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Molecular diversity analysis of pigeonpea genotypes for scar marker development

Kanchan Kumari, Thamilarasi K, J Ghosh, Vaibhav D Lohot and S Ghosal

Kanchan Kumari
Lac Production Division, ICAR-Indian Institute of Natural Resins and Gums, Ranchi, Jharkhand, India

Thamilarasi K
Lac Production Division, ICAR-Indian Institute of Natural Resins and Gums, Ranchi, Jharkhand, India

J Ghosh
Lac Production Division, ICAR-Indian Institute of Natural Resins and Gums, Ranchi, Jharkhand, India

Vaibhav D Lohot
Lac Production Division, ICAR-Indian Institute of Natural Resins and Gums, Ranchi, Jharkhand, India

S Ghosal
Lac Production Division, ICAR-Indian Institute of Natural Resins and Gums, Ranchi, Jharkhand, India

Corresponding Author:
Kanchan Kumari
Lac Production Division, ICAR-Indian Institute of Natural Resins and Gums, Ranchi, Jharkhand, India

Abstract
Pigeonpea, *Cajanus cajan* (L.) Millsp. is one of the good host plants for lac cultivation in North East India, besides being an important pulse crop. The correct identification of genotype is highly required to reap the benefits of the given pigeonpea genotype. This study was conducted in ten pigeonpea genotypes known for either grain yielding or lac yielding properties and collected from different regions of the country. The genotypes were analyzed with 20 RAPD (Random Amplified Polymorphic DNA) primers. The unweighted pair group method with arithmetic mean dendrogram constructed based on Nei's genetic distance clustered high lac yielding and high grain yielding genotypes to certain extent. Few RAPD locus, specific for certain genotypes, especially the high lac yielder, Assam local 1 were obtained. Those loci have the potential to be generated as diagnostic SCAR (Sequence Characterized Amplified Regions) markers that could be accurate and reliable marker for identification. The conversion of dominant and random RAPD markers to codominant and specific SCAR marker offers a simple and reliable tool for the genetic identification of genotypes. Molecular markers identified from these approaches that are associated with desired traits would be helpful for the selection of elite germplasm of pigeonpea for lac cultivation and high yield of pulses.

Keywords: *Cajanus cajan*, RAPD, SCAR, lac insects, genetic diversity

Introduction
Pulses are the important source of protein where intake of animal protein is less [1]. Among all pulses, pigeonpea (*Cajanus cajan* (L.) Millspaugh) is an important grain legume (family-Fabaceae) crop grown in tropical and subtropical regions of the world either as a sole crop or intermixed with cereals. Pigeonpea is an erect perennial legume bushy shrub often grown as an annual, having semi hard stem and resistant to drought and frost and endure pruning response. Glover reported that pigeonpea is an important lac host-plant in Assam, North-East India [2]. It was also reported as lac host in China, Vietnam, and Thailand [3]. Earlier, Sharma and co-workers reported that this host prefers *Kerria chinensis* species of lac insect [4]. Later on it was established that *rangeeni* strain of *Kerria lacca* (Kerr) completed its life cycle on pigeonpea in Jharkhand [5-7].

The products of lac insects, viz., resin, pigment and wax, have immense economic importance in food [8], pharmaceutical and perfume industries. Nowadays production of more resin has become a necessity due to its demand. Farmers can earn additional income from lac culture besides grain from pigeon pea. Screening of genotypes for a particular trait (high grain yield and lac yield) by traditional methods is time-consuming and relatively laborious process. The use of molecular markers in breeding programs has been proved as an efficient tool in this regard.

Among all PCR based markers, RAPD (Random Amplified Polymorphic DNA) method is frequently used in genetic diversity detection because of its simplicity, low cost and lower infrastructure requirements [9-10]. Reproducibility of this marker is low but it can be conquered by converting this marker into codominant sequence characterized amplified region (SCAR) markers. In this process, RAPD generated profile can be used to identify polymorphism, and once a set of amplified regions have been secured, SCAR markers can be developed to produce reliable unique PCR-based results from the polymorphic RAPD markers [11]. SCAR markers generally reveal higher levels of polymorphism owing to higher annealing temperatures and longer primer sequence specificity. With a SCAR marker, analysis is reduced to a simple PCR analysis using PCR primers designed from the sequence of the amplicon of RAPD fragments [12].

