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Detection of molecular divergence and development of DNA fingerprints in fieldpea cultivars

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Abstract

Studied the molecular divergence and develop DNA fingerprints in selected popular fieldpea cultivars from India. Those RAPD primers from the four sets (*viz*. OPP, OPBA, OPAQ and OPH) which showed at least 75 percent band polymorphism were selected for molecular diversity analysis. Twenty four primers generated a total of 256 amplified fragments out of which 228 (89.06%) were polymorphic. On an average, 10.67 bands were amplified per primer. Cluster analysis based upon DNA amplification polymorphism using Jaccard's similarity coefficient and UPGMA could unveil substantial amount of polymorphism among the cultivars. Genotype specific bands were represented in a diagrammatic form and can be used as a reference fingerprint. The arithmetic mean heterozygosity (H_{av}) value and marker index (MI) was found to be 0.592 and 6.317, respectively, indicating the efficiency and usefulness of RAPD as a marker system.

Key words: Fieldpea, molecular diversity, RAPD, genotype specific markers

Introduction

Fieldpea is the most widely grown cool season pulses in the world and has the highest average grain yield [1]. It is grown in over 25 million acres worldwide [2]. The third largest area in fieldpea cultivation is occupied by India after Canada and Russia [3]. It has a wide variety of uses from dry pulses to succulent fresh peas to edible podded types. Fieldpea is primarily used for human consumption and contains approximately 21-25 percent protein with high levels of amino acids (lysine and tryptophan), which are relatively low in cereal grains. Peas contain high carbohydrates, low fiber, 86-87 percent total digestible nutrients and 5 to 20 percent less trypsin inhibitors than soybean which makes them an excellent livestock feed. Fieldpea also is an excellent protein supplement in swine, cow, feeder calf, dairy and poultry rations. Peas are grown all over the Indian sub-continent and consumed mainly as green vegetable, pulses and flour. Being a leguminous crop, peas also have the capability of fixing nitrogen into the soil and thus improves nitrogen status of the soil for the succeeding crop in rotation.

Genetic diversity among the crop species in the intra or inter specific level is an inherent character contributed by evolutionary pathways. Studying the genetic polymorphism available in the gene pool in a meaningful and scientific way and its proper utilization is the base in crop improvement programme. Information on genetic diversity is also valuable for the management of collected germplasm and for their conservation strategies. Morphological markers, often fail to distinguish closely related individuals/cultivars for the masking effect of the environment. Therefore, DNA markers being independent of environmental interactions (i.e. highly heritable), unlimited in number and highly polymorphic, are considered to be the best tool for estimation of diversity or genetic relationship.

Among the various molecular markers available for estimation of genetic diversity (*viz*. RFLP, RAPD, SSR, AFLP etc.), RAPD, is a multi locus marker [4] and possess the simplest and fastest detection technology as well. It has been widely used for diversity analysis in several crop plants. In the present investigation,

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incorporation of widely adapted fieldpea cultivars in India originated from diversified pedigree and geographical distribution has been made for the purpose of molecular diversity analysis using highly polymorphic RAPD primers. In this context, the efficiency of the RAPD marker system to identify the informative cultivars has also been judged.

Materials and methods

Twenty four widely adapted, high yielding, morphologically diverse and popular cultivars of fieldpea grown in different agro climatic zones of India were collected from the core collections maintained at Indian Institute of Pulses Research, Kanpur, India and selected for molecular diversity studies (Table 1).

Leaf samples of each cultivar were collected from young seedlings grown from breeders seeds in the experimental field. Isolation of DNA was done based upon a protocol without use of liquid nitrogen [5]. Quality of DNA was checked in 0.8% agarose gel electrophoresis and quantity was measured using uncut lambda (λ) DNA as standard (300 ng/µl). Dilution of the DNA solution was done in $T_{10}E_1$ buffer to a concentration of approximately 25 ng/2 µl for use in PCR analysis.

A total of 30 RAPD primers from four kits (*viz*. OPP, OPAQ, OPBA and OPH) of Operon Technologies, Alameda, CA, USA were selected because of their reproducible amplification pattern in RAPD reaction in constant experimental condition. After analyzing the DNA amplification profile produced by these 30 primers, highly polymorphic 24 primers, which produced at least 75% polymorphic bands (Table 2), were again screened for molecular diversity analysis. Polymerase chain reactions (PCR) were carried out in a mixture of 25 µl contained 25 ng of genomic DNA template, 0.6 U of *Taq* DNA polymerase (Bangalore Genei, Bangalore, India), 0.3 µM of decamer RAPD primer, 2.5 µl of 10 X PCR assay buffer (50 mM KCl, 10 mM Tris-Cl, 1.5 mM $MgCl_2$) and 0.25 µl of pooled dNTPs (100 mM each of dATP, dCTP, dGTP and dTTP from Fermentas Life Sciences, USA). PCR cycle conditions were as follows: initial denaturing step at 94°C for 3 min followed by 44 cycles of 94°C for 1 min, 37°C for 1 min and 72°C for 2 min. In the last cycle, primer extension at 72°C for 7 min was provided.

