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Research paper

Population structure and genetic diversity analysis in faba bean (*Vicia faba* L.) germplasm using SSR markers

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ABSTRACT

In the present study, population structure and genetic diversity were studied among 100 faba bean germplasm lines along with three checks using 29 SSR (microsatellite) markers and morphological traits. ET218734, ET226572, and ET218734 genotypes exhibited maximum seed yield and high mean performance for various yield-attributing traits. The D^2 statistics analysis grouped genotypes into five clusters. SSR marker analysis revealed, on average, 2 alleles per locus ranging from 2 to 3 among the studied genotypes. Polymorphic information content (PIC) for these markers ranged from 0.063 to 0.492 with an average of 0.37. Population structure analysis classified the studied genotypes into three major clusters. The Molecular Diversity Distance was found to be greatest among Cluster III and Cluster I (0.289), while the lowest was among Cluster II and Cluster I (0.148). The allele frequency divergence (expected heterozygosity) was found highest on Cluster II (0.164), and the lowest was on Cluster III (0.096).

***Key words*:** Faba bean, Genetic diversity, Population structure, Polymorphism information content, Molecular variance.

INTRODUCTION

Faba bean (*Vicia faba* L.) is one of the most noteworthy potential legume crops in India. It belongs to the family fabaceae with its diploid chromosome number 2n=2x=12. It is an autogamous plant with partial out cross ranging from 20-80% (Saharifi *et al*., 2015). It originated in Mediterranean countries and Afghanistan. Nowadays, faba beans are being cultivated globally because of their high protein content and the presence of other crucial nutrients. It is one of the earliest crops grown and consumed by man, especially in developing countries and as animal feed in developed countries (Tiwari and Singh, 2019). It ranks fourth among the pulse crops globally after chickpea, common bean and peas. In India, faba bean is a *rabi* crop which is grown mostly in Assam, Bihar, Madhya Pradesh, Rajasthan, West Bengal and Uttar Pradesh.

Faba bean is also referred as bakala bean, broad bean, horse bean, pigeon bean, bell bean, tick bean and field bean. It is rich in protein (24-33%), starch (45%), cellulose (3%), sugar (3%), oligosaccharides (6.5%), and minerals. It also has high amounts of lysine and contains levodopa, a dopamine precursor that has the capacity to treat Parkinson's disease. It also contains several essential vitamins, tremendous amount of antioxidants, and high amounts of vicine and convicine, which are medicinally important antinutritional compounds. It is normally consumed as a vegetable, which can be fresh, green, dried, or canned. In India, it is an underutilised crop as the production is potentially low and only the local collection is being grown. The study of the genetic variability available in the germplasm is the first initial requirement of a crop improvement program. The extent of genetic variability present in faba bean germplasm was studied in terms of PCV, GCV, heritability and genetic advance. The molecular markers are important tools for identifying genetic variability and determining the fingerprinting of the germplasm lines. Various molecular markers have been employed for the analysis of genetic variability and divergence in the faba bean crop (Abid *et al.*, 2015). SSR markers, also known as microsatellite markers, are highly polymorphic, co-dominant, and have high allelic diversity. SSR markers were successfully utilised in faba beans to determine genetic diversity and DNA fingerprinting. The main objective of the present investigation was to assess the molecular genetic divergence and determine the population structure of faba bean germplasm.

MATERIALS AND METHODS

Plant material

A total of 100 faba bean germplasm lines along with three checks (Vikrant, Giza-4, and Rebya-40) were used for the present study (Table 1). These germplasm lines were provided by ICAR-NBPGR under the AICRP project. The present investigation was carried out at the research cum institutional farm of Raj Mohini Devi College of Agriculture and Research Station, Ambikapur, Chhattisgarh during *Rabi* 2020–21.

DNA extraction

DNA was isolated from the tender leaves of each germplasm by using the modified CTAB method of DNA extraction. Isolated DNA was quantified by gel electrophoresis and then diluted with Sigma (sterilised) water to make the final concentration 50 ηg/µl. PCR amplification was performed in a 20 µl reaction mixture containing 2 µl of template DNA (100 ηg/µl), 2.5 ul of 10x PCR buffer, 1.5 ul of 1.0 mM dNTPs, 1ul of 5ρmol forward and reverse primers each, 0.5µl of 5U/µl *Tag polymerase,* and 13.5µl of nanopure water each.