This methodology has been already used in pigeon pea for identification of *Fusarium* wilt resistance gene and sterility mosaic disease resistance gene and plume moth resistance gene [13-15]. Several other molecular markers such as RFLP, AFLP, microsatellite markers and Diversity Array Technology were also employed for genetic variability and phylogenetic studies in pigeonpea [16-19]. In this study, we used dominant RAPD markers to assess the genetic diversity among the released and trial pigeonpea genotypes which were collected from different parts of India and maintained at lac host gene bank of ICAR-IINRG, Ranchi.

Materials and Methods

Sample collection: Leaves of ten germplasm *viz*. Birsa Arhar 1 (c), Bahar (c), Assam local 1, Assam local 2, RCMP 2, RCMP 5, IPA 8-2, IPA 9-1, KA 9-2 and MAL 13 were collected from the lac host gene bank of ICAR-IINRG, Namkum, Ranchi, Jharkhand, India. Germplasm were distinctly different from each other in respect of their origin (Table 1). Selection of genotypes is based upon its capacity for high yield of grains and / or lac yield.

DNA isolation: Total genomic DNA was isolated from the young leaves using CTAB method with slight modification done to remove the interfering polyphenolic compounds and other polysaccharides [20]. DNA concentrations were measured by using UV spectrophotometer and then diluted with 1X TE buffer to a final concentration of 50 ng/µl. Quality checking of genomic DNA was done on a 0.8% agarose gel.

PCR amplification: The following protocol was adapted for amplification of the genomic DNA. Master mix included dNTPs (0.4 mM each), Taq Buffer (1X), $MgCl_2$ (3 mM), *Taq* DNA Polymerase (1U/reaction), forward primer and reverse primer (10 µM each), template DNA and the final volume was made to 20 µl using sterile MB grade water for each reaction. The primers used in the study for RAPD analysis were OPD series (1-20) obtained from Operon Technologies (Table 2). Amplification were carried out in a thermo-cycler (Applied Biosystem Veriti, CA, USA) with an initial denaturation at 94 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing (selected according to gradient PCR) for 30 sec and extension at 72 °C for 30 sec and final extension for 10 min at 72 °C. The amplicons were analyzed on 1.5% agarose gels. The amplicons were then visualized under UV light on a trans illuminator and documentation of the samples was done on Fluor Chem HD2 (Protein Simple).

Data analysis: Out of 20 random 10 mer oligonucleotide primers used in this study only ten primers amplified good and reproducible fragments with ten pigeonpea genotypes. PCR amplified products were scored as 1 (present) or 0 (absent). The binary data were subjected to statistical analyses using POPGENE software [21]. Analysis of molecular variance (AMOVA) was used to calculate variation among and within genotypes by using the program GenAlEx version 6.5 [22].

Resolving power (Rp) which shows the ability of the most informative primers to differentiate between the genotypes was assessed according to Prevost and Wilkinson, (1999) using: $Rp = \sum Ib$ where Ib is the band informativeness with $Ib= 1 - [2 \times (0.5-p)]$ and where p is the proportion of genotypes containing the band [23]. The resolving power is based on the distribution of detected bands within the sampled genotypes. Effective multiplex ratio (EMR) and marker index (MI) for both marker systems were calculated in order to measure the usefulness of the marker system according to Powell [24]. The multiplex ratio (MR) was estimated by dividing the total number of bands amplified by the total number of assays. The effective multiplex ratio (EMR) is the number of polymorphic fragments detected per assay. Marker index (MI) was calculated by multiplying the average heterozygosity (Hav) with EMR [24].

