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Molecular Characterization of Cowpea Genotypes for Genetic Diversity using RAPD and ISSR Techniques

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ABSTRACT

Background: Cowpea [*Vigna unguiculata* (L.) Walp] is an important pulse crop with wide nutritional and agronomic value. Genetic diversity is essential for its improvement and molecular markers such as RAPD and ISSR provide valuable information for assessing phylogenetic relationships and species distinctiveness.

Methods: In the present investigation, 78 cowpea germplasm lines, including three check varieties, were evaluated in a randomized complete block design with three replications. Out of these, 48 diverse genotypes were selected for molecular characterization using 22 primers (10 RAPD and 12 ISSR). DNA was extracted from young leaves and subjected to PCR amplification. Nine RAPD primers generated 1180 scoreable bands, while eight ISSR primers amplified 1215 bands across the 48 genotypes.

Result: The polymorphic information content (PIC) values for RAPD primers ranged from 0.27 (RAPD-1) to 0.41 (RAPD-4), with a mean of 0.35. ISSR primers showed PIC values between 0.23 (UBC 815) and 0.42 (UBC 835), with a mean of 0.35. Similarity coefficients among genotypes based on RAPD markers ranged from 0.58 to 1.00, while ISSR-based similarity values ranged from 0.55 to 0.97 with a mean of 0.729. Dendrogram analysis grouped the genotypes into two major clusters. Combined RAPD and ISSR data produced similarity coefficients between 0.58 and 0.94 and the integrated dendrogram revealed 78-83% similarity with individual marker systems. The results indicate a high degree of genetic variation among the cowpea accessions, which can be effectively utilized in pre-breeding programs to broaden the genetic base and facilitate selection of superior lines.

Key words: Genetic diversity, ISSR marker, Polymorphism, RAPD marker, Similarity coefficient, *Vigna unguiculata*.

INTRODUCTION

Cowpea [*Vigna unguiculata* (L.) Walp.] (2n = 22) is an important pulse crop belonging to the often cross-pollinated category. It plays a vital role in global food security and human nutrition due to its rich nutritional and nutraceutical properties (Olabanji *et al*., 2018). Cowpea is an annual herb with a strong tap root system and spreading growth habit. It is a multipurpose crop used as food for humans, feed and fodder for livestock and as green manure. The crop also provides livelihood opportunities for farmers and small-scale entrepreneurs (Moolendra *et al*., 2018). Cowpea is popularly known as southern pea or black-eyed pea.

Globally, cowpea was cultivated on about 15 million hectares in 2022, with more than 90 per cent of the area located in Africa (FAOSTAT, 2023). World production was approximately 9.8 million tonnes, with Niger and Nigeria being the major producers, occupying nearly 6 and 4.8 million hectares, respectively (FAO, 2023). In India, cowpea is grown on about 1.5 million hectares with an annual production of 2.21 million tonnes and an average productivity of 683 kg ha^{-1} (Kaushik *et al*., 2016). The crop is widely cultivated in states such as Maharashtra, Gujarat, Rajasthan, Punjab, Haryana, Madhya Pradesh, Kerala, Karnataka, Andhra Pradesh and Tamil Nadu. Chhattisgarh is one of the important cowpea-growing states, contributing about 0.81 per cent of national area and 0.25 per cent of production, with approximately 43.13 thousand hectares under cultivation and an average productivity of 314 kg ha^{-1} (Agriculture Department, Government of Chhattisgarh; FAOSTAT, 2023).

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Genetic diversity is the major factor influencing population improvement and selection efficiency. Assessment of genetic variability in cowpea is essential for developing improved varieties with higher yield and stress tolerance. However, cultivated cowpea possesses a narrow genetic base due to its predominantly self-pollinating nature and domestication bottlenecks (Govindaraj *et al*., 2015). Therefore, evaluation of genetic diversity is crucial for effective conservation and utilization of genetic resources (Khan *et al*., 2012).

