Egypt. J. Agron. **Vol. 47,** No. 4, pp: 835 - 840 (2025)

Egyptian Journal of Agronomy

http://agro.journals.ekb.eg/

Molecular Characterization of Pigeon Pea (*Cajanus cajan* (L.) Millsp.) by RAPD and ISSR Markers

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PIGEON PEA (*Cajanus cajan* [L.] Millsp.) plant is an important legume crop. The study aimed to molecular characterization of *C. cajan* [L.] Millsp plant (leaf and seed) by RAPD and ISSR loci as marker-assisted selection (MAS) in the plant breeding programs. The five out of ten RAPD markers gave 23 amplicons, an average of 4.6 per primer. Primer RAPD-C1 gave the most number of amplified fragments (seven bands). In contrast, primer RAPD-B9 displayed the least number of PCR products (two bands). For seed, the seven out of ten RAPD primers that yielded 26 PCR products ranged from 220 bp (primer RAPD-A13) to 1360 bp (primers RAPD-A5 and RAPD-B4), with a mean of 3.71 per primer. Both primers RAPD-A5 and RAPD-B4 showed the greatest number of fragments (five). On the contrary, primer RAPD-C17 showed the smallest number of fragments (two). According to ISSR analysis from leaf, five out of eight ISSR markers gave 23 products with an average of 4.6 per primer. Both primers ISSR HB-12 and ISSR HB-14 appeared the highest number of fragments (six). Contrarily, primer HB-11 showed the lowest number of amplicons (two). For pigeon pea seed, five out of eight ISSR markers scored 20 amplified fragments, with a mean of four per primer. Primer ISSR-89B showed the maximum number of bands (six). In contrast, both primers ISSR-14A and ISSR-44B, revealed the minimum number of fragments (three). Therefore, RAPD and ISSR loci are considered diagnostic markers for the pigeon pea plant. They could be applied in the breeding programs for the quick identification of plants and hybrids during hybridization techniques and for protection of breeder's rights.

Keywords: DNA-based markers, molecular markers, pigeon pea.

Introduction

Pigeon pea (*Cajanus cajan* [L.] Millsp.) (2n = 22) is one of the most important legume crop extensively and it is grown in Africa and Asia in different environments (Mligo et al., 2001; Sarkar et al., 2020). It belongs to the family *Leguminosae*. Pigeon pea has the capability to grow under low humidity content, making it a significant yield in arid regions. It contains several essential nutrients, e.g., vitamins A, B, and C, 14-30% protein, 57-59% carbohydrate, and 1-9% fat per 100 gram/seed (Ojo et al., 2023). All plant parts (leaf, seed, root, stem, and flower) are used for the medical purposes (Abebe et al., 2022) (Fig. 1). In addition, its capability for fixation of N2 from air and formation of root nodules that contain rhizobium, which improves soil fertility, and for reforestation particularly in dry regions (Fernandes Júnior et al., 2012). Climate change has become a major challenge affecting agricultural practices. Therefore, developing crop varieties with enhanced tolerance to these conditions is essential. So, C. cajan has many features compared with other leguminous plants, like being tolerant to drought, suitable for all types of soil, resistant to fall, and the pod has not cracked easily. Besides, pigeon pea can live in arid regions because it can acclimate to the long rooting system (Cook et al., 2005). A wide range of genetic markers has been developed and applied to investigate the genotypic diversity of plant germplasm, including pigeon pea. The use of DNA-based marker techniques in plant breeding programs can highly ease the movement of genes through genotypes and analysis of polygenic properties (Malik et al., 2000; Abdel Sattar et al., 2024). Random amplified polymorphic DNA (RAPD) and Inter simple sequence repeats (ISSR) loci have high possibility of finding molecular markers for plant breeding programs. RAPD is one of the molecular methods for using specific markers to determine genetic variability. This method includes a single primary link with random nucleotide segments (Williams et al., 1990; Rehan and Kamara 2016; Coskun and Gulsen 2024). ISSR markers use anchored primers to amplify simple sequence repeats without the need for sequence data (Tikendra et al., 2019; Ramadan et al., 2025). This marker is more trusted compared with the RAPD marker and produces greater number of polymorphism (Qian et al., 2001). ISSR markers could be successfully applied to estimate genetic variability and cluster analysis of different crops (Souframanien and Gopalakrishna, 2004). Some troubles can produce when extraction of DNA from leaves due to the

