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Analysis of Genetic Variability among Red Cowpea Genotypes by using ISSR Markers

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ABSTRACT

Cowpea (*Vigna unguiculata* (L.) Walp. (2n=22) is an early, multipurpose and the most widely adapted, versatile and nutritious grain legume crop; belongs to family Leguminosae. Lack of genetic variability for economically important traits is one of the reasons attributed for the very little progress in the crop improvement of cowpea. Estimation of genetic variability present in the available gene pool can be estimated with the help of molecular markers with more accuracy and more reliability as compared to the conventional methods in order to identify the genetically divergent parents for hybridization. The Polymorphic Information Content (PIC) value observed between 0.1 to 1.0. The PIC value is directly proportional to the information contained in the primer. Different ISSR primers showed different levels of polymorphism among 32 genotypes. Average PIC value recorded by the primers was 0.843. Almost all the polymorphic primers were able to discriminate the genotypes and varieties studied for diversity analysis by ISSR primers, certain primers discriminated genotypes and varieties specifically with presence of a unique amplicon. Genetic similarities among 32 genotypes of red cowpea were estimated from Jacquard's coefficients and genetic relationships were determined from the dendrogram constructed using the unweighted pair group method with arithmetic average (UPGMA) technique. The similarity co-efficient ranged from 0.111 (between PCP-1106 and KBC-WS-1) to 0.684 (between GC-8910 and JLCP-23) indicated the distinctness of these genotypes.

Keywords Red cowpea, ISSR, Polymorphism, Polymorphic Information Content, Jacard's similarity co-efficient.

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Table 1. List of cowpea genotypes with their sources.

Sl. No.	Code No.	Name of genotype	Pedigree	Source
1.	02	V-8981	Mutant Pusa Falguni	A. R. S., Pandharpur
2.	03	GC 8910	GC-2 × PGCP-1	A. R. S., Pandharpur
3.	06	V-240	Mutant Pusa Falguni 1	A. R. S., Pandharpur
4.	07	TC 210 82	EC 394763 × EC 394736	A. R. S., Pandharpur
5.	10	KBC-WS-1	Local Selection Nashik	A. R. S., Pandharpur
6.	11	4-40-1	Selection from Goa	A. R. S., Pandharpur
7.	12	CD 209	Re- 101 × Ajmear Selection	A. R. S., Pandharpur
8.	13	CP-210	De – 15	A. R. S., Pandharpur
9.	14	JLLP-5-1	Local selection Jalgaon	A. R. S., Pandharpur
10.	20	TPTC-1	EC 394 760 × EC 394 740	A. R. S., Pandharpur
11.	21	TC-2010 82	-	A. R. S., Pandharpur
12.	22	V-8981-2	Vausan	A. R. S., Pandharpur
13.	23	DCP-12	C-152 x GL	A. R. S., Pandharpur
14.	24	JLCP-23	Local Selection Jalgaon	A. R. S., Pandharpur
15.	25	GC-0502-1	GC- 515 x PGCP-2	A. R. S., Pandharpur
16.	26	JLCP-37	Local Selection Jalgaon	A. R. S., Pandharpur
17.	28	Phule Pandhari (red cowpea)	VCM-8 x V575	A R. S., Pandharpur
18.	32	IC 25 9104	Madurai	NBPGR, New Delhi
19.	34	IC 25 90 69	Delhi	NBPGR, New Delhi
20.	35	IC 25 32 77	Delhi	NBPGR, New Delhi
21.	36	IC 20 27 86	Goa	NBPGR, New Delhi
22.	44	EC 1099 81	Nigeria	NBPGR, New Delhi
23.	47	EC-6346 42	Taiwan	NBPGR, New Delhi
24.	49	EC -135 35	Japan	NBPGR, New Delhi
25.	50	EC -240 631	Philippines	NBPGR, New Delhi
26.	53	EC -16 966	USA	NBPGR, New Delhi
27.	59 a	Goa 1	Local Collection	Collection from local farmer
28.	59 b	Goa 2	Local Collection	Collection from local farmer
29.	60	PCP- 1106	Breeding line of Phule Pandhari	NBPGR, New Delhi.
30.	61	Vetore local	Local Collection	Collection from local farmer
31.	62	Konkan Sadabahar (local check)	Releazed variety	Dr. BSKKV, Dapoli.
32.	63	Konkan Safed (local check)	Released variety	Dr. BSKKV, Dapoli.