Molecular marker techniques such as RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter Simple Sequence Repeat) are widely used for assessing genetic diversity and establishing phylogenetic relationships. RAPD markers are simple, rapid and cost-effective tools for detecting genetic variation among cultivars (Paiva *et al*., 1994; Subramanian *et al*., 2000; Dwivedi *et al*., 2001). ISSR markers target microsatellite regions and provide highly reproducible and polymorphic multilocus profiles. DNA polymorphism-based molecular techniques have become essential for characterizing germplasm and identifying genetic variation in breeding materials. The development of molecular tools that directly detect DNA-level variation has significantly improved the efficiency and accuracy of genetic diversity analysis in crops lacking extensive classical genetic information (Nath *et al*., 2017; Asadova *et al*., 2024; Ofem *et al*., 2025).

MATERIALS AND METHODS

Genotype collection

78 germplasm lines in all (Table 1), including three check varieties, were assessed using a randomized block design with three replicates at Research cum Instructional Farm, Department of Genetics and Plant Breeding, College of Agriculture, Indira Gandhi Krishi Vishwavidalaya, Raipur, Chhattisgarh. Out of these only 48 selected diverse genotypes of cowpea were analyzed to assess the molecular diversity.

DNA extraction and electrophoresis

From each of the 48 genotypes, young and healthy leaves were harvested independently and the modified mini prep CTAB procedure was used to extract the genomic DNA. Place the chopped leaf pieces in a 2.0 ml Eppendorf tube, add 700 µl of CTAB buffer and then leave it at 4°C for three to four hours. Next, Put the leaf in a water bath at 65°C for 20 minutes, grind it if necessary, add extra CTAB buffer, add 700 µl of chloroform: iso amyl alcohol (24:1), vortex the sample and centrifuge it for 20 minutes at 14,000 rpm. Next, move the top layer of clear material into a fresh 1.5 ml Eppendorf tube, add 700 µl of ice-cold isopropanol in and 175 µl of potassium acetate and incubate for two hours at 4°C. After 20 minutes of centrifuging at 14,000 rpm and discarding the supernatant, wash the pellet with 50 µl of 70% ethanol and centrifuge it once more for 10 minutes after the centrifugation air dry the pellet and add 100 µl of TE buffer to dissolve the pellet. The Nano Drop Spectrophotometer was used to quantify the DNA samples. 2.0 µl of sample was taken and placed on the Nano Drop followed by closing the lid, enter 1st blank (TE) whose range should be 0.0 to 0.1. After quantification, the final DNA concentration for PCR amplification was 50 ηg/µl after the DNA was diluted with TE or sterile water (nuclease free water). 1 µl of diluted template DNA of each genotype was dispensed at the bottom of PCR plate (AXYGEN) for both RAPD and ISSR primers. About 19 µl to each sample and the PCR was performed.

Initial denaturation at 93°C for 3 min and again denaturation step is carried out for 1 min followed by annealing for 1 min at 36°C. The amplification was completed with 2 min final extension for 72°C. The electrophoresis of the amplification products was performed on a 1.2% agarose gel that was produced in 1X TAE buffer and stained with ethidium bromide. After electrophoresis, the products are visualized under UV transilluminator and recorded under GEL-DOC system.

Molecular marker RAPD and ISSR assay

Nine RAPD primer and eight ISSR primers were used for PCR reaction (Table 2).

RESULTS AND DISCUSSION

The PIC (Polymorphic information content) index indicated extent of polymorphic bands generated by a primer has been used extensively in many genetic diversity studies. It depends on the number of detectable alleles and distribution of their frequency and is equivalent to gene diversity. The abundant allelic variation is desirable for rapid genotyping in large populations with limited primers, since it favors the attainment of high PIC, especially if the markers are associated with gene of interest. The moderate values of PIC for the RAPD primers and ISSR primers could be attributed to the diverse nature of the cowpea genotypes and/or highly informative RAPD and ISSR markers used in this study. A total number of 22 primers (RAPD 10 primers, ISSR 12 primers) were used to estimate genetic variability between cowpea genotypes (Table 2). Out of 10 RAPD markers 9 RAPD marker and 08 out of 12 ISSR markers are amplified in all genotypes. A total 1180 bands were observed among the 48 cowpea genotypes based on RAPD analysis with 9 primers. There were 1215 scoreable bands in all from ISSR markers. The likelihood that polymorphism would occur between two randomly chosen genotypes at a given locus is determined by using the PIC value as a measure of variability at that locus. With an average of 0.35, the estimated PIC value varied from 0.27 for primer RAPD 1 to 0.41 for RAPD 4 on the other hand, the average PIC for ISSR primers was 0.35, with a range of 0.23 for primer UBC 815 to 0.42 for primer UBC 835. With an average of 131.11 bands per primer, the number of scoreable markers generated varied from 41 to 226.