Table 1. Faba bean genotypes used in the study

S.N.	Genotypes	S.N.	Genotypes	S.N.	Genotypes
1.	ET226408	36.	ET226478	71.	ET226502
2.	ET226409	37.	ET226479	72.	ET226563
3.	ET226410	38.	ET226482	73.	ET226571
4.	ET226411	39.	ET226485	74.	ET226570
5.	ET226413	40.	ET226486	75.	ET226505
6.	ET226414	41.	ET226487	76.	ET226497
7.	ET226415	42.	ET226488	77.	ET226484
8.	ET226416	43.	ET226489	78.	ET226481
9.	ET226417	44.	ET226490	79.	ET226498
10.	ET226418	45.	ET226491	80.	ET218745
11.	ET226419	46.	ET226493	81.	ET218778
12.	ET226420	47.	ET226494	82.	ET218741
13.	ET226421	48.	ET226495	83.	ET218746
14.	ET226422	49.	ET226496	84.	ET218770
15.	ET226423	50.	ET226499	85.	ET218786
16.	ET226424	51.	ET226564	86.	ET218734
17.	ET226425	52.	ET226555	87.	ET218719
18.	ET226426	53.	ET226567	88.	ET218739
19.	ET226427	54.	ET226569	89.	ET218772
20.	ET226428	55.	ET226568	90.	ET218738
21.	ET226429	56.	ET226566	91.	ET218773
22.	ET226430	57.	ET226572	92.	ET218775
23.	ET226431	58.	ET226573	93.	ET218769
24.	ET226432	59.	ET226506	94.	ET218776
25.	ET226459	60.	ET226500	95.	ET218765
26.	ET226461	61.	ET226557	96.	ET218764
27.	ET226463	62.	ET226561	97.	ET218767
28.	ET226464	63.	ET226559	98.	ET218759
29.	ET226465	64.	ET226560	99.	ET218787
30.	ET226468	65.	ET226507	100.	ET218783
31.	ET226472	66.	ET226504		
32.	ET226473	67.	ET226503		**Checks**
33.	ET226474	68.	ET226501	1.	VIKRANT
34.	ET226475	69.	ET226562	2.	GIZA-4
35.	ET226477	70.	ET226565	3.	REBYA-40

Source: NBPGR, New Delhi

After initial denaturation for 5 minutes at 94°C and each cycle comprised denaturation at 94^{0}C for 30 seconds, annealing at 55^{0}C for 30 seconds, extension at 72^{0}C for 45 seconds, and finally final extension at 72^{0}C for 7 minutes at the end of 35 cycles. PCR amplification of SSR markers was performed in the FISHER THERMAL CYCLER using the PCR conditions (Table 2). The PCR products were mixed with bromophenol blue gel loading dye, and the 1.2% agarose gel was used for better separation and visualisation of the PCR-amplified SSR product. The gel was visualised by the E-BOX CX5.TS (20M) gel picture taken by the gel documentation unit.

Table 2. PCR condition and temperature profile used for amplification of microsatellite markers

S.N.	Steps	Temperature (°C)	Cycles	Duration
1.	Initial Denaturation	94	1	5.0 Min.
2.	Denaturation	94	40	30.0 Sec.
3.	Annealing	50-60		30.0 Sec.
4.	Extension	72		45.0 Sec.
5.	Final Extension	72	1	7.0 Min.
6.	Storage	4	1	∞

PCR amplification and SSR analysis

Twenty-nine primer pairs were tested. These standard loci have been used in characterization of faba bean genetic diversity in several germplasm collections (Gong *et al.,* 2010; Ma *et al.,* 2011). The 1.2% agarose gel was used for better separation and visualisation of the PCR-amplified SSR product. The gel was visualised by the E-BOX CX5.TS (20M) gel picture taken by the gel documentation unit.

Allele scoring

The clear and unambiguous bands of markers were scored using 50bp DNA ladder. Markers were scored for the presence or absence of the corresponding band among the genotypes. The score 1 and 0 indicates the presence and absence

of the bands respectively. In case of a data matrix comprising of '1' and '0' were formed depending on the character and this data matrix was subjected to further analysis. The banding pattern of genotypes, developed by each set of primer was scored separately.

Genetic diversity

Estimation of genetic diversity was done in the software Structure ver. 2.3.4, such as allele frequency, number of alleles per locus, heterozygosity, and polymorphism information content (PIC) for a group of accessions. For each marker, allele frequency represents the prevalence of a certain allele. The fraction of heterozygous individuals in a population is known as heterozygosity. The polymorphic information content (PIC) is a measure of allelic diversity and was calculated as PIC = $1-\Sigma(pi^2)$, where pi is the frequency of the i^{th} allele detected in all individuals of the populations (Nei, 1973; Rafalski *et al.,* 1996).