INTRODUCTION

Cowpea (*Vigna unguiculata* (L.) Walp. (2n=22) is an early, multipurpose and the most widely adapted, versatile and nutritious grain legume crop; belongs to family Leguminosae. It is commonly known as 'vegetable meat'. It is one of the most important food legume crop plant of great socio-economic, cultural, nutritional importance and a valuable component of the traditional cropping systems in the semi-arid tropics covering Asia, Africa and Central America. (Shanko *et al.* 2014).

In India, it is mostly grown in Kerala, Madhya Pradesh, Maharashtra, Karnataka, Andhra Pradesh, Tamilnadu, Uttar Pradesh, Punjab and Delhi (Anonymous 2015). The area under total pulses in India during the year 2019-20 was of 28.34 million ha with the production of 23.15 million tonnes and productivity of 817 kg/ha (Anonymous 2020).

In Maharashtra, the area under pulses was 43,16,966 ha with the production of 38,49,048 tonnes and the average productivity of 644.5 kg/ha (Anonymous 2020b). In Konkan region, pulses were cultivated on an area of 49,16,043 ha with the

Table 2. Primer wise amplification and per cent polymorphism shown by 11 polymorphic ISSR markers.

Sl. No.	Primer	Total no. of bands	No. of poly-mor-phic bands	No. of mono-mor-phic bands	% Poly-mor-phism
1	UBC-815	56	24	32	42.85
2	UBC-818	61	61	0	100
3	UBC-834	46	46	0	100
4	UBC-841	125	125	0	100
5	UBC-857	69	69	0	100
6	UBC-876	77	77	0	100
7	UBC-879	73	73	0	100
8	UBC-884	111	111	0	100
9	UBC-886	100	100	0	100
10	UBC-889	130	50	80	38.46
11	UBC-891	89	89	0	100
Total		937	825	112	981.31
Average		85.18	75	10.18	89.21

Table 3. Primer wise range of amplification and Polymorphism Information Content (PIC) in red cowpea genotypes.

Sl. No.	Primer	Range of Amplification (Kb)	PIC
1	UBC - 815	0.500-2.492	0.851
2	UBC - 818	0.486-0.817	0.782
3	UBC - 834	1.036-1.952	0.935
4	UBC - 841	0.411-1.829	0.888
5	UBC - 857	0.450-2.049	0.892
6	UBC - 876	0.530-1.536	0.914
7	UBC - 879	0.736-1.863	0.931
8	UBC - 884	0.457-2.093	0.779
9	UBC - 886	0.621-2.203	0.786
10	UBC - 889	0.607-1.226	0.748
11	UBC - 891	0.897-2.596	0.771
Range	0.411- 2.492		
Average			0.843

production of 47,94,614 tonnes and average productivity of 753.28 kg/ha (Anonymous 2021).

Lack of genetic variability for economically important traits is one of the reasons attributed for the very little progress in the crop improvement of cowpea. It is a self-pollinated crop and efforts to evolve high yielding genotypes are mainly done by exercising selection in segregating generations. In India, cowpea improvement is restricted to assembling a limited number of germplasm and hybridization among randomly chosen parental lines with narrow genetic base. Hence, it is advocated that extensive hybridization involving large number of parents of diverse origin be adopted to synthesize a broad based gene pool. The ability to accumulate the variability by recombination and isolation of desired genotypes from segregating population hold the key to success of any crop improvement program. (Kurer *et al*. 2010).

The use of SSR markers as a tool to detect polymorphism between the cultivated varieties and landraces of cowpea and to identify the extent of genetic variation with respect to quantitative traits provides insight into the diversity of crop varieties and their potential contributions. Utility of microsatellite markers for assessment of genetic diversity among cultivars and their wild relatives has been demonstrated in many crops including soybean, maize, wheat, rice and sorghum. The usefulness of SSR markers in assessing the level of genetic diversity in wild and cultivated cowpeas in recent past was reported by many scientists (Uma *et al.* 2016). Estimation of genetic variability present in the available gene pool can be estimated with the help of molecular markers with more accuracy and more reliability as compared to the conventional methods in order to identify the genetically divergent parents for hybridization.