PCR products mixed with 1/10th volume of gel loading dye, were electrophoretically separated on a 1.5% agarose gel containing ethidium bromide using 1X TBE buffer (pH 8.0). The amplified products were visualized and photographed under UV light source using gel documentation system. O'Gene Ruler^{TM} 100 bp DNA Ladder Plus (Fermentas Life Sciences, USA) was used as molecular weight marker for the purpose of detection of molecular weight of the amplified products produced in the fieldpea genotypes.

DNA bands were scored '1' for its presence and '0' for its absence for each primer genotype combination. These binary data matrix was then utilized to generate genetic similarity data among fieldpea cultivars. Jaccard's similarity coefficient was employed for the estimation of genetic similarity between the varieties (Table 3). Based on these binary data, UPGMA (unweighted pair group method using arithmetic averages) clustering was carried out by applying the software NTSYS-pc [6]. Strength of the clusters was supported and evaluated by bootstrap analysis using Win Boot software [7]. One thousand samples were generated by re-sampling with replacement of characters within the combined 1/0 data matrix. The expected heterozygosity for a genetic marker (H_n) was calculated by $H_n = 1\text{-}pi^2$ (pi is the allele frequency of the ith allele [8]. H_{av} (the arithmetic mean heterozygosity) value was calculated by employing the formula $H_{av} = H_n/n$ [9], where, n = number of markers or loci analysed. The average heterozygosity for polymorphic markers $(Hav)_p$ was derived as $(Hav)_p = H_n/np$ (np = no. of polymorphic markers or loci). The value of the marker index (MI) was calculated as $MI = E\,(Hav)_p$ (E is effective multiplex ratio and measured by nβ where β is the fraction of polymorphic marker or loci).

Results and discussion

Detection of polymorphism and efficiency of RAPD

A total of 256 unambiguous and clear bands were scored from RAPD amplification of 24 cultivars of fieldpea, out of which 228 (89.06%) bands were polymorphic, reason being incorporation of highly polymorphic and informative primers in the present study. Average number of 10.7 bands were obtained per primer and amplification produced ranged in size from 200 bp (by the primers OPBA 10 and OPH 20) to 3200 bp by the primer OPP 16. Maximum number of 22 amplification products were obtained with the primer OPP 13, followed by 16 products with primer OPP 14 and 15 products each with primers OPBA 09 and OPH 02. Minimum number of 5 RAPD products were generated with primer OPAQ 16. Fifty percent primers (12/24) produced DNA bands more than the average value of 10.7. A minimum of 75% band polymorphism was obtained by OPAQ 06 (6/8 polymorphic bands), followed by 80% polymorphism each with OPP 08 (8/10 polymorphic bands) and OPAQ

Table 1. Characteristics and distribution of fieldpea genotypes employed for molecular diversity analysis

Sl. No.	Name	Morphology	Pedigree	Distribution in India
1.	KFP 103	Tall type, large seed, light green leaves	KPMR 83 X KPMR 9	North India
2.	PG 3	Dwarf type, blue-green leaves	T 163 X Bonnevilla	North India
3.	Rachna	Tall type, green leaves	T 163 X T 10	North-east and central India
4.	IPF 99-25	Tall type, large seed, green leaves	PDPD 8 X Pant P 5	Central India
5.	Pant P 5	Tall type	T 10 X T 163	North India
6.	JM 6	Tall type	Local yellow Botri x (6588-1 x 46 C)	Central India
7.	Jayanti	Dwarf type, leaflet less	HFP 4 x PG 3	Haryana state
8.	KPMR 522	Dwarf type, leaflet less	KPMR 156 x HFP 4	North India
9.	KPMR 144-1	Dwarf type, leaflet less	Rachna x HFP 4	U.P. state
10.	DMR 7	Tall type	6587 x L 116	North India
11.	Ambika	Tall type	DMR 22 x HUP 7	Central India
12.	VL 1	Tall type	Selection from Miller	North Hill region
13.	VL 3	Dwarf type, blue-green leaves, dual purpose	Old Sugar X Wrinkled Dwarf	North Hill region
14.	B 22	Tall type, blue flower, blue-green leaves	Selection of local material from Berhampore (State of West Bengal, India)	West Bengal
15.	JP 885	Tall type and erect	(T 163 x 6588-1) x 46C	Central India
16.	Swati	Dwarf type, leaflet less	Flavanda x HFP 4	U.P. state
17.	Subrita	Tall type	Rachna x JP 885	M.P., Chhatisgarh
18.	HUP 2	Tall type, leaflet less	(Alfaknud x C 5064) x S 143	North-east India
19.	DDR 44	Dwarf type, large seed, short duration	HFP 4 x KPMR 157	Delhi state
20.	HFP 8909 (Uttara)	Dwarf type, leaflet less	EC 109185 x HFP 4	North India
21.	KPMR 400	Dwarf type, leaflet less	Rachna x HFP 4	Central India
22.	HFP 4	Dwarf type, leaflet less	T 163 x EC 109196	North and Central India
23.	HUDP 15	Dwarf type, short duration, leaflet less	(PG 3 x S143) x FC 1	North-east India
24.	IPFD 99-13	Dwarf type, leaflet less	HFP 4 x LFP 80	Central India

16 (4/5 polymorphic bands). A total of 7 primers showed 100% polymorphism, where as 3 primers showed polymorphism with 90% or above (Table 2). DNA amplification pattern as detected by some of the RAPD primers in fieldpea cultivars has been provided in Fig. 1.

