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Research Note

Evaluation of genetic diversity in ricebean [*Vigna umbellata* (Thunb.) Ohwi and Ohashi] germplasm using SSR markers

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Abstract
Genetic improvement mainly depends on the extent of genetic variability present in the population. The molecular marker is a useful tool for assessing genetic variations and resolving cultivar identities. The objective of this study was to evaluate the genetic divergence of 40 germplasm lines of ricebean. Forty-four SSR markers were used in the present study, out of which only seven were found to be polymorphic. The dendrogram based on SSR marker analysis grouped the 40 ricebean genotypes into 2 clusters, where cluster I was the largest with 37 genotypes. STRUCTURE analysis for the estimation of population structure divided 40 genotypes into 4 groups in which P1 group consisted of 5 genotypes, P2 with 23 genotypes, P3 with maximum number of 7 genotypes, whereas, P4 consisted of 5 genotypes. The genetic variation revealed by SSR analysis will be used in the future breeding programme of ricebean for the selection of diverse parents for hybridization.

Key words
Ricebean, Genetic variation, SSR markers, Hybridization, Genetic divergence

Ricebean [*Vigna umbellata*, (Thunb.) Ohwi and Ohashi], one of the underutilized pulse is a multipurpose grain legume crop mainly cultivated for food, fodder and green manure. The nutritional quality of ricebean appears to be the best among all the traditional pulses due to higher amount of proteins, essential amino acids, essential fatty acids and minerals. It contains about 20% protein which is enriched with essential amino acids like methionine, tryptophan, lysine, tyrosine and valine. Its favorable protein content ensure higher digestibility and therefore, makes it a appropriate food item for children, women and older people with weak digestion.

Being nutritionally superior and having high production potential, ricebean has a large scope in future to meet the demand for pulses. Though diverse landraces with different plant and seed morphologies have been locally produced and maintained by farmers, still the genetic improvement of this crop is slow due to inadequate information of genetic stocks and paucity of this information about existing ones. Thus, systematic efforts are needed to characterize the germplasm for their sustainable utilization in breeding programmes. The variability studies conducted on available germplasm in this crop so far are largely based on morphology, agronomic behavior, and biochemical traits. There is a limitation on this traditional characterization as it is associated with a low level of polymorphism (Yamaguchi, 1992; Lumpkin & McClary, 1994). Many of the traits are polygenic and influenced by environmental conditions and therefore, are difficult to evaluate with accuracy. Molecular markers, by virtue of their abundance and consistency across environments, provide reliable means for assessment of genetic diversity.

In the present programme of study, SSR markers were used for diversity analysis of 40 germplasm lines of ricebean. Because SSRs are multi-allelic, they have high potential for use in evolutionary studies and studies regarding genetic diversity and relationships (Gracia *et al.*, 2004). At present, microsatellites are one of the most promising molecular-marker types able to identify or differentiate genotypes within a species. Their codominant inheritance, high level of polymorphism and easy handling make them extremely useful for many different applications. SSR markers have successfully been used by other workers in ricebean. Kaga *et al*. (2005) studied genetic diversity of ricebean gene pool as assessed by SSR markers and observed that SSR markers were efficient in revealing genetic diversity among the ricebean genotypes. Similarly, Wang *et al.* (2016) did analysis of simple sequence repeats in ricebean using an SSR-enriched library and designed a series of primer pairs based on SSR flanking sequences and validated a subset of primer pairs.

A total of 40 ricebean genotypes were used in the study (Table 1). These genotypes were raised in the field for the evaluation of various qualitative and quantitative traits. DNA was extracted from the young leaves using CTAB method (Dellaporta *et al*., 1983). 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 10X PCR buffer (10mM Tris-HCl, 50mM 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 S1000^{TM} Thermal Cycler (BIO-

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RAD). Amplification conditions were an initial denaturation at 94°C for 5 min and 39 cycles at 94°C for 30 seconds, 57°C for 30 seconds, 72°C for 1 min, followed by 5 min at 72°C. The amplification products were electrophoresed in 3 % agarose gel (HIMEDIA) and stained with ethidium bromide (0.5 µg/ml). The gels were visualized and photographed using the Gel-Documentation Unit.

The DNA profiles of genotypes were scored for the presence or absence of each band of a particular molecular weight for different primers. A binary data matrix with '1' indicating the presence of a particular molecular weight band and '0' indicating its absence was generated separately for each primer. The binary data were used to generate a similarity matrix using Jaccard's coefficient in SIMQUAL programme of NTSYS-pc package (Rohlf, 1993). Cluster analysis of genotypes based on similarity values was done by unweighted paired group arithmetic mean method (UPGMA) in SAHN programme of NTSYS-pc package to construct a dendrogram.