MATERIALS AND METHODS

A total of 32 genotypes used in the present investigation the details of the germplasm and its source is given in Table 1.

Molecular analysis

The molecular analysis was carried out in the laboratory of Plant Biotechnology Center, College of Agriculture, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli, Dist. Ratnagiri (MS) during the year 2019-20 and subjected to molecular analysis through ISSR markers in order to check the genetic distance among them.

Table 4. List of primers discriminating genotypes with a unique amplicon.

Sl. No.	Primer	Genotype/ varieties	Size Kb	No. of geno-types
1.	UBC-815	V-8981	0.984	5
		TC-2010 82	1.026	
		EC-135 35	1.032	
		Goa1	1.048	
		IC 25 90 69	1.139	
2.	UBC-834	IC 20 27 86	1.500	5
		PCP-1106	1.528	
		IC 25 90 69	1.557	
		GC-0502-1	1.683	
		TC-2010 82	1.925	
3.	UBC-841	EC-16 966	1.014	1
4.	UBC-857	IC 20 27 86	0.676	2
		JLCP-23	0.776	
5.	UBC-876	IC 20 27 86	0.693	
		IC 25 9104	0.758	
		GC-0502-1	0.802	
		V-8981	0.837	
		Goa 2	0.839	
		CD 209	0.895	6
6.	UBC-879	IC 25 90 69	0.736	
		IC 25 9104	0.877	
		IC 25 32 77	0.905	
		4-40-1	0.913	
		JLLP-5-1	0.928	
		V-8981	0.938	
		Goa 1	0.975	
		Goa 2	0.960	
		KBC-WS-1	0.975	9
7.	UBC-884	IC 25 90 69	0.756	
		KBC-WS-1	0.963	2
8.	UBC-886	EC-240 631	1.048	
		Phule Pandhari	1.104	
		CP-210	1.590	
9.	UBC-889	IC 25 90 69	0.666	
		Konkan Safed	0.747	
		Goa 2	0.820	
10.	UBC-891	IC 25 90 69	1.000	
		CP-210	1.243	
		V-8981	1.255	
		TC-2010 82	1.331	
		Goa 2	1.438	6

Table 5. Clustering pattern of 32 genotypes in red cowpea.

Cluster			Number of genotypes	Name of the genotype
I			1	IC 25 90 69.
	IIA	IIAa	3	Kokan Safed, IC 20 27 86,
		IIAb		PCP-1106.
II			6	EC-6346 42, Vetore local, TPTC-1, EC 1099 81, IC 25 9104, GC-0502-1
		IIBa	1	TC-2010 82.
		IIBb	21	Phule Pandhari, EC-16 966, DCP-12, Konkan Sadabahar, V-240, CD 209, GC 8910, JLCP-23, JLCP-37, Goa 2 V-8981-2, TC 210 82, KBC-WS-1, 4-40-1, EC-135 35, EC-240 631, IC 25 32 77, CP-210, JLLP-5-1, Goa 1, V-8981.

Extraction of genomic DNA

Plant material

In the present experimental study, all the thirty two genotypes of red cowpea including three check varieties were sown in the pots. The leaf samples were collected from 5-6 days old seedlings for the extraction of genomic DNA.

Procedure for extraction of genomic DNA

The DNA was isolated by following the protocol of Doyle and Doyle (1990) i.e. rapid method with slight modifications of buffer composition and concentration. The young newly emerged leaves were collected and sterilized with 70% ethanol to avoid the contamination. Purification of DNA was done to remove RNA and proteins which were the major contaminants. RNA was removed by RNase treatment and proteins were removed by Proteinase-K treatment.

DNA quantification by using agarose gel electrophoresis

Concentration of DNA in the sample was determined by agarose gel electrophoresis with uncut lambda DNA on 0.8% agarose gel and by comparison of the intensity of band staining with ethidium bromide.

DNA amplification

The isolated and quantified DNA sample was subjected to DNA amplification with the help of 15 ISSR primers.

