Such multivariate clustering techniques have proven to be effective in identifying patterns of genetic divergence and have been widely employed in legume crops (Murthy and Arunachalam, 1966). The observed clustering patterns are consistent with previous findings by Wang *et al*., (2001), who reported significant

variation in adzuki bean germplasm collections, reflecting the crop's broad genetic base. Clusters I and III showed the greatest inter-cluster distance, indicating their potential utility as parental lines in hybridization programs due to their ability to produce superior recombinants and exhibit greater heterosis (Anderson *et al*., 1993; Bhatt, 1970; Mohammadi and Prassana, 2005). In contrast, the minimal inter-cluster distance between Clusters II and III suggests a potentially reduced diversity, which may limit their utility for generating novel variability. Such insights are crucial for parental selection strategies in breeding programs aimed at maximizing genetic gain (Franco *et al*., 2005; Singh *et al*., 2013). Accessions in Cluster I, specifically IC-485385, IC-341937, EC-340286 and EC-340251, were notable for their resistance to cercospora leaf spot, a key foliar disease affecting adzuki bean productivity. These accessions also exhibited favourable agronomic traits, including early maturity, reduced plant height, higher pod number per plant, increased thousand seed weight and significant seed yield per plant. Similar associations between disease resistance and superior agronomic performance have been reported in other legumes (Dwivedi *et al*., 2008), highlighting the importance of integrating both traits in breeding targets. Conversely, accessions such as EC-340274, EC-59489, EC-340281 and EC-240246 were highly susceptible to cercospora leaf spot, reinforcing the need for incorporating resistance screening in early stages of germplasm evaluation (Rubiales *et al*., 2015). To unravel the genetic basis of cercospora leaf spot resistance, a mapping population could be generated using resistant and susceptible accessions from genetically divergent clusters, as proposed by Singh *et al*., (2021), to facilitate the identification of quantitative trait loci (QTLs) associated with resistance. The strategic use of multivariate analysis plays a vital role in identifying divergent parents, conserving genetic resources and guiding effective hybridization strategies (Rao, 1952).

None of the SSR primers used in the present study exhibited heterozygosity values exceeding 0.5, suggesting a low probability of heterozygosity at the marker loci among the studied adzuki bean accessions. This observation is consistent with the sparse genetic diversity detected in the current investigation and aligns with the principles outlined by Nei (1973). Similarly, the Polymorphic Information Content (PIC) values for all markers were below 0.5, indicating relatively low informativeness of the SSR markers employed. These values were notably lower than those reported by Wang *et al*., (2012), who evaluated 158 adzuki bean accessions using 85 polymorphic SSR markers and observed higher PIC values, reflecting greater genetic variability. However, comparable findings of low PIC values were reported by Chen *et al*., (2015) using EST-SSRs derived from transcriptome data of adzuki bean, emphasizing the variability in marker performance depending on their origin and design. Despite the low PIC values observed, SSR markers remain valuable due to their high reproducibility, co-dominant inheritance and relative conservation across species (Mason, 2015). These attributes make SSRs reliable tools for detecting genetic variation, and the polymorphic loci identified in this study may be linked to functional genes or quantitative trait loci (QTLs) (Gupta *et al*., 2009 and Chankaew *et al*., 2014). Low PIC values are associated with loci having very high or very low allele frequencies, resulting in reduced allelic variation and lower discriminating power for genetic diversity analysis (Grativol *et al*., 2011).

The partitioning of adzuki bean accessions into distinct and discernible clusters in the present study reflects significant genetic divergence among them. Each cluster comprised both indigenous accessions collected from various parts of the Himalayan region of India and exotic germplasm sourced globally. Similar patterns of genetic erosion in domesticated populations were previously noted by Stachel *et al*., (2000) and Wang *et al*., (2019), attributed to bottlenecks and selection pressures. In contrast, wild adzuki bean accessions exhibited greater variability and are increasingly recognized as vital reservoirs of novel alleles for use in breeding programs (Kaga *et al*., 1998; Chankaew *et al*., 2014). These findings underscore the importance of conserving and characterizing wild and underutilized germplasm, which can significantly contribute to broadening the genetic base of cultivated adzuki bean. However, despite the promising potential of such resources, genetic studies on adzuki bean remain limited. Most research efforts have been constrained to low-density genetic mapping and limited marker coverage, as noted by Wang *et al*., (2009). This has resulted in a slower pace of gene discovery and trait association in adzuki bean compared to other legumes such as soybean and common bean (Varshney *et al*., 2012). Integrating high-throughput genotyping, GWAS, and genomic selection can accelerate the identification of key loci for yield, disease resistance and abiotic stress tolerance, thereby enhancing adzuki bean improvement (Varshney *et al*., 2014; Raggi *et al*., 2019).

The substantial admixture observed among accessions across clusters indicates frequent natural outcrossing, hybridization and gene flow, contributing to broader genetic diversity and the generation of

segregating progenies for crop improvement (Barrett, 2002; Xu *et al*., 2008). This diversity facilitates the identification and introgression of desirable alleles from diverse genetic backgrounds and enhances the efficiency of association mapping and genome-wide association studies (GWAS) by increasing allelic variation and improving the detection of trait-associated loci (Garris *et al*., 2005; Flint-Garcia *et al*., 2005; Zhao *et al*., 2011). Similar population structure has been reported in adzuki bean, with distinct subgroups identified using SSR and SNP markers, often corresponding to geographic origin and domestication history (Chen *et al*., 2015; Liu *et al*., 2020). Furthermore, the genetic divergence between wild and cultivated accessions highlights the importance of wild germplasm for broadening the breeding pool (Kaga *et al*., 2008). These findings support germplasm conservation, genomic selection and the development of resilient, high-yielding cultivars (Varshney *et al*., 2014; Upadhyaya *et al*., 2006).

In the present study, clustering of adzuki bean accessions was independent of geographic origin, with indigenous and exotic accessions distributed across the same subpopulations. Similar patterns have been reported in *Vigna* species, where gene flow, seed exchange and historical introductions obscure geographic structuring (Phansak *et al*., 2005). The observed clustering reflects the combined effects of domestication, selection and environmental factors on genetic diversity (Kaga *et al*., 2008; Xu *et al*., 2010). Identification of genetically distinct purebred lines provides valuable resources for developing representative core collections, thereby maximizing genetic diversity while minimizing redundancy (Brown, 1989; Upadhyaya *et al*., 2003). Previous studies identified the Himalayan region as a major reservoir of genetic diversity in wild adzuki bean (Xu *et al*., 2008; *Kaga et al*., 2008). Consistent with these findings, many native accessions in the present study originated from the Himalayan foothills, highlighting their potential as valuable donors of stress tolerance, disease resistance and yield-related traits for future breeding and conservation programs (Tomooka *et al*., 2002; Maxted *et al*., 2012).

Conclusion

This study offers a comprehensive assessment of the genetic diversity in adzuki bean using SSR markers, revealing two major gene pools among accessions collected from diverse global regions. The SSR markers identified provide valuable tools for future germplasm characterization, genetic mapping and trait association studies. Distinct genetic backgrounds observed in accessions from regions such as India, Mexico and the USA highlight underutilized diversity that can be harnessed to broaden the genetic base in breeding programs. These findings lay a foundation for the development of improved cultivars with enhanced adaptability, yield and stress resilience. Future research could focus on integrating genomic selection, identifying functional genes linked to agronomic traits and exploring gene-environment interactions to accelerate the breeding of climate-resilient adzuki bean varieties.

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