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Received: 06/11/2024; Accepted: 02/9/2025
DOI: 10.21608/agro.2025.333401.1555

presence of pigments, phenols, and secondary metabolites of plants. So, we have to search alternative methods, e.g., seed, stem, flower, and root to perform the first step of molecular marker studies. Therefore, this study aimed to molecular identification of *C. cajan* [L.] Millsp plant (leaf and seed) by RAPD and ISSR loci as marker-assisted selection (MAS) in the plant breeding programs to enable the rapid identification of desirable genetic traits in plants during the breeding process.

Materials and Methods

Plant materials

The plant materials used in this study is a group of plants collected from Alnobaria region, El Beheira, Egypt during May 2022. The plants were identified by Dr. Mohamed Al-Gebaly. It was necessary to cultivate these plants to know their morphological and crop characteristics to increase the value of scientific and applied research for plant breeders, as you mentioned in this paper.

Isolation of DNA

Total DNA was isolated from leaf and seed (100 mg) of *C. cajan* [L.] Millsp plant as described by Dellaporta et al. (1983).

RAPD and ISSR assays

The ten RAPD and eight ISSR primers were used to characterize pigeon pea plant **(Tables 1 and 2).** The amplification reaction was carried out in a DNA thermocycler (Biometra, Germany) according to the protocol described by Williams *et al*. (1990) and Zietkiewicz et al. (1994). The PCR amplification was performed in a 25 µl reaction volume containing the following: 2.5 µl of dNTPs (2.5 mM), 1.5 µl of $MgCl_2$ (25 mM), 2.5 µl of 10x buffer, 2.0 µl of primer (2.5 µM), 2.0 µl of template DNA (50 ng/µl), 0.3 µl of *Taq* polymerase (5 U/µl) and 14.7 µl of sterile ddH_2O. The reaction of RAPD was subjected to one cycle at 95°C for 5 minutes, followed by 35 cycles at 94°C for 30 seconds, 37°C for 30 seconds, and 72°C for 30 seconds, then a final cycle of 72°C for 12 min. For ISSR, reactions were performed with the following temperature profiles: an initial denaturation step of 94°C/4 min followed by 40 cycles consisting of a denaturation step of 94°C/30 s, a primer annealing step of 52°C/45 s, and an extension step of 72°C/2 min. The last cycle was followed by 72°C/7 min for final extension.

Gel electrophoresis

The amplified DNA (15 µl) for all samples was electrophoresed on 1% agarose containing ethidium bromide. (0.5 µg/ml) in 1X TBE buffer at 75 constant volt, and determine with a UV transilluminator. The size of each fragment was estimated with reference to a size marker of 100 bp DNA ladder (BioRoN, Germany).