Molecular markers provide a powerful tool for the proper characterization and management of germplasm. In the present programme of study, SSR markers are used for diversity analysis of 40 germplasm lines of ricebean. Due to their high allelic diversity and codominant nature, the SSR loci are also well suited to study the population structure (Garris *et al.,* 2005). In the present study, a total of 44 SSR primers were screened for PCR amplification on 40 genotypes. Out of these 7 primers were found polymorphic (Table 2). SSR polymorphism in 40 genotypes revealed by primer *CEDAAG002*, M = 100 bp DNA ladder is shown in fig. 1. Polymorphic information content (PIC) value, a parameter associated with the discriminating power of markers, ranged from 0.146 to 0.307 with an average of 201 per primer. Effective multiplex ratio (EMR) ranged from 2 to 3 with an average of 2.28 per primer, while Marker Index (MI) ranged from 0.341 to 0.704 with an average of 0.457 per primer. High value of MI, which is considered to be overall measure of efficiency to detect polymorphism for markers, is derived from its high polymorphism, EMR and PIC values.

Based on polymorphism exhibited by SSR markers, dendrogram was constructed using Jaccard's similarity coefficient, the genotypes were grouped into two main clusters (Fig. 2). Cluster I comprised of 37 genotypes *viz*., LRB-478, LRB-474, LRB-473, LRB-476, LRB-449, LRB-447, LRB-475, LRB-464, LRB-311, LRB-498, LRB-455, IC-395028, IC-23512, IC-538983, IC-419518, IC-359282, IC-524082, IC-421926, IC-538870, UURB-17, RBHP-401, RBHP-43, RBHP-38, RBHP-403, RBHP-404, RBHP-405, RBHP-406, RBHP-407, RBHP-408, RBHP-409, RBHP-410, RBHP-411, RBHP-412, RBHP-413, RBHP-414, PRR-2 and UURB-2014-I whereas, only 3 genotypes were placed in cluster II *viz*., IC-538878, PRR-I and PRR-2007-2 (Table 3).

The ricebean genotypes of different regions were also subjected to STRUCTURE analysis for estimation of population structure using a panel of seven SSR markers. The STRUCTURE analysis divided the population into four main groups. (Figure 3), but the differentiations at K = 4 were almost consistent with pedigree knowledge with few exceptions. Thus, the pedigree information was used to guide the division of P1, P2, P3 and P4 groups combining with the cluster membership. The P1 group consisted of 5 genotypes, P2 with 23 genotypes, P3 with maximum number of 7 genotypes, whereas, P4 consisted of 5 genotypes (Table 4).

Characterization of diversity present among the genotypes is of immense importance in any crop improvement program for judicious choice of parents and efficient handling of segregating populations. The genetic information provided by morphological traits has limitations which can be overcome by molecular techniques such as ISSR. Since molecular markers are considered to be more stable under a non-labile genetic system, they have been used in many crops to obtain results with higher precision in breeding programmes. In the present study, SSR markers have been used to assess genetic variability among ricebean genotypes. The SSR technique has been successfully applied earlier to assess molecular polymorphism in ricebean by number of workers (Tian *et al*., 2013, Singh *et al*., 2014 and Wang *et al*. 2016). Given the paucity of research on molecular aspects of ricebean, characterization and assessment of its diversity could have great significance in designing breeding and germplasm conservation strategies**.**

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Table 1. List of germplasm lines of ricebean evaluated under the present study