Results and Discussion

Though, in NCBI whole genome sequence of pigeonpea is available, the RAPD generated SCAR markers has great importance in the identification of a specific genotype which contains explicit characters such as pest and disease resistance. RAPD generated SCAR markers are the most suitable tools which could be used for its identification in short time in a single reaction. The fundamental prerequisite for marker assisted selection in a plant breeding programme is the identification of a linked marker and an efficient means for screening huge populations in a reproducible way. In previous work, number of tightly linked markers were identified in pigeonpea that would easily transfer desired genes to popular cultivars using marker-assisted breeding. The RAPD markers have been converted into a simple SCAR marker for large-scale application in marker-assisted breeding. The conversion of a linked marker to SCAR has been applied successfully in a number of crops, like common bean [25], rice [26], tomato [27] and soyabean [28].

Polymorphism and Marker Efficiency: Out of the twenty RAPD primers screened, ten primers produced unambiguous profiles for all ten genotypes. Hence, those ten RAPD primers were used for genetic diversity in selected genotypes of pigeonpea. The amplification profiles generated by 10 mer RAPD primers have been shown in Figure 1. Total 68 distinct bands/fragments were generated by the ten primers; out of which 39 bands were found to be polymorphic and 29 bands were monomorphic. Hence the total polymorphism percentage was found to be 58%, whereas Prajapati and Malviya obtained 65.42% and 80% polymorphism in pigeonpea, respectively [29-30]. Low level of polymorphism (indication of narrow genetic base) in cultivated germplasm was also reported by many pigeon pea workers [31]. Number of bands produced by primers ranged from 1 to 14 with an average of 6.8 bands per primer (Table 3). The molecular size of the amplified PCR products ranged from 182 bp (OPD10) to 3000 bp (OPD10 and OPD11). Two RAPD markers OPD10 and OPD18 produced maximum number of 10 bands, while OPD 13 produced only single band. Hundred percent

polymorphism was shown by the primer OPD20; OPD11 and OPD10 produced polymorphism of 92.85 and 80%, respectively. The RAPD marker OPD8 gave the lowest polymorphism i.e. 16%. Therefore, according to the results obtained, the primers used in this study were highly informative markers with the exception of OPD13 and OPD15.

Identification of genotypes specific bands from RAPD markers: Some genotypes specific RAPD bands were also identified, which were found in one genotype but absent in other genotypes. The reproducibility of the amplification profile was assured by repeating each reaction twice under the similar lab conditions. These primers (OPD10, OPD11 and OPD20) were selected for distinct genotypes specific markers development in pigeonpea (Table3). The RAPD primer OPD10 generated total ten bands. Among them five bands $OPD10_{3000,}$ $OPD10_{805}$, $OPD10_{671}$, $OPD10_{255}$ and $OPD10_{182}$ were specific for the single genotype Assam local 1. Another primer OPD11 produced maximum number of bands (14) in which only one band was monomorphic and rest of the bands were polymorphic. The percentage of polymorphism shown by OPD11 was also higher (92.85%). Among fourteen bands, four bands were unique for the genotype Assam local 1. These results showed that the genotype- Assam local 1 was genetically different from other genotypes. This germplasm showed its wild nature because number of loci was distinct with respect to other nine genotypes. The primer OPD20 showed its uniqueness for the genotype RCMP 2 (Figure 1). The loci $OPD20_{612}$, $OPD20_{450}$, $OPD20_{391}$ were generated by the RAPD primer OPD20. Among ten primers used in this study, OPD10, OPD11 and OPD20 can be used for the development of genotypes specific markers development. Further experiments are required for the identification of these genes which could be associated with some traits. This type of linkage of a marker to a gene can be used for indirect selection of traits.

Analysis of Molecular Variance (AMOVA) is a method of estimating population differentiation directly from molecular data. Analysis of molecular variance (AMOVA) was performed to assess the variation among and within the genotypes, using the genetic binary matrix generated by RAPD markers. The partitioning of the genetic variability among the ten genotypes was shown in Table 5. These genotypes were grouped according to their origin (in each group or/ population contain two genotypes), the genetic differentiation among the populations was 3%. Individuals within the groups or genotypes contributed 97% of total variability (Figure 2).