Results

RAPD-PCR

DNA was extracted from the leaf of *C. cajan* (L.) Millsp plant. The DNA concentration was more reliable for analysis by RAPD-PCR using ten random decamer primers. Five out of ten RAPD primers gave 23 DNA products with different molecular sizes, ranging from 240 bp (primer RAPD-E15) to 1130 bp (primer RAPD-C9), an average of 4.6 each primer (**Figs. 2 and 3**). Primer RAPD-C1 gave the most number of amplified amplicons (seven bands of 300; 350; 400; 470; 700; 730, and 980) bp, followed by primers RAPD-C9and RAPD-E15 (five bands) with molecular sizes of (350; 400; 450; 680; and 1130) bp, (240; 300; 600; 700; and 800) bp, respectively. In addition, primer RAPD-C13 scored four bands of 300; 350; 450; and 700 bp. In contrast, primer RAPD-B9 displayed the least number of PCR products (two amplicons of 530 and 1000 bp). For seed, the seven out of ten RAPD primers that yielded 26 PCR products ranged from 220 bp (primer RAPD-A13) to 1360 bp (primers RAPD-A5 and RAPD-B4), with a mean of 3.71 per primer. Both primers RAPD-A5 and RAPD-B4 showed the greatest number of amplicons (five) of (300; 460; 640; 1000; and 1360) bp and (300; 460; 640; 1000; and 1360) bp, respectively. Followed by primer RAPD-B9 and primer RAPD-C5 (four bands) of (350; 450; 620; and 700) bp, (300; 500; 600; and 820) bp, respectively. Furthermore, primers RAPD-A13 and RAPD-E15 scored three amplicons of (220; 300; and 460) bp and (300; 450; and 620) bp, respectively. Finally, primer RAPD-C17 showed the smallest number of bands (two) of (300 and 400) bp (**Figs. 2 and 3**).

Fig. 1. Shows the morphological shape of leaves and flowers (a) and mature pods (b) of pigeon pea plant (https://tropicalforages.info/text/entities/cajanus_cajan.htm).

Table 2. The ISSR primers used in the study.

No	Name	Sequence	No	Name	Sequence
1	HB-08	5` GAGAGAGAGAGAGG 3`	6	14A	5`CTC TCT CTC TCT CTC TTG 3`
2	HB-09	5′ GTGTGTGTGTGTGC 3`	7	44B	5` CTC TCT CTC TCT CTC TGC 3`
3	HB-11	5` GTGTGTGTGTGTTGTCC3`	8	89B	5` CAC ACA CAC ACA GT 3`
4	HB- 12	5′CACCACCACGC 3`			
5	HB-14	5′ CTCCTCCTCGC 3`			

Table 1. The RAPD primers used in this study.

NO.	Name	Sequence	No.	Name	Sequence
1	OP-A05	5′ AGGGGTCTTG 3`	6	OP-B09	5` CTCACCGTCC 3`
2	OP-A13	5′ CAGCACCCAC 3`	7	OP-C01	5` TTCGAGCCAG 3`
3	OP-B04	5′ GATGACCGCC 3`	8	OP-C09	5′ CTCACCGTCC 3`
4	OP-C05	5`GATGACCGCC 3`	9	OP-C13	5` AAGCCTCGTC 3`
5	OP-C17	5` TTCCCCCCAG 3`	10	OP-E15	5` TCGGCG ATAG 3`

ISSR-PCR

The genetic identification of the pigeon pea plant was determined by the isolation of DNA from the leaf, and then amplification of DNA by eight ISSR-PCR primers. The five ISSR markers out of eight recorded 23 products ranged from 300 bp (primer ISSR HB11) to 900 bp (primer ISSR HB-14), with an average 4.6 per primer (**Figs. 4 and 5**). Both primers ISSR HB-12 and ISSR HB-14 appeared to have the highest number of amplicons (six) of (400; 450; 540; 640; 700; and 800) bp and (440; 480; 640; 700; 860; and 900) bp, respectively. Followed by, primer ISSR HB-9 displayed five PCR products with molecular sizes of (330; 520; 700; 800; and 860) bp. Besides, primer ISSR HB-8 gave four amplicons of (430; 500; 640; and 800 bp). Contrarily, primer HB-11 showed the lowest number of bands (two) of (300 and 680) bp (**Figs. 4 and 5**). For pigeon pea seed, five out of eight ISSR markers scored 20 amplified bands, ranging from 240 bp (primer ISSR-89B) to 860 bp (primer ISSR HB-12), with a mean of four per primer. Primer ISSR-89B showed the maximum number of amplicons (six) with molecular sizes of (240; 280; 300; 370; 450; and 500) bp. Followed by, primers ISSR HB-9 and ISSR HB-12 gave four bands of (450; 500; 700; and 830 bp) and (350; 400; 500; and 860 bp). In contrast, both primers ISSR-14A and ISSR-44B revealed the minimum number of bands (three) of (400; 450; and 530 bp) and (300; 370; and 450 bp), respectively (**Figs. 4 and 5**).