Table 1: List of 78 cowpea genotypes collected from different locations of the Chhattisgarh state.

Genotype/check	District	Block	Village	Latitude	Longitude
BL-1	-	National check	-	-	-
BL-2	-	Zonal check	-	-	-
MFC-8-14	-	National check	-	-	-
MFC-09-3	Mandya	-	-	-	-
MFC-09-4	Mandya	-	-	-	-
MFC-09-13	Mandya	-	-	-	-
TSFC-12-15	Hyderabad	-	-	-	-
UPC-1501	Pantnagar	-	-	-	-
UPC-5286	-	National check	-	-	-
UPC-628	-	Zonal check	-	-	-
Vellyani-1	Vellyani	-	-	-	-
RCC 1	Durg	Durg	Dhanora	21°08′36.6324″	81°19′28.8552″
RCC 2	Odisha	Odisha	Gaijore	22°01′33.0726″	84°03′42.0003″
RCC 3	Rajnandgaon	Dongargarh	Musra	21°09′25.7544″	80°52′43.2732″
RCC 4	Rajnandgaon	Dongargarh	Saloni	21°14′41.3232″	80°51′15.8292″
RCC 5	Koria	Koria	Barbaspur	23°17′52.0584″	82°23′49.6248″
RCC 6	Rajnandgaon	Dongargarh	Saloni	21°15′02.0592″	80°51′00.9972″
RCC 7	Odisha	Odisha	Ghnatabund	22°01′36.3036″	84°04′37.0065″
RCC 8	Kondagaon	Kondagaon	Bhiragaon	19°36′10.0404″	81°38′00.3804″
RCC 11	Kondagaon	Kondagaon	Gulbha	19°36′49.3344″	81°36′43.7508″
RCC 19	Odisha	Odisha	Titheitangar	22°00′50.0076″	84°03′27.198″
RCC 21	Lacknow	Lacknow	Lacknow	26°50′48.0984″	80°56′46.1976″
RCC 22	Mirjapur	Mirjapur	Mirjapur	25°07′00.8832″	82°34′24.2904″
RCC 23	Odisha	Odisha	Masanikani	22°01′30.8604″	84°02′01.608″
RCC 27	Rajnandgaon	Khairgarh	Rahud	21°22′38.1864″	81°07′49.2312″
RCC 28	Rajnandgaon	Chouki	Chouki	20°46′39.6804″	80°44′52.0584″
RCC 29	Rajnandgaon	Chouki	Chouki	20°44′25.2312″	80°44′45.8808″
RCC 30	Rajnandgaon	Chouki	Chouki	20°44′20.6088″	80°44′11.274″
RCC 31	Durg	Dhamdha	Dhamdha	21°27′22.2264″	81°20′03.7032″
RCC 32	Durg	Dhamdha	Funda	21°20′50.6544″	81°16′18.1416″
RCC 33	Durg	Dhamdha	Dhamdha	21°24′19.0476″	81°21′45.6696″
RCC 35	Bemetara	Bemetara	Saja	21°39′02.4732″	81°19′17.3532″
RCC 36	Bemetara	Bemetara	Beeja	21°39′34.6392″	81°24′33.7608″
RCC 37	Bemetara	Bemetara	Kodwa	21°38′57.8796″	81°21′55.5588″
RCC 38	Rajnandgaon	Dongargarh	Shivpuri	21°14′18.0816″	80°49′48.846″
RCC 39	Rajnandgaon	Dongargarh	Dhara	21°15′17.2764″	80°50′19.1292″
RCC 40	Rajnandgaon	Dongargarh	Achholi	21°11′42.9828″	80°47′06.6264″
RCC 41	Rajnandgaon	Dongargarh	Saloni	21°14′50.0496″	80°51′08.2584″
RCC 42	Rajnandgaon	Dongargarh	Dhara	21°15′01.0152″	80°51′12.5856″
RCC 43	Narayanpur	Narayanpur	Kokadi	19°43′51.3260″	81°12′23.2140″
RCC 44	Narayanpur	Narayanpur	Kokadi	19°43′54.1483″	81°12′32.1120″
RCC 45	Narayanpur	Narayanpur	Palki	19°43′34.1486″	81°12′21.3418″
RCC 46	Narayanpur	Narayanpur	Palki	19°43′31.1844″	81°12′13.2012″
RCC 47	Narayanpur	Narayanpur	Palki	19°43′31.1556″	81°12′13.3164″
RCC 48	Narayanpur	Narayanpur	Palki	19°43′31.1740″	81°12′13.3853″
RCC 49	Narayanpur	Narayanpur	Palki	19°43′31.1556″	81°12′13.3164″
RCC 50	Narayanpur	Narayanpur	Palki	19°43′31.1483″	81°12′13.2627″
RCC 51	Narayanpur	Narayanpur	Kokadi	19°43′49.1844″	81°12′27.1692″
RCC 52	Narayanpur	Narayanpur	Bharmbeda	19°43′53.5476″	81°11′46.3848″
RCC 53	Beejapur	Bhairamgarh	Mangapetha	19°00′36.0864″	81°03′41.9976″