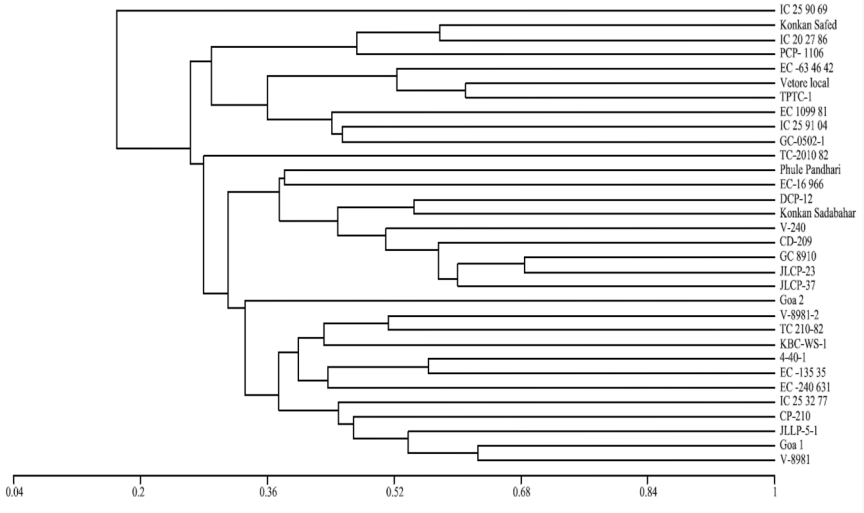

Fig. 1. Dendrogram constructed using Jaccard's similarity coefficient using ISSR markers.

The amplified products in ISSR reaction were separated by electrophoresis in 2% agarose gel (SRL, India), containing Ethidium Bromide in 1X TAE Buffer (pH 8.0) and separation were carried out by applying constant voltage of 100 volts for 1 hour. The standard DNA ladder used was Φ ×174/Hae III digest. PCR and gel electrophoresis were carried out two times and only reproducible patterns were used for data analysis.

Photography and gel documentation

The gels were photographed under UV light using Pentax K 312 nm camera. The images of gels were also taken by the documentation systems (Uvi-Tech. Fire reader, Cambridge, England) and saved in computer for further analysis.

Statistical analysis

ISSR markers across the genotypes were scored for their presence (1) or absence (0) of bands for each primer. The binary data so generated was used to estimate the levels of polymorphism by dividing the number of polymorphic bands by the total number of scored bands. Jaccard's similarity coefficients for each pairwise comparison between germplasm were calculated and similarity co-efficient matrix was generated. This matrix was subjected to Unweighted Pair Group Method for Arithmetic Average analysis (UPGMA) to construct a dendrogram. The similarity co-efficient analysis and dendrogram construction were carried out by using MVSP-A Multivariate Statistical Package-5785 (Version 3.1).

Distance matrix and dendrogram was constructed based on diversity coefficient generated from pooled data by using unweighted pair group method of arithmetic means (UPGMA), a computer program for distance estimation. Other parameters computed were,

$$\text{Total number of polymorphic bands} = \frac{\text{(Per cent polymorphism bands)}}{\text{(Total number of bands)}} \times 100$$

RESULTS AND DISCUSSION

To study the genetic diversity among the 32 geno-

types of red cowpea DNA was isolated using Doyle and Doyle (1990) method of isolation, followed by PCR amplification and gel electrophoresis to obtain different banding patterns of the genotypes under study using fifteen ISSR primers. Out of fifteen primers used for amplification, eleven primers showed analyzable banding pattern in the agarose gel. PCR amplification was good when annealing temperature was set to 40–60ºC as per previous report from Kelkar *et al.* (2017).

The data given in Table 2 revealed the primer wise information regarding total number of bands, number of polymorphic bands and number of monomorphic bands produced. On the basis of polymorphism obtained, the per cent polymorphism exhibited by the 11 polymorphic primers is mentioned in Table 3. Out of the 11 ISSR primers, 9 exhibited 100% polymorphism whereas primers UBC-815 and UBC-889 exhibited 42.85% and 38.46% polymorphism respectively with 856 amplicons at 8 to 14 different loci per primers. A total of 937 bands were generated by 32 genotypes under study with an average of 85.18 bands per primer. The total number of polymorphic bands yielded was 825 with an average of 75 bands. The total number of monomorphic bands was 112 and on an average, 89.21 % polymorphism was observed as a result of primer amplification. The Polymorphic Information Content (PIC) value observed between 0.1 to 1.0. The PIC value is directly proportional to the information contained in the primer. Different ISSR primers showed different levels of polymorphism among 32 genotypes. Average PIC value recorded by the primers was 0.843 (Table 3).