Population structure

A distance-based method and a model-based approach were employed to evaluate genetic structure. Structure ver. 2.3.4 software was used as a model-based approach (Pritchard *et al.* 2000). This method detected the real number of subpopulations (K) using following criteria: the likelihood of admixture and the correlation between allele frequencies. The run length was specified as being made up of 100,000 burning periods and 100,000 replications. Plotting the average estimate of the log posterior probability of the data L (K) against the specified K value allowed us to calculate the ideal K value. The highest value of L (K) was used to determine the actual number of subpopulations.

Data analysis

Based on the algorithm, SSR genotype data was processed for the purpose of determining population structure and genetic diversity. A 1/0 matrix was developed based on the presence or absence of alleles for the 29 markers. For each of the SSR markers, only repeatable and consistent SSR fragments were used to determine whether a marker was present (1) or absent (0).

The polymorphism information content (PIC) of each pair of SSR primers was calculated using the formula

$$\text{PIC} = 1 - \left(\sum_{i=1}^{n} p_i^2\right) - \left(\sum_{i=1}^{n-1}\sum_{j=i+1}^{n} 2q_i^2 q_j^2\right)$$

Where n is the number of alleles (marker), qi is the ith allele frequency, and qj is the jth allele frequency.

RESULTS AND DISCUSSION

Morphological characterization

The primary goal of varietal characterization is to identify the presence of traits that indicate the existence of a specific genotype. Characters that are used to differentiate cultivars should be able to provide specific description and recognition, and they are only considered important when they are not subject to environmental stimulus. Morphological characterization showed a wide range of variation; 15 morphological characters were evaluated for

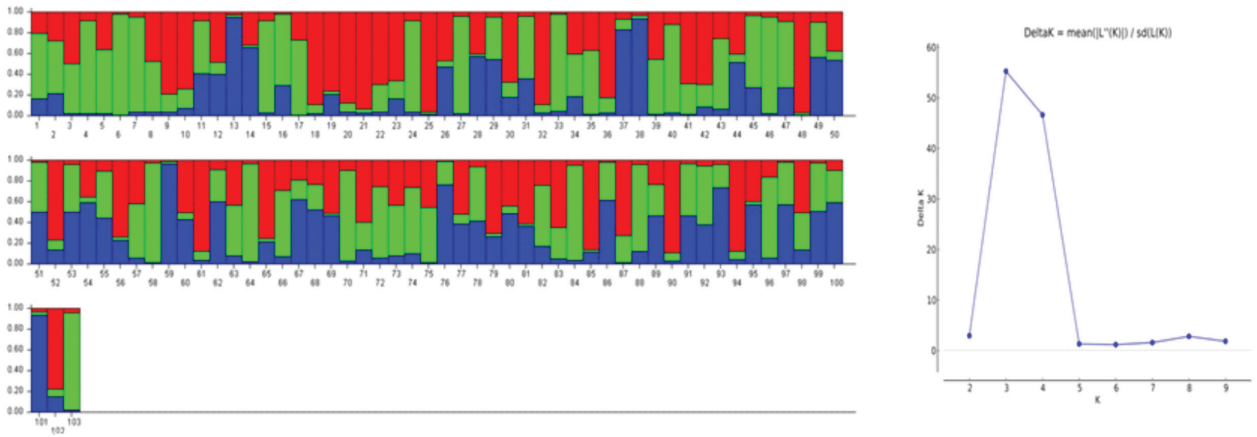

Fig. 1. Population structure of 103 germplasm based on 29 SSR markers (K= 3) and graph of estimated membership fraction for K= 3. The maximum of adhoc measure ΔK determined by structure harvester was found to be K=3.

morphological characterization. The existing genetic variability in faba beans will serve as the foundation for developing a breeding programme. There are few reports on morphological variation in the faba bean. There will be a higher likelihood of producing a desired type with greater variability. For polygenetic character, selection based on phenotype would be ambitious. The findings of the morphological characterization are given in Table 3.