The resolving power (Rp), marker index (MI), average band in formativeness (AvIb) and Effective multiplex ratio of each polymorphic RAPD primer was presented in Table 6. Resolving power depended on the distribution of the alleles within genotypes. The Rp and MI values varied from 0.4 to 3.6 and from 0.12 to 10.07. The primer OPD7 showed highest Rp (3.6) and the primer OPD8 showed lowest Rp (0.4) values. Choudhury & co-workers also reported low maker index (5.027) in pigeonpea using RAPD analysis [32]. The effective multiplex ratios (EMR) analysis was found to range between 0.12 and 12.07, respectively. The primer OPD7 showed highest AvIb (0.45) while OPD8 showed lowest AvIb of 0.06. Comparative analyses in pigeonpea using RAPD and ISSR primers, Rani *et al*. (2015) showed that Rp value of 3.7 for ISSR markers and 3.3 for RAPD primers [33].

UPGMA (Unweighted Pair Group Method with Arithmetic Mean) dendrogram give us information about the genetic distances and evolution of particular genotype. According to the dendrogram generated on the basis of Nei's analysis (1973) all the genotypes were divided into two subgroups (Figure 3) [34]. The smaller subgroup comprised of single genotypes Assam local 1. The larger subgroup contained remaining nine genotypes Birsa Arhar 1, IPA 8-2, RCMP 5, MAL 13, IPA 9-1, KA 9-2, Bahar, Assam local 2 and RCMP 2. Maximum diversity exists between the two pairs of genotypes (RCMP 2 and Assam local 1) and (KA 9-2 and Assam local 1). And the genotype IPA 8-2 was genetically closer to Birsa Arhar 1 and RCMP 5 (Table 4). The most divergent plants could be further used for plant breeding and hybridization programme. Dendrogram also separated all the genotypes on the basis of lac and grain yield. Ranking [Grain Yield-(g)/ plant and Scraped lac/ plant (g)] of all genotypes were done according to Ghosh *et al*., 2014.

Four genotypes Bahar, Assam local 2, RCMP 2 and Assam local 1, the high lac yielders accumulated in one sub cluster and the high grain yielding genotypes, Birsa Arhar 1, IPA 8-2, MAL 13, IPA 9-1 and KA 9-2 with exception of RCMP 5 were accumulated in another sub cluster. The primers used in this study distinguished genotypes for grain yield and lac yield and separated diversified germplasm Assam local 1 from rest of the genotypes but it failed to distinguish them on geographical distance. Thus Assam local 1 may be used in further breeding programme for widening the genetic distance among grain yielding genotypes and selection of elite segregants for higher lac with the help of two RADP markers OPD10 and OPD11. Few potential loci were obtained which could be converted into SCAR for further characterization of the given germplasm. In summary, clear and reproducible banding patterns were obtained from these three RAPD markers (OPD10, OPD11 and OPD20), which allowed precise identification of certain genotypes used in the present study.

Table 1: Details of *Cajanus cajan* gemplasm used for genetic diversity analysis and field evaluation for grain and lac yield[6].

Germplasm	Origin	Adaptation	Maturity	Grain Yield-(g)/ plant	Rank in grain yield	Scraped lac / plant (g)	Rank in lac yield
1. Birsa Arhar 1 (c)	BAU, Ranchi	Jharkhand	Medium	20.6	6	16.1	8
2. Bahar (c)	Bihar	Bihar, UP, Jh	Late	21.0	5	23.1	3
3. Assam local 1	Assam	Assam	Late	14.5	10	24.8	2
4. Assam local 2	Assam	Assam	Late	20.5	7	28.6	1
5. RCMP 2	Manipur	Manipur	Medium	18.9	8	19.7	6
6. RCMP 5	Manipur	Manipur	Medium	16.6	9	21.5	4
7. IPA 8-2	IIPR Kanpur	NEPZ	Late	22.7	4	15.9	9
8. IPA 9-1	IIPR Kanpur	NEPZ	Late	27.0	2	20.9	5
9. KA 9-2	AICRP-PP	NEPZ	Late	36.6	1	18.4	7
10. MAL 13	BHU, Varanasi	UP, Bihar, Jha	Late	23.3	3	12.8	10