Discussion

Assessing the genetic diversity of a crop species is an essential prerequisite for its genetic improvement. In pigeon pea, several studies have investigated diversity using different approaches, including morphological variation, biochemical profiling, and molecular marker analyses. Although morphological traits can be influenced by environmental conditions, morphological characterization remains the fundamental step in germplasm description and classification (Upadhyaya et al., 2009; Fiacre et al., 2018).

Extraction of genomic DNA is considered an important step in molecular marker studies. Thus, some plants contain the cell wall, phenolic compounds, tannins, pigments, polysaccharides, and secondary compounds. These compounds make isolation of DNA more difficult. Therefore, many studies were conducted to the isolate genomic DNA from different parts of plants such as seed, flower, fruit, and root (Yin et al., 2011; Karaaslan et al., 2014). In this study, extraction of genomic DNA from leaf and seed of *C. cajan* (L.) Millsp. plant was estimated as described by Dellaporta et al. (1983). This method was suitable for RAPD and ISSR assays.

In the current investigation, five out of ten RAPD primers gave 23 DNA products with different molecular sizes, ranging from 240 bp (primer RAPD-E15) to 1130 bp (primer RAPD-C9), an average of 4.6 each primer. Primer RAPD-C1 gave the most number of amplified fragments (seven), followed by primer RAPD-C9 and primer RAPD-E15 (five). Contrarily, primer RAPD-B9 displayed the least number of PCR products (two). For seed, the seven out of ten RAPD primers gave 26 PCR products ranged from 220 bp (primer RAPD-A13) to 1360 bp (primers RAPD-A5 and RAPD-B4), with a mean of 3.71 per primer. Both primers RAPD-A5 and RAPD-B4 displayed the greatest number of amplicons (five). In contrast, primer RAPD-C17 showed the smallest number of bands (two). Welsh and McClelland, (1990) and Arif and Khan, (2009) mentioned that the RAPD-PCR

technique is rapid, less time-consuming, less expensive, simple, and no need for genetic data about the plant. In addition, sequence-based analyses usually do not succeed in discrimination between species because of the significant identity between their DNA sequences in the amplified segment.