Table 3. Morphological descriptors and their frequency (%) in different categories for different characters

Traits	Category	No. of genotype	Frequency %
Early plant vigour	1 Poor	14	13.59
	2 Good	60	58.25
	3 Very good	29	28.16
Plant habit	1 Determinate	41	39.81
	2 Semi determinate	21	20.39
	3 Indeterminate	41	39.80
Flower ground colour	1White	93	90.29
	3 Violet	10	9.71
Wing petal colour	1 Uniformly white	3	2.91
	2 Spotted	100	97.09
Leaflet shape	1 Narrow	56	54.37
	2 Intermediate	30	29.13
	3 Rounded	17	16.50
Leaflet size	1 Small	46	44.66
	2 Medium	38	36.89
	3 Large	19	18.45
Stem colour	1 Light green	76	73.79
	2 Dark green	27	26.21
Stem pigmentation	0 Absent	83	80.58
	1 Weak	4	3.88
	3 Strong	16	15.53
Pod angle/ attitude	1 Erect	100	97.09
	2 Horizontal	3	2.91
Pod colour	1 Light yellow	72	69.90
	2 Dark brown	31	30.10
Pod distribution on the stem	1 Mainly basal	36	34.95
	2 Uniform	17	16.51
	3 Mainly terminal	50	48.54
Pod shape	1 Flattened non constricted,	30	29.13
	2 Flattened constricted	73	70.87
Pod shattering	1 Low	62	60.19
	2 Medium	28	27.19
	3 High	13	12.62
Seed coat colour	5 Light green	9	8.74
	6 Dark green	34	33.01
	7 Light brown	46	44.66
	8 Dark brown	14	13.59
Seed shape	1 Flattened	42	40.78
	2 Round	60	58.25
	3 Angular	1	0.97

Similar results have been reported by Yilmaz *et al.* (2020), Kumar and Kaushik (2020) and Abid *et al.* (2015). They observed similar results for most of the characters, such as pod shape, flower colour, plant vigour, leaf colour, and inflorescence colour.

Genetic diversity, heritability and genetic advance

Estimated PCV and GCV help to clearly understand the various genotypes. The phenotypic and genotypic coefficients of variation for all 11 characters have been given in Table 4. High magnitudes of phenotypic and genotypic coefficients of variation were recorded for traits such as seed yield per plant (PCV = 65.38%, GCV = 64.52%), number of pods per plant (PCV = 43.47%, GCV = 42.96%), pod length (PCV = 32.47%, GCV = 32.35%), and pod width (PCV = 31.96%, GCV = 31.79%). This shows that there is a higher selection response for these characteristics. Moderate phenotypic and genotypic coefficients of variation were recorded for traits such as 100 seed weight (PCV = 27.79%, GCV = 27.70%), stem thickness (PCV = 26.66%, GCV = 26.27%), number of seeds per pod (PCV = 25.52%, GCV = 24.95%), plant height (PCV = 24.50%, GCV = 24.46%), and number of branches per plant (PCV = 20.95, GCV = 20.77%). Some characters exhibited low phenotypic and genotypic coefficients of variation; these traits are days to 50% flowering (PCV = 8.09%, GCV = 7.56%) and days to 80% maturity (PCV = 3.64%, GCV = 3.29%).

Higher estimates of GCV and PCV indicate the high variability of characters in the germplasm. The characteristics that showed high GCV and PCV are of commercial importance, and there is scope for refinement of this character via selection. The high-value GA for these characters is regulated by additive genes, and selection will be gratifying for the further refinement and enhancement of such characters. All 11 quantitative characters studied were highly heritable with the highest heritability recorded for plant height and the lowest on days to 80% maturity. Genetic advance as a percentage of the mean was observed high on the characters seed yield per plant, number of pods per plant, pod length and pod width. These results were similar with findings of Chaudhary *et al.* (2018) and Afeta *et al.* (2020).

Correlation coefficient

The correlation study revealed that the seed yield per plant was found to be highly significant (at the 5% level of significance) and to have a

Table 4. Genetic parameters of variability for yield and attributes in faba bean

Characters	General mean	Range		PCV (%)	GCV (%)	h^2 (%)	Genetic advance	GA as % of mean
		Min	Max					
Plant height (cm)	82.13	37.00	150.20	24.50	24.46	99.67	40.75	50.30
No. of branches per plant	5.29	3.20	9.20	20.95	20.77	98.27	2.23	42.41
Stem thickness (cm)	0.60	0.28	1.16	26.66	26.27	97.04	0.32	53.30
Pod length (cm)	5.30	2.60	9.23	32.47	32.35	99.32	3.47	66.42
Pod width (cm)	1.10	0.41	1.70	31.96	31.79	98.95	0.71	65.15
No. of pods per plant	9.04	4.20	27.00	43.47	42.96	97.62	7.69	87.43
No. of seeds per pod	2.45	1.40	3.60	25.52	24.95	95.59	1.23	50.26
Days to 50% flowering	55.97	48	65	8.09	7.56	87.78	8.10	14.47
Days to 80% maturity	128.97	121	138	3.64	3.29	81.38	7.88	6.10
100 Seed weight (g)	43.08	25.63	76.25	27.79	27.70	99.30	24.41	56.84
Seed yield per plant (g)	11.04	2.67	46.11	65.38	64.52	97.41	14.12	131.18