Table 2: Characteristics of primers used for studies

Sl. No.	Name of primer	Primer sequence (5' - 3')	Annealing Temperature (^{0}C)
1	OPD-5	5'-ACCGCGAAGC-3'	48
2	OPD-6	5'-ACCTGAACGG-3'	44
3	OPD-7	5'-TTGGCACGGG-3'	46
4	OPD-8	5'-GTGTGCCCCA-3'	44
5	OPD-10	5'-GGTCTACACC-3'	44
6	OPD-11	5'- AGCGCCATTG -3'	44
7	OPD-13	5'-GGGGTGACGA-3'	44
8	OPD-15	5'-CATCCGTGCT-3'	44
9	OPD-18	5'-GAGAGCCAAC-3'	44
10	OPD-20	5'-ACCCGGTCAC -3'	44

Table 3: Results obtained with RAPD primers

Sl. No.	Name of primer	No. of RAPD bands generated	No. of monomorphic bands	No. of polymorphic bands	Polymorphism (%)	Range of the amplified fragments (bp)	Unique loci	
							Genotypes	Size (bp)
1	OPD-5	5	4	1	20	1000-1600		
2	OPD-6	4	3	1	25	714-2350		
3	OPD-7	8	3	5	62.5	662-2000		
4	OPD-8	6	5	1	16.66	212-1081		
5	OPD-10	10	2	8	80	182-3000	Assam local 1 Assam local 1 Assam local 1 Assam local 1 Assam local 1	3000 805 671 255 182
6	OPD-11	14	1	13	92.85	194-3000	Assam local 1 Assam local 1 Assam local 1 Assam local 1	2830 1025 917 609
7	OPD-13	1	1	0	0	1100		
8	OPD-15	3	3	0	0	550-1400		
9	OPD-18	10	7	3	30	400-2000		
10	OPD-20	7	0	7	100	391-1500	RCMP 2 RCMP 2 RCMP 2	612 450 391
Total		68	29	39	58			

