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Amplified Fragment Length Polymorphism based Genetic Diversity of Tropical Legume: *Lablab purpureus* var. typicus

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ABSTRACT

Background: *Lablab purpureus* L. is a legume appropriate to grow in tropical environment as it is adaptable to a broad temperature spectrum, rainfall and altitudes. It develops well under humid and warm conditions at temperatures ranging between 18°C and 30°C. It is a drought hardy crop grown in humid and semi-arid regions. The species is propagated by seed and can be sown alone or intercropped; planted near hedges or near other less leafy plants to climb on. *Lablab purpureus* L. has a flowering response of short-day, with early and late flowering types. Some landraces flower after sowing as early as 55 days. It is predominantly self pollinated although some out crossing is known to occur.

Methods: In this study, *Lablab purpureus* L. genetic diversity were analyzed utilizing AFLP markers on ten Indian *Lablab purpureus* L. accessions at Department of Botany, Bundelkhand University, Jhansi during 2019-20. Five primers set were used for checking the diversity in *Lablab purpureus* L. and the marker performance were measured by using 4 parameters *i.e.* PIC, Mean resolving Power, Resolving Power and MI.

Result: A total of 52 bands were generated, of which 45 bands (88.46%) were polymorphic which varies from 6 (M-CAT/E-ACT) to 13 (M-CAC/E-ACC, M-CAC/E-ACT) with mean of 9.00 bands per primer. The PIC value ranges between 0.55 and 0.76 with a mean value of 0.64. The average value of matrix index is 3.04. Higher the MI, better the marker is. The value of RP ranges between 2.60 and 7.80 with a mean value of 4.68. In the UPGMA dendrogram, the 10 accessions were separated into two main clusters with all the primers used. In this study, we have shown that molecular markers can be used successfully to understand the genetic structure of the accessions of *Lablab purpureus* L. and identified a series of sequence-specific markers that potentially could be used in *Lablab purpureus* L. genetic mapping analyses. Since *Lablab purpureus* L. has been considered as a promising crop because of its wide spectrum of adaptability to different ecological conditions than other legumes, participatory evaluation at an early breeding stage could shape the variety being developed to increase the productivity and other traits as well as for utilization as food.

Key words: AFLP, Genetic diversity, *Lablab purpureus* L., Marker, Polymorphic.

INTRODUCTION

The high protein content and atmospheric nitrogen fixing capacities of grain legumes in world agriculture are important. The pulses represent the only concentrated type of dietary proteins for many developing countries. As far as the developed countries are concerned, legumes are the key source of protein, being good, biologically valuable animal feed. Indians generally favour vegetarian food and the pulses are the major source of protein. Most of the country is in rain fed condition and the pulses are well balanced in different mixed cropping or intercropping rotations.

Lablab purpureus L. also known by the name of *Lablab purpureus* (L.) is found to be inhabiting the areas of India or South-East Asia (Murphy and Colucci, 1999). It is found to be originated from Asia and is also known to be planted for long time as well. India grows a wild variety of *lablab*. *Lablab purpureus* L. was possibly taken to the Africa's tropical part from where it might got spread across various other parts of the world including Sudan, Malaysia, Caribbean, Papua New Guinea, Egypt, Mainland China, Indonesia and Philippines.

More research is being done to expand both technical and practical knowledge about the bean, so that its full potential can be realised. The majority of research on enhancing lablab as a food crop is now taking place in Asia, with only a small amount of work being done in Africa (Maass *et al.* 2010). The levels of genetic diversity in current germplasm and breeding stocks determines how far genetic improvement can be made (McCouch, 2004). Phenotypic features can be used to measure genetic diversity, however they are heavily impacted by the environment and do not accurately reflect genetic relatedness between accessions.

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Molecular markers are increasingly employed to supplement phenotypic and protein-based indicators in order to detect genetic variation and aid in the management of plant resources (Virk *et al.* 1995; Song *et al.* 2003). Different marker systems have been used to identify genetic diversity in *Lablab purpureus* L.