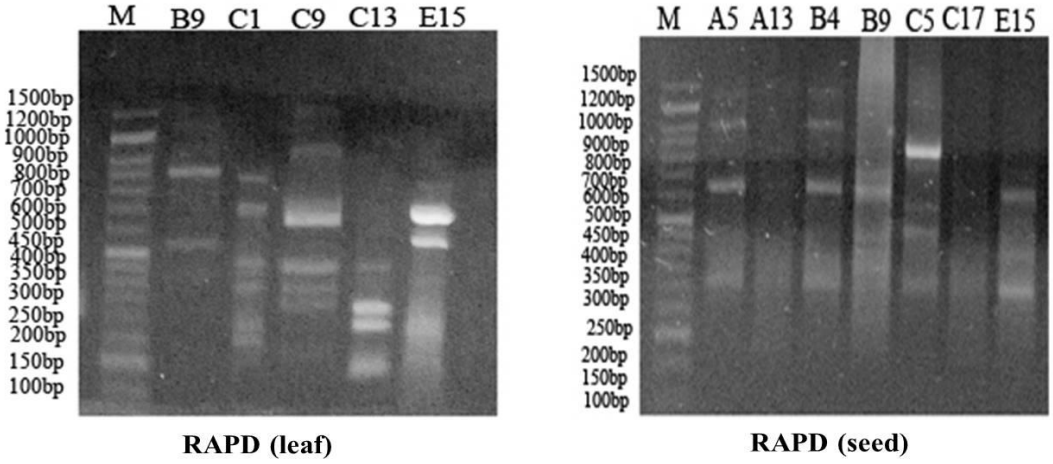

Fig. 1. Molecular characterization of leaf and seed from *C. cajan* (L.) Millsp plant using five (B9, C1, C9, C13, and E15) and seven (A5, A13, B4, B9, C5, C17, and E15) RAPD markers, respectively. Lane M: 100 bp DNA ladder.

No.	MS (bp)	RAPD markers (leaf)					RAPD markers (seed)						
		B9	C1	C9	C13	E15	A5	A13	B4	B9	C5	C17	E15
1	1360												
2	1130												
3	1000												
4	980												
5	820												
6	800												
7	730												
8	700												
9	680												
10	640												
11	620												
12	600												
13	530												
14	500												
15	470												
16	460												
17	450												
18	400												
19	350												
20	300												
21	240												
22	200												

Bands appeared only in leaf Bands appeared only in seed Common bands MS= Molecular sizes

Fig. 2. Heat map representation of RAPD-PCR banding patterns in leaf and seed of pigeon pea plant.

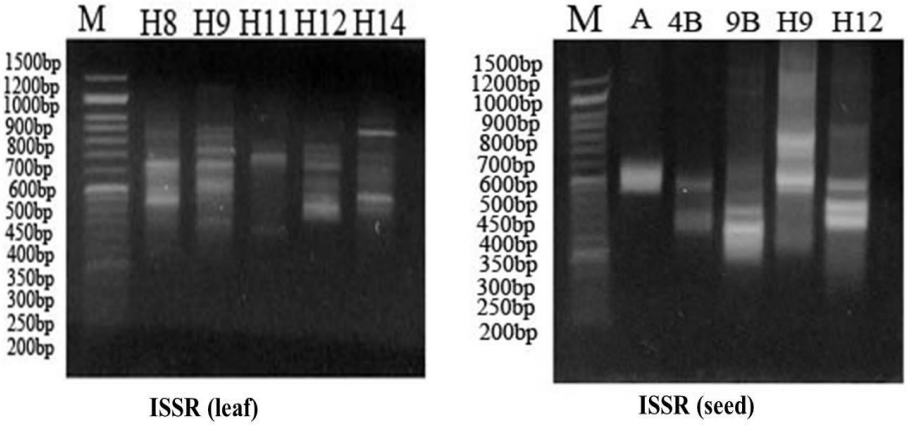

Fig. 3. Molecular characterization of leaf and seed from *C. cajan* (L.) Millsp plant using five (H8, H9, H11, H12, and H14) and (A, 4B, 9B, H9, and H12) ISSR markers, respectively. Lane M: 100 bp DNA ladder.