Table 1: Continue...

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RCC 54	Beejapur	Beejapur	Santoshpur	19°00′26.0586″	81°03′41.2308″
RCC 55	Beejapur	Bhopalpatannam	Gotaiguda	18°50′29.1264″	80°22′34.8276″
RCC 56	Beejapur	Bhopalpatannam	Rampeta	18°45′28.5372″	80°07′46.6188″
RCC 57	Beejapur	Bhopalpatannam	Metlacheru	18°45′01.0332″	80°19′26.7312″
RCC 58	Beejapur	Beejapur	Manjiguda	18°48′55.3536″	80°46′54.8868″
RCC 59	Beejapur	Beejapur	Manjiguda	18°48′52.1250″	80°46′47.4783″
RCC 60	Beejapur	Bhopalpatannam	Maddeu	18°47′02.0660″	80°00′48.5731″
RCC 61	Beejapur	Beejapur	Meengachal	18°54′18.3132″	80°57′44.0604″
RCC 62	Beejapur	Beejapur	Meengachal	18°54′20.5452″	80°57′40.0446″
RCC 63	Beejapur	Beejapur	Meengachal	18°54′19.5192″	80°57′44.3052″
RCC 64	Beejapur	Bhopalpatannam	Meenur	18°46′21.8496″	80°28′15.6864″
RCC 65	Beejapur	Bhopalpatannam	Gorla	18°47′21.0768″	80°28′07.2516″
RCC 66	Beejapur	Beejapur	Pegdapalli	18°48′09.7272″	80°31′00.1056″
RCC 67	Beejapur	Bhopalpatannam	Meenur	18°47′09.1824″	80°27′16.5492″
RCC 68	Beejapur	Bhopalpatannam	Chadanagiri	18°49′52.1328″	80°25′50.1096″
RCC 69	Beejapur	Bhopalpatannam	Meenur	18°46′29.3988″	80°29′10.2588″
RCC 70	Beejapur	Bhopalpatannam	Chadanagiri	18°49′50.0844″	80°25′52.8924″
RCC 71	Beejapur	Bhopalpatannam	Kesaigunda	18°47′34.6668″	80°30′14.6052″
RCC 72	Beejapur	Beejapur	Itapal	18°48′19.0962″	80°46′36.6564″
RCC 73	Bastar	Bastar	Sargipal	19°03′52.0128″	82°00′17.6904″
RCC 74	Rajnandgaon	Rajnandgaon	Garatar kala	21°08′37.7088″	80°58′18.0624″
RCC 75	Rajnandgaon	Rajnandgaon	Khapri kala	21°09′13.4424″	80°59′20.4792″
RCC 76	Rajnandgaon	Rajnandgaon	Garatar kala	21°08′34.5408″	80°58′20.0226″
RCC 77	Rajnandgaon	Rajnandgaon	Khapri kala	21°09′08.5464″	80°59′21.0984″
RCCV5	Raipur	IGKV, Raipur	Dept. Hort.	21°14′14.3556″	81°42′30.978″
RCCV46	Raipur	IGKV, Raipur	Dept. Hort.	21°14′14.3556″	81°42′30.978″
RCCV48	Raipur	IGKV, Raipur	Dept. Hort.	21°14′14.3556″	81°42′30.978″
RCCV49	Raipur	IGKV, Raipur	Dept. Hort.	21°14′14.3556″	81°42′30.978″

Table 2: Total number of bands and PIC value of RAPD and ISSR markers.