AFLPs were used to illustrate genetic diversity in laboratory accessions from other countries; India commonwealth scientific and industrial research organization (CSIRO) 103 accessions to germplasm (Maass *et al.* 2005), 62 land breeds obtained from southern India (Venkatesha *et al.* 2007) and 40 other accessions in India (Patil *et al.* 2009). The research cited by the different scientists has analysed the genetic variability among various accessions that are dissimilar from the present accessions collected for the analysis. However, the current work establishes the effectiveness of different DNA markers for analysis through AFLP markers which were ignored by previous experts. A similarity matrix data among the studied accessions along with dendogram is considered a unique feature in the current work. Therefore, current study was carried out to analyse genetic relatedness and heritability of unexplored *Lablab purpureus* L. bean accessions at the molecular level, to construct a phylogenetic tree and band matrix similarity and polymorphism data of AFLP marker in genetic divergence analysis.

MATERIALS AND METHODS

Material used for the study

For the present Investigation, ten accessions of *Lablab purpureus* L. namely VRSEM- 847, VRSEM-739, VRSEM-799, VRSEM-757, VRSEM-776, VRSEM-733, VRSEM-730, VRSEM-746, VRSEM-843, VRSEM-702 procured from Indian Institute of Vegetable Research, Varanasi were used for studying the genetic divergence among them in order to characterize and identify possible duplicated accessions of *Lablab purpureus* L. were assessed by AFLP and efficient DNA fingerprinting tool.

Extraction of DNA

Leaves were used by CTAB process for extraction of DNA, with little change in the procedure (Reif *et al.* 2003). Around 150 mg of leaves with extraction buffer (1 ml) were crushed by using mortar and pestle. Extraction buffer was then applied to tubes already having crushed leaf. With normal mixing, the tubes were incubated for 45 minutes at 65°C. Isoamyl alcohol: Chloroform (1:24) was added in equal volume and mixed for 5 minutes, following centrifugation for 15 minutes at 14000 rpm. 2/3 amount of isopropanol was added to the supernatant. Centrifuged for 10 minutes at 14000 rpm and drained the supernatant and washed the pellet with 70% ethanol containing DNA and suspended in 100 µl sterile Distilled Water. 10-15 µl RNAse incubated at 65°C for 30 minutes. Qualitative DNA assessment was conducted on 0.8 per cent agarose gel by running sample and quantification was made by recording its absorbance at 260 nm and 280 nm.

AFLP analysis

The AFLP analysis was done by the methods given by Zabeau (1993) with modifications as prescribed by Waugh (1994) published in technical manual of IAEA (2002). Sequence of the adapters used in the current study is mentioned as follows:

Adapter	Sequence
Eco RI adapter	5′- CTC GTA GAC TGC GTA CC-3′
	5′- AAT TGG TAC GCA GTC TAC-3′
Mse I adapter	5′- GAC GAT GAG TCC TGA G-3′
	5′-TAC TCA GGA CTC AT-3′

Restriction digestion of genomic DNA

The reaction for restriction is as follows: 10x Assay buffer (10 µl), 18 µl of sample Genomic DNA (500 ng), 1 µl Eco RI (10U/ µl), 1 µl of MseI (10U/ µl) and 100 µl of water (AFLP grade). Contents were mixed gently and incubated for 2 hrs at 37°C. Following the restriction, the reaction mixture was incubated at 70°C for 15 minutes to inactivate the enzymes. DNA was eluted in 25 µl of Elution buffer or suspended the precipitated DNA in 25 µl of autoclaved distilled water.

Ligation of adapters

In the presence of T4 DNA ligase, Mse I and Eco RI adapters were ligated in an adapter ligation solution to digested fragments. The reaction were as follows: 12.5 µl of 2.0x quick ligase assay buffer, 7.5 µl of digested DNA (Purified), 2.0 µl of Eco RI adapter, 2.0 µl of Mse I adapter, 1.0 µl of T4 DNA ligase with final volume of 25.0 µl. After mixing, the components were incubated at room temperature for 1 hr. The adapter ligated DNA was diluted in the ratio 1:4 and taken for the preamplification step.