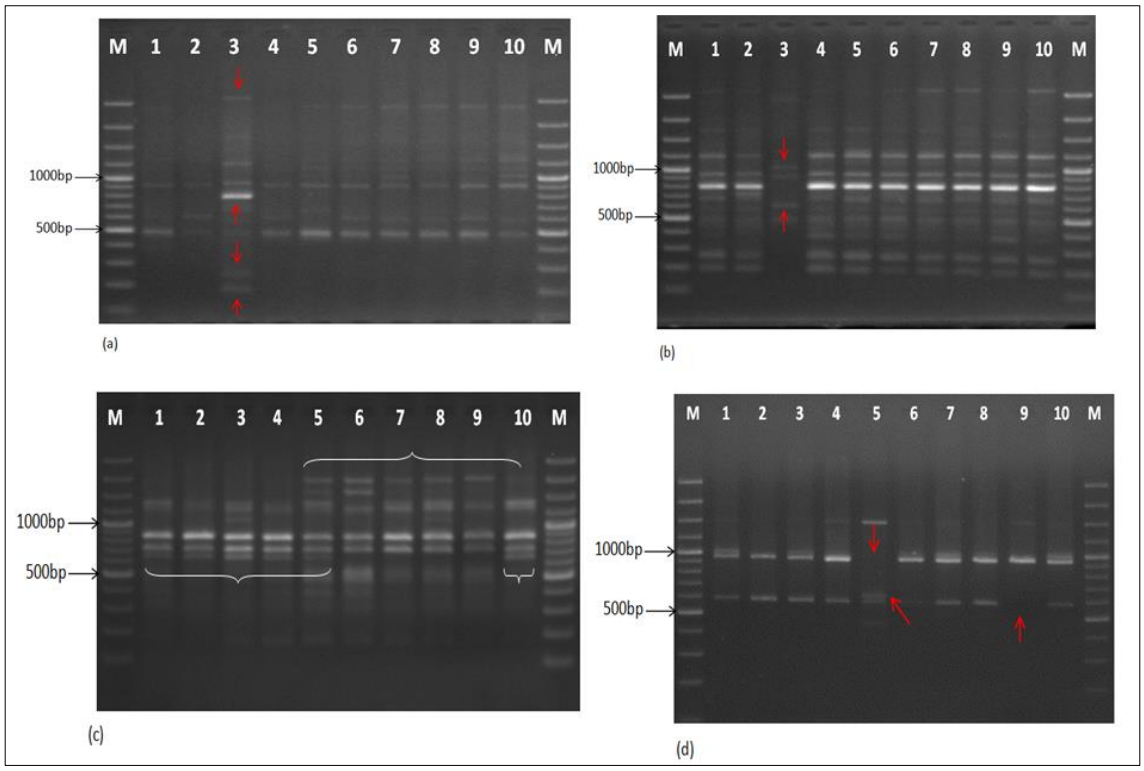

Fig 1(a, b, c, d): Amplification profile of 10 Pigeonpea genotypes with (a) OPD 10 (b) OPD 11 (c) OPD 7 (d) OPD 20 primers. Lane 1= Birsa Arhar 1, Lane 2= Bahar (C), Lane 3= Assam local 1, Lane 4= Assam local 2, Lane 5= RCMP 2, Lane 6= RCMP 5, Lane 7= IPA 8-2, Lane 8= IPA 9-1, Lane 9= KA 9-2, Lane 10= MAL 13, M= Molecular weight markers: 100 bp plus DNA Ladder (Thermo Scientific)

Table 4: Nei's genetic identity (above diagonal) and genetic distance (below diagonal) among the different genotypes of pigeonpea

Pop ID	BirsaArhar 1	Bahar (C)	Assam local 1	Assam local 2	RCMP 2	RCMP 5	IPA 8-2	IPA 9-1	KA 9-2	MAL 13
Birsa Arhar 1 ©	xxxx	0.8971	0.6324	0.9265	0.8382	0.9412	0.9559	0.8824	0.8676	0.9265
. Bahar ©	0.1086	xxxx	0.6471	0.9412	0.7647	0.8676	0.8824	0.8382	0.8235	0.8529
Assam local 1	0.4583	0.4353	xxxx	0.6471	0.5294	0.5735	0.6176	0.6029	0.5294	0.6471
Assam local 2	0.0764	0.0606	0.4353	xxxx	0.7941	0.8971	0.8824	0.8676	0.8529	0.8824
RCMP 2	0.1765	0.2683	0.6360	0.2305	xxxx	0.8382	0.8235	0.8088	0.8235	0.8235
RCMP 5	0.0606	0.1420	0.5559	0.1086	0.1765	xxxx	0.9559	0.9118	0.9265	0.8971
IPA 8-2	0.0451	0.1252	0.4818	0.1252	0.1942	0.0451	xxxx	0.8971	0.8824	0.9412
IPA 9-1	0.1252	0.1765	0.5059	0.1420	0.2122	0.0924	0.1086	xxxx	0.8971	0.8971
KA 9-2	0.1420	0.1942	0.6360	0.1591	0.1942	0.0764	0.1252	0.1086	xxxx	0.8529
MAL 13	0.0764	0.1591	0.4353	0.1252	0.1942	0.1086	0.0606	0.1086	0.1591	xxxx

Table 5: Summary of analysis of molecular variance (AMOVA)

Source	df	SS	MS	Est. Var.	Percentage
Among Pops	4	25.000	6.250	0.175	3%
Within Pops	5	29.500	5.900	5.900	97%
Total	9	54.500		6.075	100%