Name of primers	Number of band	PIC value
RAPD 1	52	0.27
RAPD 2	137	0.38
RAPD 3	112	0.37
RAPD 4	157	0.41
RAPD 5	41	0.28
RAPD 6	123	0.39
RAPD 7	141	0.36
RAPD 8	191	0.38
RAPD 9	226	0.30
UBC 809	193	0.38
UBC 810	330	0.30
UBC 815	33	0.23
UBC 834	206	0.40
UBC 835	206	0.42
UBC 836	102	0.39
UBC 840	57	0.33
UBC 842	88	0.30

The similarity coefficient analysis was performed unit UPGMA coefficient (NT SYSPC 2014, software) based on 9 RAPD markers ranged from 0.58 to 1.00 (Fig 1). The genotype RCC63 and RCC64 showed the highest similarity index (1.00) and CP1 and CP41 showed the lowest similarity index (0.58). The mean similarity index was 0.79. RAPD markers to generate a dendrogram of cowpea genotypes. The dendrogram separated cowpea genotypes and showing two clusters; the cluster I (24 genotypes) divided into the two sub cluster A (4 genotypes) and B (20 genotypes). Sub cluster A further divided two cluster a‘ and a‘‘ with cluster a‘ containing only one genotypes RCC47 and Cluster a‘‘ having 3 genotype RCC35, RCC51, RCC40. Sub cluster B included two cluster b‘ and b‘‘. Cluster b‘ have 3 genotype Vellyani-1, RCC5 and RCC6 whereas 17 genotypes containing in cluster b‘‘. Among the 48 genotypes, eight ISSR markers yielded 1215 scoreable bands. With an average of 151.88 bands per primer, the number of scoreable markers generated varied from 57 to 330 (Similar results also reported by Nounagnon *et al.,* 2024 and Munyao, 2023). Whereas for ISSR marker ISSR primer the similarity coefficients for the cowpea genotypes based on 1215 ISSR markers ranged between 0.55 and 0.97 (Fig 2). The mean similarity index was 0.729. The genetic similarity matrix was used to obtain dendrogram through UPGMA based cluster analysis showing two clusters; the cluster II (24 genotypes) further divided sub

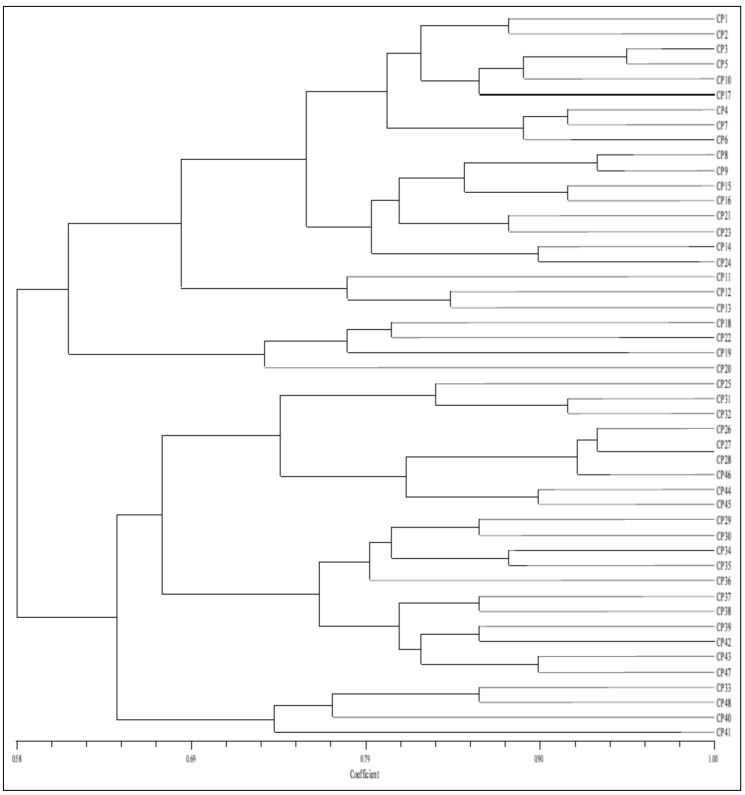


Fig 1: UPGMA based clustering of 48 cowpea genotypes using genetic distance matrix for RAPD markers.