Pre-amplification reactions

Preamplification was done using primers with a single specific nucleotide at the 3′ end corresponding to the Eco RI and Mse I adapters. The reaction was constituted as: 0.5 µl of diluted Template DNA (from above step), 2.0 µl of pre-Amp primer mix, 2.5 µl of 10x NEB Taq Assay Buffer, 1.0 µl of dNTPs (2.5 mM each), 0.5 µl of NEB Taq DNA polymerase with final volume of 25 µl. Mixed gently and collected the reaction by brief centrifugation. By using the following parameters, the samples were pre-amplified for thermocycling: 25 cycles for 30s at 94°C; for 30s at 55°C; for 2 minutes at 72°C and a final extension for 5 minutes at 72°C. Perform 1:20 dilution of the pre-selective PCR products and used for subsequent selective amplification step.

Selective AFLP amplification

For selective amplification, the reaction was as follows: 2.0 µl of diluted Template DNA (from previous step), 2.0 µl of selective primer Mix, 2.5 µl of 10x NEB Taq Assay Buffer, 1.0 µl of dNTPs (2.5 mM each), 0.5 µl of NEB Taq DNA polymerase, 17 µl of water for final volume. Mixed gently and placed the PCR tubes in PCR programmed with the following conditions: 11 cycles for 30s at 94°C; for 30s at

55°C; 72°C decreased by 0.7°C at each successive cycle for 1 minute at 65°C and 24 cycles for 30s at 94°C; for 30s at 55°C; for 1 min at 72°C. Finally, the amplified products were electrophoresed in a 6% denaturing polyacrylamide gel and auto radiographed (Sambrook *et al.* 1989).

Data analysis and polymorphic information content

Photograph was taken by the digital camera of the gel documentation unit to score banding results. Bands were shown according to their molecular size (length of polynucleotide amplified). To evaluate the molecular scale, each sample of primer was filled by 1 kb DNA Ladder. The distance covered by bands generated from the well was converted into molecular sizes in relation to molecular marker weight. Absence of band was scored as '0' and existence of band as '1'. Lightly seen bands weren't scored however measured for scoring if found equivalent to major band. To check the existence of the bands and to assess the reproducibility, all the tested primers were repeated three times.

Genetic profile of *Lablab purpureus* L. were estimated by the performance of the primers which were calculated by means of following four parameters *i.e.* Polymorphic information content (Sultana *et al*. 2000), Mean resolving power (Tian *et al*. 2005), Resolving power (Tian *et al.* 2005) and Matrix index (Gnanesh *et al.* 2005).

RESULTS AND DISCUSSION

AFLP analysis

In this analysis, the bands of DNA were amplified from 5 sets of AFLP primer against *Lablab purpureus* L. From the molecular weight of 121 base pair to 979 base pair, sets of different primers revealed a total of fifty-two bands indicating 88.46 percent polymorphism (45 bands). The polymorphism varies between 71 per cent and 100 per cent. Banding arrangement generated with sets of primer M-CAC/E-ACC and M-CAC/E-ACT generated the largest no. of bands i.e. 13 over set of primer M-CAT/AGC *i.e.* 12 bands while M-CAT/E-ACT and M-CAC/E-AGC sets of primer revealed a total of seven bands (Table 1).

The twelve bands generated with M-CAT/E-AGC primer set exhibited seventy-five per cent of polymorphism. The uppermost band generated at about nine hundred and seventy nine basepair and lowest at about one hundred and seventy five base pair. Thirteen bands generated with M-CAC/E-ACC primer set exhibited a hundred per cent of polymorphism. The uppermost band generated at about nine hundred and fifty five base pair whereas the lowest at about one hundred and forty one base pair. Against M-CAT/E-ACT primer set, seven bands were generated; of which polymorphic bands are six thus revealing eighty-six per cent polymorphism. The uppermost band generated at about six hundred and ninety four base pair whereas the lowest at about two hundred and twenty five base pair. The pattern of banding generated with M-CAC/AGC primer set revealed seven bands having seventy-one per cent polymorphism among the accessions considered. The uppermost band generated at about nine hundred and twenty three base pair and the lowest at about two hundred and eighty one base pair. Finally, the pattern of banding generated with M-CAC/E-ACT primer set intensified thirteen bands with hundred percent polymorphism rate. The uppermost band generated at about nine hundred and thirty base pair and the lowest at about one hundred and twenty one base pair (Figure1).