[PIC= 1-(Total number amplicons /Total number of genotypes)^2

Genetic relationship among genotypes

Genetic similarities among 32 genotypes of red cowpea were estimated from Jacquard's coefficients and genetic relationships were determined from the dendrogram constructed using the unweighted pair group method with arithmetic average (UPGMA) technique. The similarity co-efficient ranged from 0.111 (between PCP-1106 and KBC-WS-1) to 0.684 (between GC-8910 and JLCP-23) indicated the distinctness of these genotypes.

Cluster analysis

In the present study, 32 genotypes were subjected to cluster analysis for assessing the molecular diversity based on UPGMA analysis. The clustering pattern and the dendogram constructed using Jaccard's similarity coefficient across the 32 red cowpea genotypes is presented in Fig. 1 (dendrogram).

The cluster analysis band on the similarity co-efficient clearly distinguished all the 32 genotypes into two main groups. The clustering pattern is mentioned in Table 6. The first cluster contains unique genotype IC 25 90 69 which showed difference from other 31 genotypes. Second cluster further divided into two sub-clusters IIA and IIB. Sub-cluster IIA again divided into two sub-clusters IIAa and IIAb. Sub-cluster IIAa contain three genotypes viz., Kokan Safed, IC 20 27 86 and PCP-1106. Sub-cluster IIAb contain six genotypes viz., EC-6346 42, Vetore local, TPTC-1, EC-1099 81, IC 25 9104 and GC-0502-1. Sub-cluster IIB again divided into two sub-clusters IIBa and IIBb. The sub-cluster IIBa expressed single genotype i.e. TC-2010 82. Sub-cluster IIBb contained 21 genotypes viz., Phule Pandhari EC-16 966, DCP-12, Konkan Sadabahar,V-240, CD 209, GC 8910, JLCP-23, JLCP-37, Goa-2, V-8981-2, TC 210 82, KBC-WS-1, 4-40-1, EC-135 35, EC-240 631, IC-25 32 77, CP-210, JLLP-5-1, Goa 1 and V-8981.

Assessment of genetic diversity of cowpea accessions using molecular markers is imperative for their genetic improvement and conservation. Use of efficacious markers to obtain the required knowledge of the genetic diversity within the local and regional germplasm collections can enhance overall effectiveness of cowpea improvement program (Igwe *et al.* 2017).

Understanding the genetic variability within a species, as assessed by the proximity and diversity between genotypes is an essential prerequisite in hybridization and plant improvement. Such knowl-

edge not only permits the identification of divergent and complementary genotypes that can be used as progenitors in a breeding program but also increases the chance of selecting elite genotypes in segregating generations (Mendes *et al.* 2015).

Molecular markers are valuable tools in the investigation of genetic variability within a plant collection or among wild species. Amongst available markers, inter-simple sequence repeats (ISSR) have been widely used by virtue of the repeatability and reproducibility of the banding patterns obtained and the simplicity of the techniques involved. A number of reports have been published on the application of ISSR in the determination of genetic diversity in cowpea (Ghalmi *et al*. 2010, Santos *et al.* 2020).

For exploring the diversity of crop plants a range of DNA markers, viz., AFLP, DAMD, ISSR, ITS, and RAPD have already been used. Among these, ISSR is a reproducible, semi arbitrary primed PCR method that uses inter simple sequence repeats as primers. Any prior genome sequence information is not required for ISSR markers. ISSR markers are highly variable, highly reproducible and ubiquitously distributed across the genome and cost effective. All these characteristics make ISSR an ideal genetic marker (Joshi *et al.* 2018).