positive correlation with the number of pods per plant (0.886) and plant height (0.711). The value of r was 0.347. The estimation of the correlation coefficients and the different characters of the faba bean are given in Table 5. The findings confirm the findings of previous workers Mulualum *et al*. (2013), Kalia *et al*. (2003) and Kumar *et al*. (2020). Analysis of correlation revealed that days to 50% flowering, days to 80% maturity, pod length, pod width, 100 seed weight, plant height, and stem thickness were highly positively correlated with each other, indicating that the correlations are interdependent. Selection of these traits will lead to indirect selection of interdependent characters.

Genetic diversity and population structure analysis

For information on population structure in faba bean germplasm based on allelic frequency, we used approaches as described by Pritchard *et al.* (2000). The analysis of 100 germplasms revealed that the value of K ranged between 2 and 3. The optimal value is 3 (k = 3), according to the likelihood provided by structure. According to the measure of K, which was discovered to be k = 3 (Fig. 2),

Table 5. Estimation of genotypic (G) and phenotypic (P) correlation coefficient among various characters in faba bean

Character		No. of branches per plant	Stem thickness (cm)	Pod length (cm)	Pod width (cm)	No. of pods per plant	No. of seeds per pod	Days to 50% flowering	Days to 80% maturity	100 Seed weight (g)	Seed yield per plant (g)
Plant height (cm)	(G)	0.497**	0.716**	0.470**	0.458**	0.627**	0.487**	-0.342**	-0.316**	0.449**	0.711**
	(P)	0.497**	0.706**	0.469**	0.456**	0.617**	0.481**	-0.341**	-0.319**	0.447**	0.704**
No. of branches per plant	(G)	1.0000	0.341**	0.332**	0.276**	0.526**	0.118	-0.245*	-0.285*	0.191	0.514**
	(P)	1.0000	0.332**	0.330**	0.276**	0.513**	0.129	-0.243*	-0.304*	0.192	0.515**
Stem thickness (cm)	(G)		1.0000	0.333**	0.359**	0.460**	0.353**	-0.205*	-0.132*	0.340**	0.463**
	(P)		1.0000	0.328**	0.353**	0.438**	0.362**	-0.202*	-0.113*	0.332**	0.457**
Pod length (cm)	(G)			1.0000	0.749**	0.560**	0.756**	-0.082	-0.168	0.848**	0.654**
	(P)			1.0000	0.739**	0.555**	0.738**	-0.082	-0.159	0.839**	0.644**
Pod width (cm)	(G)				1.0000	0.535**	0.699**	-0.014	-0.028	0.751**	0.522**
	(P)				1.0000	0.520**	0.686**	-0.012	-0.025	0.748**	0.517**
No. of pods per plant	(G)					1.0000	0.579**	-0.067	-0.054	0.557**	0.886**
	(P)					1.0000	0.550**	-0.066	-0.052	0.550**	0.871**
No. of seeds per pod	(G)						1.0000	0.029	0.042	0.807**	0.573**
	(P)						1.0000	0.029	0.041	0.784**	0.576**
Days to 50% flowering	(G)							1.0000	0.935**	-0.028	-0.122
	(P)							1.0000	0.925**	-0.028	-0.120
Days to 80% maturity	(G)								1.0000	-0.032	-0.153
	(P)								1.0000	-0.031	-0.152
100 Seed weight (g)	(G)									1.0000	0.636**
	(P)									1.0000	0.632**

Note: ** significant at 1% level; * significant at 5% level.

Table 6. Details of SSR loci used for genotyping in the 103 germplasm lines and their genetic diversity parameters.