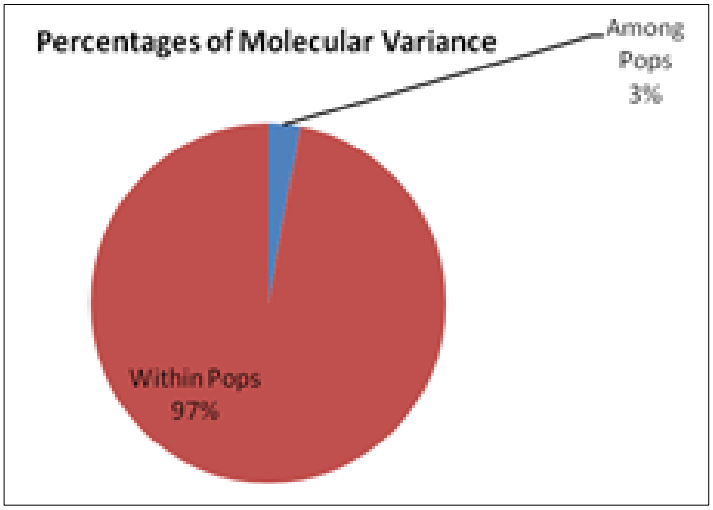

Fig 2: Summary of analysis of molecular variance (AMOVA) for the partitioning of RAPD variation among and within populations based on 68 bands.

Table 6: Resolving power (RP); Effective multiplex ratio (EMR); average band in formativeness (Ib aveg) and Marker index (MI) of RAPD primers among the Pigeonpea genotypes.

Sl. No.	Name of primer	R_P	EMR	MI	AvIb
1	OPD-5	0.8	0.20	0.15	0.16
2	OPD-6	0.4	0.25	0.17	0.1
3	OPD-7	3.6	3.12	2.66	0.45
4	OPD-8	0.4	0.16	0.13	0.06
5	OPD-10	2.2	6.40	5.22	0.22
6	OPD-11	3.4	12.07	10.92	0.24
7	OPD-13	0	0	0	0
8	OPD-15	0	0	0	0
9	OPD-18	1.6	0.9	0.80	0.16
10	OPD-20	2.6	7	5.53	0.37

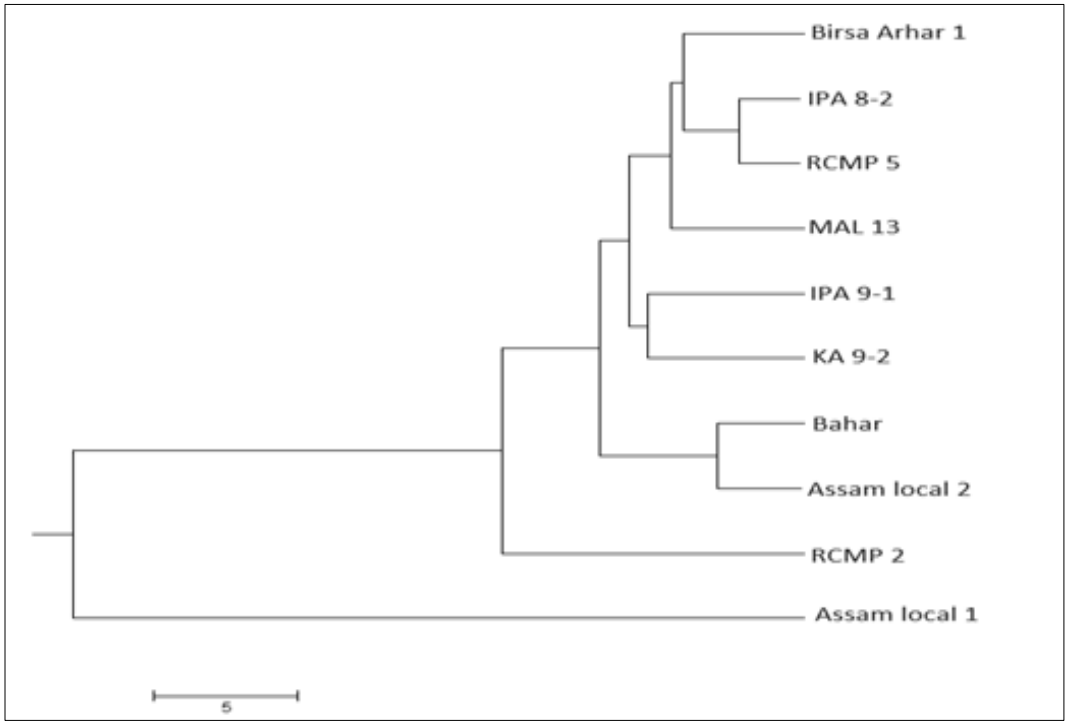

Fig 3: Dendrogram showing genetic relationship among various pigeonpea genotypes

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