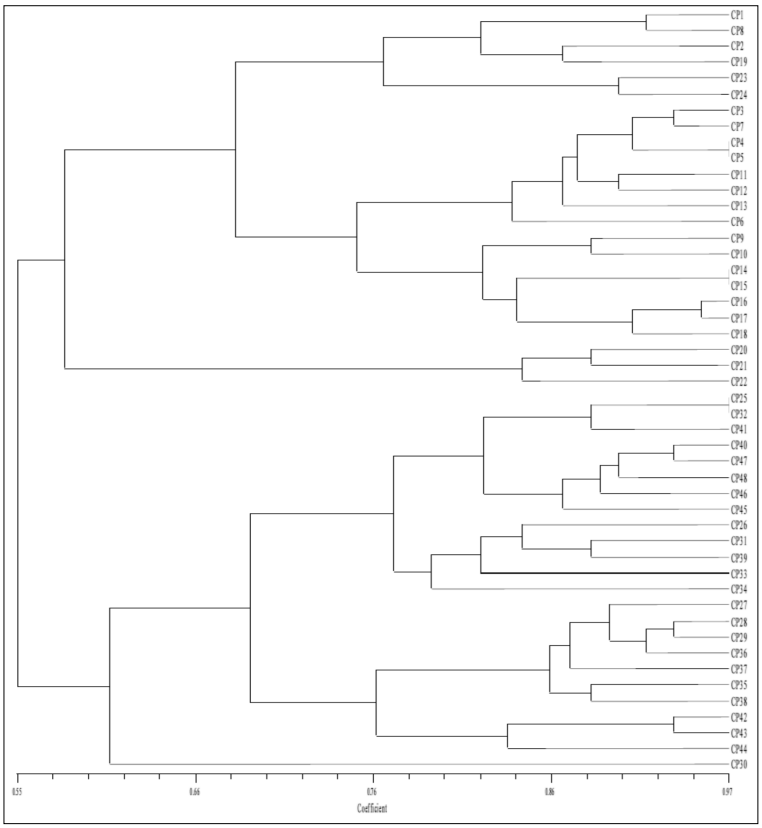


Fig 2: UPGMA based clustering of 48 cowpea genotypes using genetic distance matrix for ISSR markers.

cluster C (1 genotype) and D (23 genotypes). Sub cluster C have only one genotype RCC66. Sub cluster D included two cluster d' and d''. Cluster d' contained the 10 genotypes and the cluster d'' included 13 genotype. Cluster I (24 genotypes) further divided into two sub cluster A (3 genotypes) and B (21 genotypes). The sub cluster A classified into two cluster a' and a''. Cluster a' included only one genotype RCC51 and cluster a'' have two genotype RCC47, RCC49. Sub cluster B also further divided into two cluster b' and b''. The cluster b' containing 15 genotypes, whereas b'' containing 6 genotypes MFC-09-3, BL1, BL2, RCC40, RCC47 and RCC40. Highest genetic distance between genotype MFC-8-14 and TSFC-12-15 and the least distance UPC-5286 with MFC-09-3, RCC7 with RCC8 and RCC61 with RCC68 (0.97) In general, the genetic similarity between the majorities of the genotypes analyzed was quite high. The similarity coefficient analysis was performed using UPGMA coefficient (NT SYSPC 2014, software) based on 9 RAPD markers ranged from 0.58 to 1.00 which indicates there was high variability among the cowpea cultivar under study. Similar results also reported by Raza *et al.* (2019) and Giachino (2019) for many studies, including population differentiation and fingerprinting. To determine the genetic similarity and dissimilarity between all the genotypes, the dendogram divided the cowpea genotypes into two clusters. Similarity coefficients for the cowpea genotypes based on 1215 bands of ISSR markers ranged between 0.55 and 0.97. Similar results also reported by Vyas *et al.* (2016); Diallo *et al.* (2024); Saxena and Rukam (2020). The mean similarity index was 0.729. Comparative analysis of RAPD and ISSR markers was done. RAPD and ISSR primers amplified an average of 131.11 and 151.88 fragments, respectively (Plate 1-8). The range of the similarity coefficient was 0.58 to 0.94. The combined cluster of ISSR and RAPD was 78-83% similar to each of the individual cluster analyses of ISSR or RAPD.