Diversity assessment assists in accession prediction and selection (Chakravarthi *et al.* 2006). Portraying diversity inside firmly related accessions is a significant method for the reasonable utilization of hereditary assets. The approach used for investigating genetic diversity is useful for distinguishing variation inside and among the *Lablab purpureus* L. AFLP (Tefera *et al.* 2006) were utilized as atomic primers in this investigation since that is amazingly reproducible (Steiger *et al.* 2002) and has been broadly used to explain structures and pathway of advancement through a variety of crop like coffee (Steiger *et al.* 2002), basic beans (Thome *et al.* 1996), azuki beans (Yee *et al.* 1999) and sweet potato (Zhang *et al.* 2000).

Our findings support the results of other workers who considered diverse AFLPs marker pairs and noticed to be of great use in studying diversity (Maass *et al.* 2005). The number of polymorphic groups acquired was the greatest contrast with the past investigation of *Lablab purpureus* L. accessions (Maass *et al.* 2005; Kimani *et al.* 2012; Venkatesha *et al.* 2010). Distinctive part factors, for example, groundwork structure, scarcely any tempering locales and so on are answerable for the distinction in the number of groups upgraded by various groundworks (Kernodle *et al.* 1993). The enhanced groups additionally had distinctive sub-atomic loads which approved the length contrasts between the destinations where groundwork ties and the template DNA bringing about shifting of bands (Devos and Gale 1992).

Table 1: Efficiency and polymorphism by the use of five AFLP primers.

Details	M-CAC/ E-ACC	M-CAC/ E-AGC	M-CAT/ E-AGC	M-CAT/ E-ACT	M-CAC/ E-ACT	Mean
Band number	13	07	12	07	13	10.20
Polymorphic band number	13	05	08	06	13	9.20
Polymorphism per cent level	100	71	75	86	100	88.46
MRP	0.56	0.33	0.39	0.37	0.74	0.48
PIC	0.61	0.76	0.69	0.58	0.55	0.64
MI	3.57	2.47	2.18	3.48	3.52	3.04
RP	5.80	2.60	3.60	2.61	7.80	4.68

Polymorphism information content and data collection

AFLP marker performance was compared by evaluating PIC that is not consistently higher for each marker. The least PIC value was shown by M-CAC/E-ACT is 0.55 and the largest by M-CAC/E-AGC is 0.76 with an average value of 0.64. The largest value of RP was shown by M-CAC/E-ACT primer set is 7.80 and the least by M-CAC/E-AGC primer set is 2.60 with an average value of 4.68. MI value ranged between 3.57 and 2.18 with an average of 3.04. M-CAC/E-ACC primer appears the largest value of 3.57 and least by primer set M-CAT/E-AGC *i.e.,* 2.18. The largest MRP value was shown by M-CAC/E-ACT primer set is 0.74 and the lowest by M-CAC/E-AGC primer set is 0.33 with an average of 0.48 (Table 1).

Genetic relationship and phylogenetic study

The genetic diversity of accessions collected from IIVR, Varanasi exhibited random pattern of distribution of two clusters revealing that the existence of genetic diversity may be used in future for furthur breeding programme to improve growth and yeild parameters among the accessions. The results on genetic diversity revealed that the diversity connection between the accessions depends on the pairwise coefficient of Jaccard's for each primer. By using markers, diversity connection and data assemble every accession in its clusters. Among the considered accessions pairwise similarity ranges between 0.89 and 0.21 with an average of 0.55 (M-CAT/E-AGC), 0.93 and 0.40 with an average of 0.66 (M-CAC/E-ACC), 1.00 and 0.44 with an average of 0.72 (M-CAT/E-ACT) and 0.89 and 0.27 with an average of 0.58 (M-CAC/E-AGC) and 1.00 and 0.31 with an average of 0.65 (M-CAC/E-ACT) (Table 2 to 6). The findings are in support of Kimani *et al.* (2012) who studied different AFLP set of primers showed genetic distance varied from 2 -142 (per cent dissimilarity ranged from 0.8-63) signifying a superior genetic diversity between the accessions of field bean.