S. No.	Genotypes	Source/Pedigree	S. No.	Genotypes	Source/Pedigree
1	LRB-478	PAU, Ludhiana	21	UURB-17	UUHF, Ranichauri
2	LRB-474	PAU, Ludhiana	22	UURB-2014-I	UUHF, Ranichauri
3	LRB-473	PAU, Ludhiana	23	PRR-2007-2	UUHF, Ranichauri
4	LRB-476	PAU, Ludhiana	24	RBHP-401	CSKHPKV, Palampur
5	LRB-449	PAU, Ludhiana	25	RBHP-43	CSKHPKV , Palampur
6	LRB-447	PAU, Ludhiana	26	RBHP-38	CSKHPKV , Palampur
7	LRB-475	PAU, Ludhiana	27	RBHP-403	CSKHPKV , Palampur
8	LRB-464	PAU, Ludhiana	28	RBHP-404	CSKHPKV , Palampur
9	LRB-311	PAU, Ludhiana	29	RBHP-405	CSKHPKV , Palampur
10	LRB-498	PAU, Ludhiana	30	RBHP-406	CSKHPKV , Palampur
11	LRB-455	PAU, Ludhiana	31	RBHP-407	CSKHPKV , Palampur
12	IC-395028	NBPGR, New Delhi	32	RBHP-408	CSKHPKV , Palampur
13	IC-538878	NBPGR, New Delhi	33	RBHP-409	CSKHPKV , Palampur
14	IC-23512	NBPGR, New Delhi	34	RBHP-410	CSKHPKV , Palampur
15	IC-538983	NBPGR, New Delhi	35	RBHP-411	CSKHPKV , Palampur
16	IC-419518	NBPGR, New Delhi	36	RBHP-412	CSKHPKV , Palampur
17	IC-359282	NBPGR, New Delhi	37	RBHP-413	CSKHPKV , Palampur
18	IC-524082	NBPGR, New Delhi	38	RBHP-414	CSKHPKV , Palampur
19	IC-421926	NBPGR, New Delhi	39	PRR-I	UUHF, Ranichauri
20	IC-538870	NBPGR, New Delhi	40	PRR-2	UUHF, Ranichauri

Table 2. List of polymorphic ricebean SSR primer sequences used in the present study

S. No.	Primers	Forward sequence	Reverse sequence
1	CEDG286	CGAGCAGAACACTGATCATG	CCTCTTAGAGGTCATTGCTC
2	CEDAAG002	GCAGCAACGCACAGTTTCATGG	GCAAAACTTTTCACCGGTACGACC
3	CEDG090	ATAAGTAGAAATTGGTTCAAATG	GGTTCGTTAAAGTAACTTTTAAT
4	CEDG021	GCAGAATTTTAGCCACCGAG	AAAGGATGCGAGAGTGTAGC
5	CEDG294	CACCTTCTTAATCTCTTCACC	GGGTTTCTCTTAATTCATTGAGTC
6	*CEDG214*	CACTCACTGCAAAGAGCAAC	CTACCTATCTGAGGGACAC
7	X2	AGGCGAGGTTTCGTTTCAAG	GCCCATATTTTTACGCCCAC

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Table 3. Grouping of ricebean genotypes into different clusters on the basis of SSR data

Clusters	No. of genotypes	Genotypes
Cluster 1	37	LRB-478, LRB-474, LRB-473, LRB-476, LRB-449, LRB-447, LRB-475, LRB-464, LRB-311, LRB-498, LRB-455, IC-395028, IC-23512, IC-538983, IC-419518, IC-359282, IC-524082, IC-421926, IC-538870, UURB-17, RBHP-401, RBHP-43, RBHP-38, RBHP-403, RBHP-404, RBHP-405, RBHP-406, RBHP-407, RBHP-408, RBHP-409, RBHP-410, RBHP-411, RBHP-412, RBHP-413, RBHP-414 , PRR-2 , UURB-2014-I
Cluster 2	3	IC-538878, PRR-I , PRR-2007-2

Table 4. Grouping of ricebean genotypes using STRUCTURE software program

Group	No. of genotypes	Genotypes
P1	5	RBHP-401, UURB-2014-1, RBHP-408, RBHP-413, RBHP-114
P2	23	LRB-478, LRB-474, LRB-473, LRB-476, LRB-449, LRB-447, LRB-475, LRB-464, LRB-311, LRB-498, LRB-455, IC-23512, UURB-17, RBHP-43, RBHP-38, RBHP-403, RBHP-404, RBHP-406, RBHP-407, RBHP-409, RBHP-410, RBHP-411, RBHP-412
P3	7	IC-35928, PRR-2 (C), IC-419518 , IC-395028, IC-421926, IC-524082, RBHP-405
P4	5	IC-538878, IC-538870, PRR-2007-2, PRR-I (C), IC-538983

Fig. 1. SSR polymorphism in 40 genotypes revealed by primer *CEDAAG002*, M = 100 bp DNA ladder