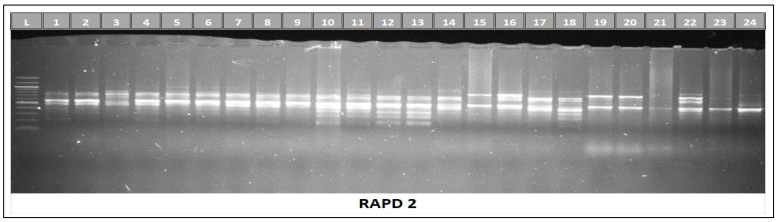


Plate 1: Polymorphic RAPD banding pattern for the locus RAPD 2, L=Molecular size ladder 100 bp (CP1 to CP24).

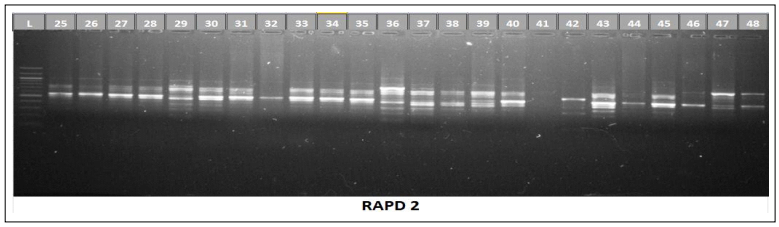


Plate 2: Polymorphic RAPD banding pattern for the locus RAPD 2, L=Molecular size ladder 100 bp. (CP25 to CP48).

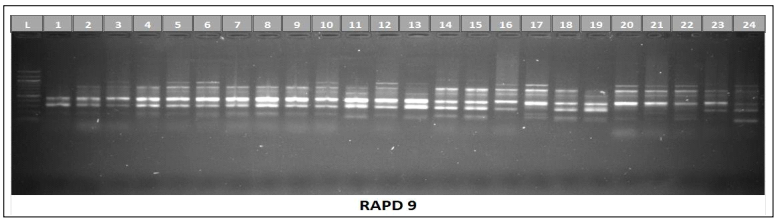


Plate 3: Polymorphic RAPD banding pattern for the locus RAPD 9, L=Molecular size ladder 100 bp (CP1 to CP24).

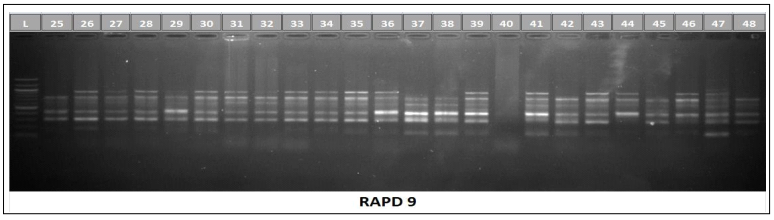


Plate 4: Polymorphic RAPD banding pattern for the locus RAPD 9, L= Molecular size ladder 100 bp (CP25 to CP48).

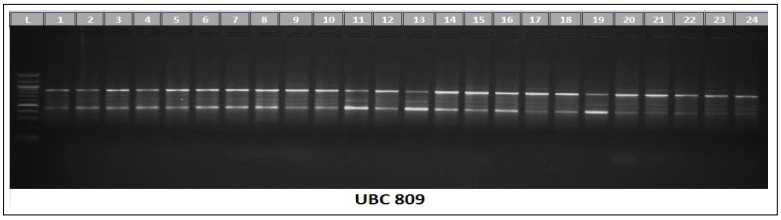


Plate 5: Polymorphic ISSR banding pattern for the locus UBC 809, L=Molecular size ladder 100 bp (CP1 to CP24).