Cluster analysis by UPGMA with the Gene Alex Version 6.2 software generates a dendogram generated from the data matrix analysis (Rai *et al.* 2008). Depends on the genetic distances with M-CAT/E-AGC primer set, the UPGMA tree method produced two separate clusters. According to the resultant phylogenetic tree (Fig 2A), accessions were separated in 2 clusters having three accessions in one which again separates into 2 while the remaining accessions (07) separate in other clusters and again divided into 2 clusters. For a set of primer M-CAC/E-ACC (Fig 2B), the neighbour-joining UPGMA method

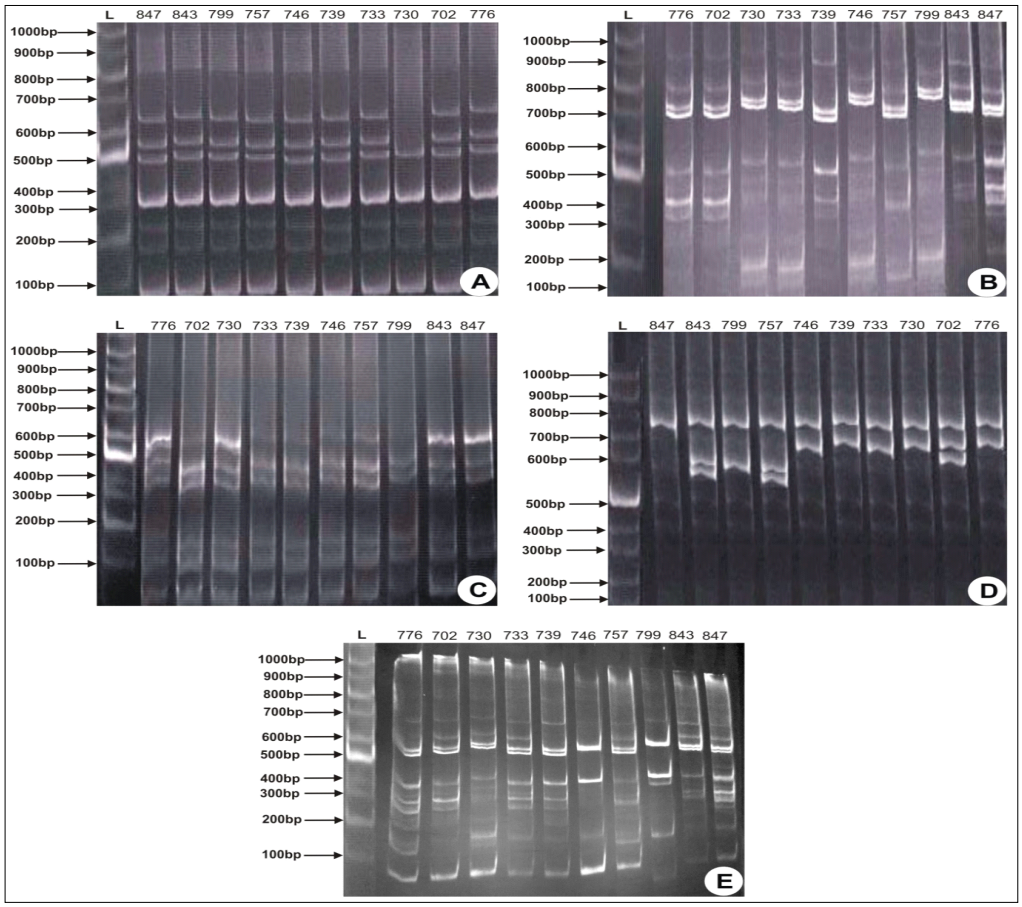

Fig 1: Banding patterns formed from *Lablab purpureus* (L.) accessions by the use of five AFLP primers (A) M-CAT/E-AGC, (B) M-CAC/E-ACC, (C) M-CAT/E-ACT, (D) M-CAC/E-AGC, (E) M-CAC/E-ACT.