Markers name		Sequence (5' to 3')	No. of alleles	Alleles size (bp)	Alleles frequency	PIC value
M41	F	CAACGCGGCAGTTAAAGAAT	3	80	0.77	0.364
	R	CAGGTATGGCTGACACCTCA		200	0.19	
				310	0.04	
VfG67	F	GTTCATCAAGCACCAATCTAAAC	2	150	0.76	0.365
	R	TCAATTTGGTTTATCTCTCTCTCT		190	0.24	
VfG87	F	AGGGCCAGCGTGATCCAATA	2	100	0.56	0.492
	R	TGGGTTGGGATCTTTTGGTTG		250	0.44	
M10	F	CCATGGTTACTGCAGTCGAA	2	50	0.74	0.385
	R	GCCGTCGATTGATTCGTATT		100	0.26	
VfG27	F	CCCAAAAAGAGACGAACTGTAT	2	100	0.65	0.457
	R	AGGGTTCATACGTTTGGCTT		180	0.35	
VfG19	F	AGCGATGGTGCTCATGCTTA	2	150	0.75	0.380
	R	TCTCTCACGGAATCACATCTTT		500	0.25	
P11	F	CGTGGTTGATTTGCTTC	2	100	0.71	0.413
	R	ACCTCCATCTTCGCCTC		250	0.29	
VfG81	F	GTCCTGGAAAAAAGAAAGAGA	2	50	0.77	0.353
	R	AAAGAAACCTCTCTCTCCAT		100	0.23	
M57	F	TGCAGAGAAGCTAAGCACCA	2	100	0.71	0.412
	R	TCGCATGGTACAGTAGCAAAA		180	0.29	
VfG10	F	ACCAAAACGCGCACTTATCA	2	150	0.71	0.412
	R	AAGAGAGAGAAGAGAGCTTC		500	0.29	
M17	F	CTCCAACGAAGGCAGAGAAG	2	100	0.80	0.317
	R	CATGATTCCCATAGCCTTGC		115	0.20	
M49	F	GCGTTATTAGGCCGCTGTAA	2	240	0.88	0.211
	R	AAAACCGTGGCTCGAATATTTA		260	0.12	
VfG1	F	TTTCAGCAAACTAGAACCAATC	2	170	0.91	0.167
	R	GGCATTCAGTTTTTACCTTGTA		195	0.09	
P27	F	CGGGTTTATTCCATCATTT	2	120	0.94	0.200
	R	CGTTATTGTTGTCGCTATTT		135	0.06	
VfG41	F	CAAGCTTGTTGAGCCAAA	2	100	0.97	0.063
	R	GAACGAGGCTCACGAAAATA		250	0.03	
VfG3	F	TTCTTTGGTCCTCTCTCTATC	2	70	0.90	0.177
	R	GCACTGTTGTTGCTGATACAA		110	0.10	

the complete germplasm line can be classified into 3 clusters, subgroups, or subpopulations. The inferred clusters were Cluster I (0.356), Cluster II (0.379), and Cluster III (0.265). The net nucleotide distance between Cluster III and Cluster I was found to be the highest (0.289), whereas Cluster II and Cluster I had the lowest (0.148). The allele frequency divergence (expected heterozygosity) was found highest on Cluster II (0.164), and the lowest was on Cluster III (0.096). Each individual was characterized by a single colour line, and each cluster was denoted by a colour. The greater area of a colour that an individual expected means the greater the opportunity that the individual belonged to the matching group. Accessions in the admixture class may have partial ancestry in more than one background or feasibly resulting from the gene flow between taxa. Relative low percentages of pure accessions in both clusters indicate high admixture and natural interbreeding of accessions. Cross-pollination of faba bean is expedited by insect pollinators like honeybees, bumble bees and diverse solitary bees. They possibly had an intricate history involving inter crossing.

Genetic diversity is the main factor behind the use of germplasm in crop improvement. A population with a lot of genetic diversity is a treasured resource for expanding the genetic base in any crop improvement attempt. Molecular markers help to recognise the level of genetic diversity that exists among faba bean accessions that can be used in breeding programmes. By analysing the population structure using molecular markers like SSRs, for instance, it is possible to determine the genetic architecture of different germplasm lines with great accuracy (Oliveria *et al.,* 2016; Rebaa *et al.,* 2017). In this study, SSR genotypic data were used to perform model-based clustering and a distance-based clustering technique to evaluate the genetic diversity among the accessions.

CONCLUSION

SSR marker approaches have offered useful information in terms of detecting polymorphism and characterising faba bean accessions. We determined the level of genetic structural variation in the accessions. Furthermore, the data may be valuable to breeders in properly selecting parents, resulting in progenies with high differentiation among them. It also enables the best possible arrangement of faba bean gene pool management and more effective sampling of available genotypic resources. Hence, further research into these accessions is possible. It might introduce new alleles into biotic or abiotic stress tolerance essential for breeding of faba bean.

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