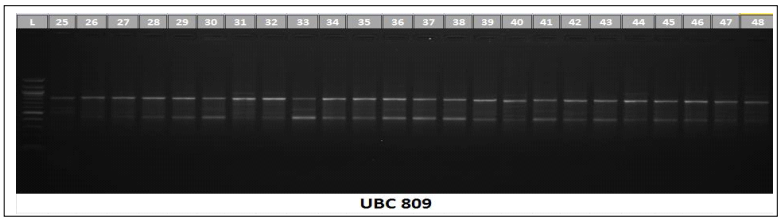


Plate 6: Polymorphic ISSR banding pattern for the locus UBC 809, L=Molecular size ladder 100 bp (CP25 to CP48).

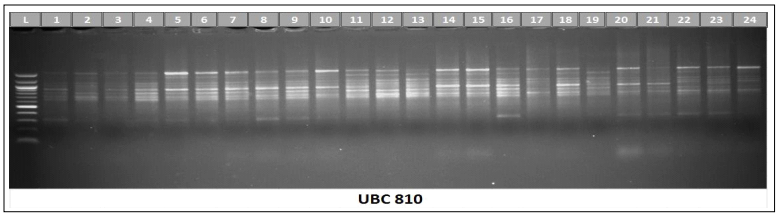


Plate 7: Polymorphic ISSR banding pattern for the locus UBC 810, L=Molecular size ladder 100 bp (CP1 to CP24).

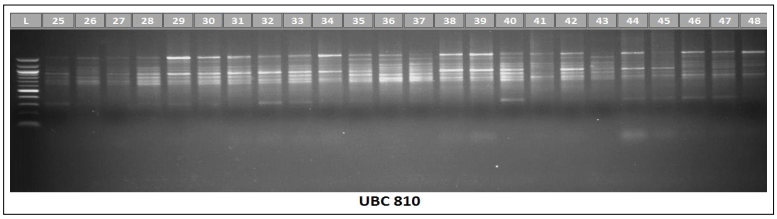


Plate 8: Polymorphic ISSR banding pattern for the locus UBC 810, L=Molecular size ladder 100 bp (CP25 to CP 48).

Similar findings also reported by Moolendra *et al.* (2018); Ba *et al.* (2004); Gajera *et al.* (2014) and Sharma *et al.* (2014) and Khare *et al. (*2024).

CONCLUSION

The likelihood that polymorphism would occur between two randomly chosen genotypes at a given locus is determined by using the PIC value as a measure of variability at that locus. Cowpea genotypes were divided into two clusters by the dendogram and the combined cluster of RAPD and ISSR was 78-83% similar to each of the individual cluster analyses (RAPD or ISSR). The estimated PIC value ranged from 0.27 for primer RAPD 1 to 0.41 for RAPD 4 with an average of 0.35. Whereas for ISSR primers PIC ranged from 0.23 for primer UBC 815 to 0.42 for UBC 835 with average of 0.35. The number of scorable markers produced per primer ranged from 41 to 226 with an average of 131.11 bands per primer. Eight ISSR markers produced a total of 1215 scorable bands among the 48 genotypes. The number of scorable markers produced per primer ranged from 57 to 330 with an average of 151.88 bands per primer. Based on the current findings, it is clear that the cowpea accessions exhibit a high degree of genetic diversity. Therefor it will be helpful in pre-breeding for generating variability for selection of superior lines.

Based on the overall results, both RAPD and ISSR markers were found to be effective in detecting genetic polymorphism among the cowpea genotypes. However, ISSR markers generated a higher average number of scorable bands and exhibited slightly higher polymorphism levels and similarity resolution compared to RAPD markers. The relatively higher PIC values and greater number of amplified fragments obtained with ISSR primers indicate that ISSR markers are comparatively more informative and reliable for assessing genetic diversity in cowpea. Identification of greater genetic distance and higher levels of polymorphism among genotypes can significantly facilitate effective hybridization. Utilization of genetically

diverse parents in breeding programs enhances the chances of obtaining superior recombinants and broadens the genetic base, thereby contributing to the development of improved cowpea varieties.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of other research institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript. This study did not involve any experiments on humans or animals. Therefore, ethical approval and informed consent were not required for the present research work.

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