Inter Simple Sequence Repeats (ISSRs) are highly polymorphic markers found in the plants, which produced large amount of genetic information needed for the diversity analysis. The ISSR markers are highly reliable, consistent, no need of genetic information and allow low cost genotyping as compared to RAPD. The latter detects nucleotide sequence polymorphisms, using a single primer of arbitrary nucleotide sequence and the former permits detection of polymorphisms in inter-microsatellite loci, using a primer designed from di-nucleotide or tri-nucleotide simple sequence repeats (Gajera *et al.* 2014). ISSR markers are arbitrary markers that target multiple genomic loci and amplify DNA segments present between two identical microsatellite regions that are opposite with each other in orientation (Igwe *et al.* 2017).

CONCLUSION

The PIC value was highly informative since it is observed between 0.748 to 0.935 and it is reported that PIC value >0.5 is highly informative, 0.25–0.50 is reasonably informative and <0.25 is slightly informative (Botstein *et al*. 1980). The dendrogram and similarity coefficient values give an idea about the nature of the individual sample in the whole sample set. The results obtained in cluster analysis proved to be important during the selection of genetically diverse parents for the breeding program.

REFERENCES

Anonymous (2015) Proceedings of the Regional Consultation on the promotion of pulses in Asia for multiple health benefits, Bangkok, Thailand, 29-30 June, (2015). Compiled and edited by Subhash Dasgupta and Indrajit Roy.

Anonymous (2020) Agricultural Statistics at a glance 2019. Government of India, Ministry of Agriculture and Farmers' Welfare, Department of Agriculture Co-operation and Farmers' Welfare, Directorate of Economics and Statistics, 2020.

Anonymous (2021) www.krishi.maharashtra.gov.in.

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. *Focus* 12 : 13—15.

Ghalmi N, Malice M, Jacquemin JM, Ounane SM, Mekliche L, Baudoin JP (2010) Morphological and molecular diversity within *Algerian cowpea (Vigna unguiculata* (L.) Walp.) landraces. *Genet*. *Resour Crop Evol* 57 : 371—386.

Igwe David Okeh, Celestine Azubike Aflukwa, Benjamin Ewa UBI, Kenneth Idika Ogbu, Omena Benard Ojuederie, George Nkem Ude (2017) Assessment of genetic diversity in *Vigna unguiculata* L. (Walp) accessions using inter-simple sequence repeat (ISSR) and start codon targeted (SCoT) polymorphic markers. *BMC Genet* 18 : 98.

Joshi Apoorva, Sharma Somya, Kumar Anand, Singh Monika, Das Pulak (2018). Intraspecific genetic diversity in different cowpea germplasm revealed by ISSR markers. *Int J Curr Biotechnol* 6 (6) : 1—5.

Kelkar VG, Sawardekar SV, Bhave SG, Gokhale NB, Rasam DV, Sawant SS (2017) Analysis of genetic variability among the finger millet germplasm by using ISSR markers. *Environ Ecol* 35 (2C) : 1233—1237.

Kurer Shashidhar, Gangaprasad S, Uma MS., Shanthakumar G, Salimath PM (2010) Research note genetic variability studies in F_2 and F_3 generations of cowpea (*Vigna unguiculata* (L.) Walp). *Elect J Pl Breed* 1 (5) : 1344—1346.

Mendes RFM, Britto FB, Freire Filho FR, Lima PS (2015) Genetic variability of indigenous cowpea genotype as determined using inter-simple sequence repeats markers. *Afr J Biotechnol* 14 (36) : 2652—2661.

Santos LFC dos, Ferrer M M, Ruenes-Morales, MR, Montanez-Escalante PI, Andueza-Noh RH, Jimenez-Osornio J

(2020) Genetic diversity and structure analysis of *Vigna-unguiculata* L. (Walp.) landraces from southeastern Mexico using ISSR markers. *Pl Genet Resources : Characterization and Utilization* 18 (4) : 201—210.

Shanko Diriba, Mebeaselassie Andargie and Habtamu Zelleke (2014) Interrelationship and path coefficient analysis of some growth and yield characteristics in cowpea (*Vigna unguiculata* L. Walp.). *Genotypes* 2 (2) : 97—101.

Uma MS, Hegde Niranjan, Hittalmani Shailaja (2016) Identification of SSR markers associated with rust resistance in cowpea (*Vigna unguiculata* (L.) Walp.) using bulk segregant analysis. *Leg Res* 39 (1) : 39—42.