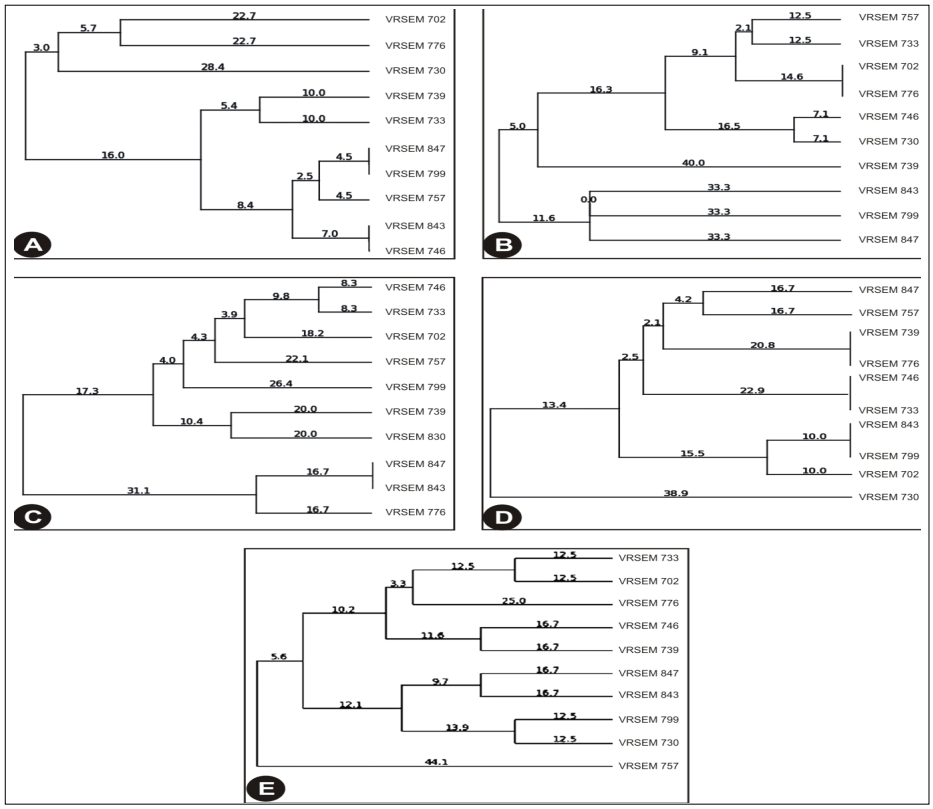

Fig 2: Dendogram obtained from five AFLP primers based on genetic distance (A) M-CAT/E-AGC, (B) M-CAC/E-ACC, (C) M-CAT/E-ACT, (D) M-CAC/E-AGC, (E) M-CAC/E-ACT.

Table 2: Data matrix similarity of primer M-CAT/E-AGC.

	847	843	799	757	746	739	733	730	702	776
847	1.00									
843	0.67	1.00								
799	0.67	0.89	1.00							
757	0.56	0.78	0.78	1.00						
746	0.67	0.89	0.89	0.78	1.00					
739	0.63	0.63	0.63	0.63	0.63	1.00				
733	0.59	0.71	0.71	0.71	0.71	0.56	1.00			
730	0.25	0.25	0.25	0.25	0.25	0.35	0.27	1.00		
702	0.22	0.22	0.22	0.22	0.22	0.21	0.24	0.50	1.00	
776	0.22	0.25	0.22	0.22	0.22	0.21	0.25	0.35	0.56	1.00

Table 3: Data matrix similarity of primer M-CAC/E-ACC.