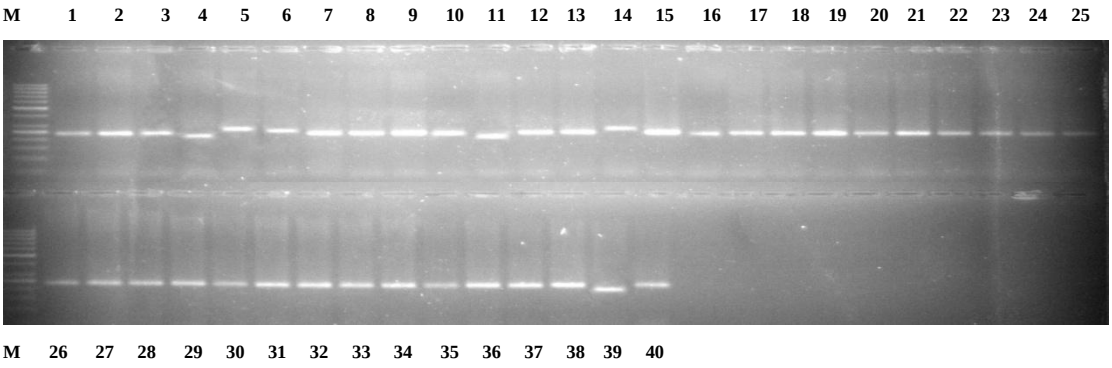

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Fig. 2. Dendrogram depicting genetic relationships among the ricebean genotypes constructed by NTSYS – PC (version 2.02)

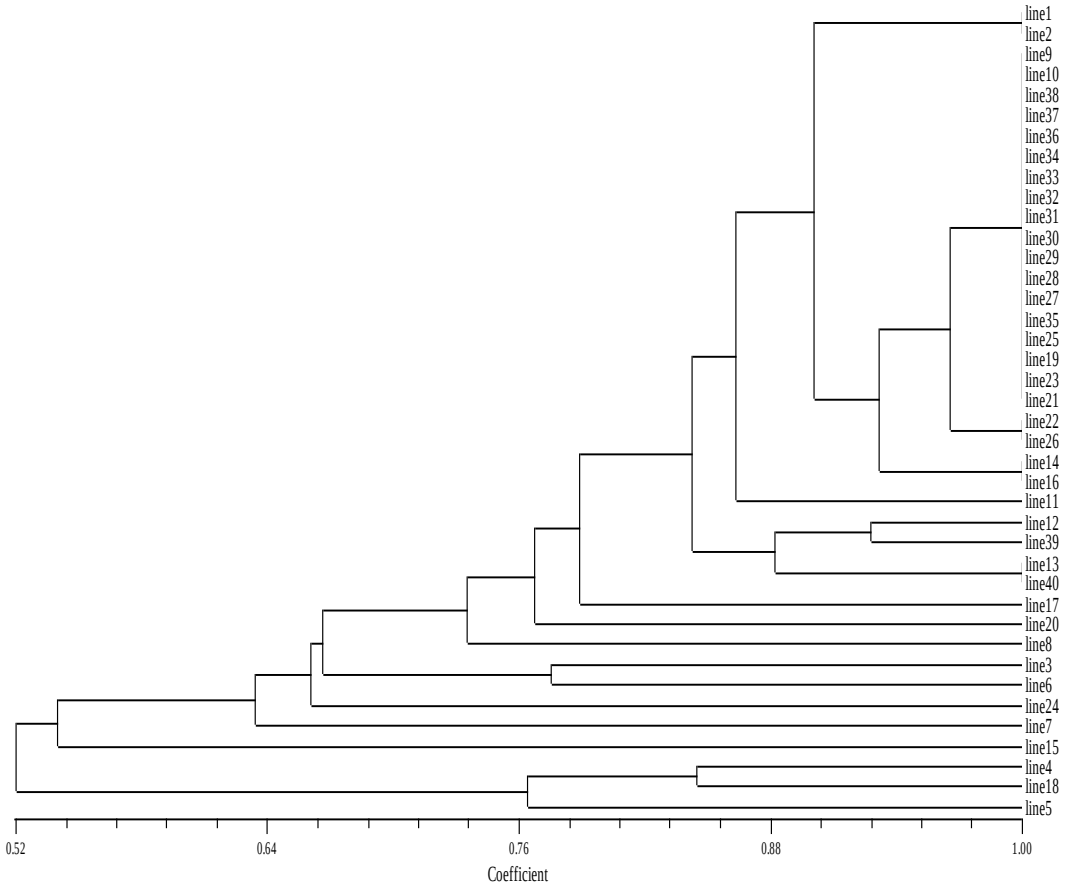

Fig. 3. Population structure of ricebean genotypes using structure software program (K=4)