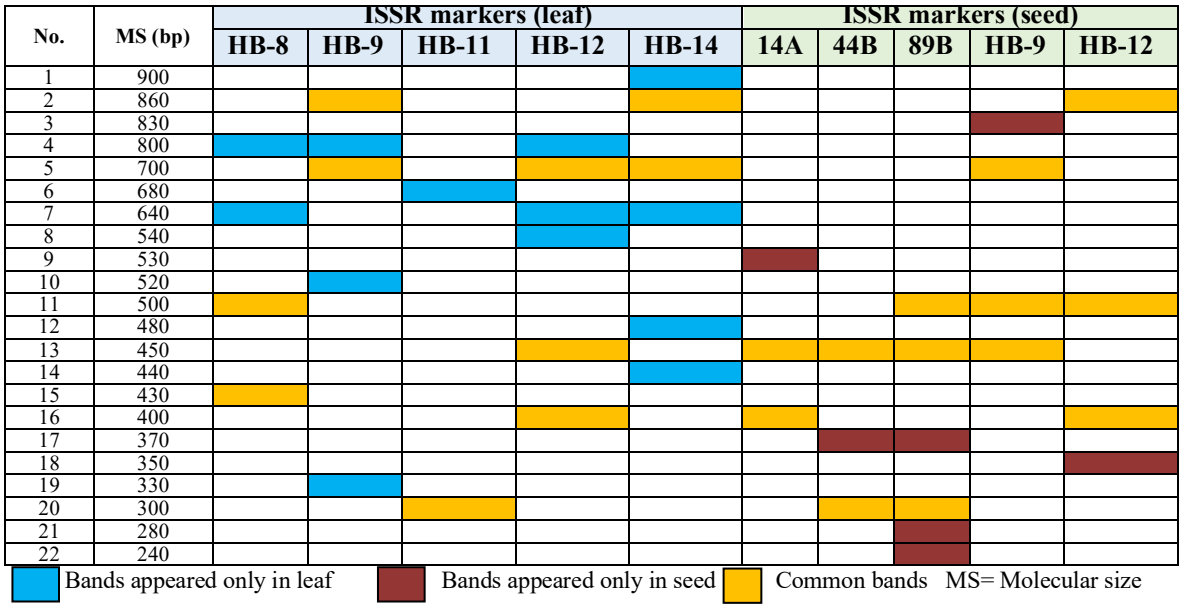

Fig. 4. Heat map representation of ISSR-PCR banding patterns in leaf and seed of pigeon pea plant.

Besides, RAPD technique linked to both coding and non-coding sequences of the genome (Vanijajiva et al., 2005). But some of the troubles with RAPD-PCR are linked to primer design, PCR conditions, and reproducibility which, is considered important factors for observing amplicons (Jones et al., 1997). It is found that if the annealing temperature inside the PCR eppendorf is similar, RAPD bands are then possibly to be reproducible (Skroch and Nienhuis, 1995).

In this work, the five ISSR loci out of eight scored 23 products, ranging from 300 bp (primer ISSR HB-11) to 900 bp (primer ISSR HB-14), with an average 4.6 per primer. Both primers ISSR HB-12 and ISSR HB-14 revealed the highest number of amplicons (six). In contrast, primer HB-11 showed the lowest number of bands (two). For pigeon pea seed, five out of ten ISSR markers scored 20 amplified fragments, ranging from 240 bp (primer ISSR-89B) to 860 bp (primer ISSR HB-12), with a mean of four per primer. Primer ISSR-89B showed the maximum number of amplicons (six). In contrast, both primers ISSR-14A and ISSR-44B exhibited the minimum number of fragments (three). ISSR assays are used in the plant genetics due to their cost-effectiveness and need less DNA quantity. In addition, ISSR assays applied in molecular characterization of plant species from trace fragments, e.g., leaf, stem, flower, root, and seed (Hawkins et al., 2015). ISSR marker is easy to use and more reproducible compared with other markers (Gonzalez et al., 2000). ISSR is nucleotide repeats is distributed through the genome and has distinctive power, which seen in the plant for the determination of genetic variability between populations, plant species, and varieties (Yuan et al., 2015).

Conclusions

Plant tissue was observed as an important source for the extraction of genomic DNA. In this study, molecular identification of *C. cajan* (L.) Millsp plant was carried out by extraction of DNA from leaf and seed of pigeon pea plant, then amplification by RAPD and ISSR loci. The five out of ten RAPD markers gave 23 amplicons of leaf, and 26 amplified fragments of seed. However, ISSR loci scored 23 bands of leaf, and 20 amplicons of seed. The diagnostic markers for plant species could be applied for the accurate and fast characterization of plant species and selection of the hybrids and hybridization programs of pigeon pea. This is important in the breeding techniques and for the protection of breeder's rights.

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