	847	843	799	757	746	739	733	730	702	776
847	1.00									
843	0.67	1.00								
799	0.67	0.50	1.00							
757	0.67	0.88	0.63	1.00						
746	0.43	0.40	0.53	0.40	1.00					
739	0.86	0.80	0.67	0.80	0.43	1.00				
733	0.67	0.75	0.63	0.75	0.53	0.80	1.00			
730	0.53	0.50	0.63	0.50	0.93	0.53	0.63	1.00		
702	0.57	0.40	0.67	0.53	0.86	0.57	0.53	0.93	1.00	
776	0.47	0.78	0.67	0.78	0.71	0.59	0.67	0.78	0.71	1.00

Table 4: Data matrix similarity of primer M-CAT/E-ACT.

	847	843	799	757	746	739	733	730	702	776
847	1.00									
843	0.89	1.00								
799	0.57	0.75	1.00							
757	0.75	0.67	0.57	1.00						
746	0.75	0.67	0.57	0.50	1.00					
739	0.75	0.67	0.57	0.50	1.00	1.00				
733	0.75	0.67	0.57	0.50	1.00	1.00	1.00			
730	0.75	0.67	0.57	0.50	1.00	1.00	1.00	1.00		
702	0.89	0.80	0.50	0.67	0.89	0.89	0.89	0.89	1.00	
776	0.67	0.60	0.50	0.44	0.89	0.89	0.89	0.89	0.80	1.00

Table 5: Data matrix similarity of primer M-CAC/ E-AGC.

	847	843	799	757	746	739	733	730	702	776
847	1.00									
843	0.71	1.00								
799	0.50	0.67	1.00							
757	0.75	0.67	0.29	1.00						
746	0.43	0.62	0.50	0.50	1.00					
739	0.67	0.59	0.38	0.63	0.43	1.00				
733	0.67	0.71	0.50	0.63	0.43	0.89	1.00			
730	0.70	0.84	0.67	0.67	0.63	0.70	0.70	1.00		
702	0.56	0.82	0.63	0.75	0.71	0.67	0.78	0.80	1.00	
776	0.74	0.56	0.47	0.59	0.27	0.74	0.74	0.67	0.53	1.00

Table 6: Data matrix similarity of primer M-CAC/E-ACT.

	847	843	799	757	746	739	733	730	702	776
847	1.00									
843	0.80	1.00								
799	0.80	1.00	1.00							
757	0.60	0.80	0.80	1.00						
746	0.60	0.80	0.80	0.80	1.00					
739	0.50	0.75	0.75	0.75	0.50	1.00				
733	0.67	0.89	0.89	0.89	0.67	0.86	1.00			
730	0.67	0.67	0.67	0.67	0.44	0.86	0.75	1.00		
702	1.00	0.80	0.80	0.60	0.60	0.50	0.67	0.67	1.00	
776	0.53	0.40	0.40	0.53	0.40	0.31	0.43	0.43	0.53	1.00

separates into 2 clusters showing 07 accessions in 1 cluster whereas, remaining accessions (03) divides in another cluster. Findings with M-CAT/E-ACT primer set (Fig 2C) formed 2 clusters only whereas with M-CAC/E-AGC primer set (Fig 2D) two separates clusters were prepared, of which nine germplasm were collected into 1 cluster and one remains suspended in its own. Lastly, two distinct clusters with M-CAC/E-ACT primer set (Fig 2E) were formed having nine and one accessions.

CONCLUSION

AFLP bands generated from PCR amplification are necessary for fingerprinting since these data can be utilised to classify novel alleles for agronomically and biochemically significant genes. The AFLP markers used in this analysis were a useful tool in Indian *Lablab purpureus* bean genetic diversity identification since there are no unique markers for *Lablab purpureus* L. In *Lablab purpureus* L. a total of all five sets of AFLP primers were found to be important and ideal for evaluating polymorphism. Study of variability in accession showed all are genetically diverse and these can be used to produce crosses and classify novel alleles for agronomically and biochemically significant genes. Furthermore, such results can further help to increase the applicability of *Lablab purpureus* L. breeding programs, when contrasted with morphological data for accessions assessment.

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