RAPD, being a popular and dominant marker, has been used for molecular diversity analysis in several crop species *viz*. rice [10], wheat [11], barley [12] and pulse crops like chick pea [13], mungbean [14] and fieldpea [15-17]. In terms of detection of polymorphism, the result obtained in the present study is highly comparable with the result obtained by Simioniuc *et al.*, [16]. Moreover, estimated genetic similarity obtained in the present study was also high (0.43 to 0.84) because of the fact that informative primers were employed in the present study. Although reproducibility of the result is a matter of concern in RAPD, it could be overcome by optimizing the experimental condition and can be used as marker of choice as evident by Baranger *et al*. [18] in *Pisum sativum*, where mean allelic frequency was found highest for RAPD than isozyme, SSR and ISSR. Additionally, genetic relatedness among

Table 2. List of highly informative RAPD primers, their amplification pattern and polymorphism

Primer	No. of polymorphic and amplified bands	Percent polymorphism (x/y) x 100	Amplification product (bp, maximum and minimum)
OPP 01	10/13	76.9	1900, 475
OPP 04	13/13	100	3000, 250
OPP 08	08/10	80	2200, 500
OPP 09	06/07	85.7	1700, 700
OPP 11	09/10	90	2800, 600
OPP 13	21/22	95.5	2700, 350
OPP 14	13/16	81.3	3100, 500
OPP 16	10/12	83.3	3200, 510
OPBA 04	12/13	92.3	2000, 275
OPBA 05	06/07	85.71	2500, 700
OPBA 09	13/15	86.7	2600, 450
OPBA 10	07/08	87.5	1950, 200
OPBA 11	12/14	85.7	2050, 375
OPAQ 06	06/08	75	1400, 450
OPAQ 09	05/06	83.3	1800, 1050
OPAQ 15	12/14	85.7	2800, 300
OPAQ 16	04/05	80	3000, 425
OPH 01	02/02	100	1450, 975
OPH 02	15/15	100	3000, 340
OPH 03	10/12	83.3	3000, 400
OPH 07	07/07	100	2350, 600
OPH 08	11/11	100	1900, 425
OPH 09	08/08	100	1350, 375
OPH 20	08/08	100	1250, 200

the genotypes identified by RAPD was found to be closely correlating with all the other marker systems in the same study, thus proving the compatibility and usefulness of RAPD.

Heterozygosity was calculated for the 256 amplified products with 24 polymorphic RAPD primers and the H_{av} and $(Hav)_p$ values were found to be 0.592 and 0.665 respectively. The marker index (MI) value was obtained to be 6.318. Identification of number of alleles at a locus and their frequency of distribution as detected by a marker system is considered to be the polymorphism in a population. Estimation of marker utility and detection of polymorphism can be quantified in terms of mean heterozygosity and marker index [9]. If the detected H_{av} value in leguminous and other crops are compared using biochemical markers like allozyme or DNA markers like RAPD, it was observed that the value obtained in the present study (0.592) was higher as compared to cowpea (0.027 using allozyme [19]), wild lentil (0.342 using allozyme [20], *Trigonella foenum–graecum* (0.203 using RAPD [21], thus supporting the utility of RAPD as a marker system for detection of molecular diversity.

Genetic similarity and phylogenic relationship

Molecular diversity between the cultivars having different morphological parameters, parentages and geographical distribution found to be varied considerably (0.43 to 0.84) as observed in the similarity coefficient (Table 3). Highest similarity (0.84) was obtained between the tall type DMR 7 and Ambika. Few combinations also showed very high genetic similarity *viz*. KPMR 400 and KPMR 144-1 (0.79), DDR 44 and KPMR 144-1 (0.78), Rachna and IPF 99-25 (0.775) and DDR 44 and KPMR 522 (0.77). Most diverse relationship was obtained between the tall land race B 22 with dwarf type HFP 4 (0.43). Low genetic similarity was also evident between the genotypes B 22/Jayanti (0.46) and B 22/HUP 2 (0.466), thus indicating B 22 as genetically most diverse genotype in the lot analysed in the present study.

The dendrogram prepared based upon the multivariate (cluster) analysis of the genetic similarity data grouped the cultivars into three major clusters (I, II and III; Fig. 2) and five genetically diverse cultivars placed them away from any cluster. The first cluster (I) is a representative of a group with specific morphological character (tall, green leaf). Three tall genotypes were not indicated in cluster I, out of which two (VL1 and HUP 2) are developed from genetically diverse exotic parents and another one (B 22) is a land race collection from eastern part of India and showed highest genetic distance with most of the genotypes. The second cluster (II) consisted of five dwarf genotypes with green and semi-leafless character whereas cluster III also consisted of five dwarf genotypes where T 163, PG 3 and HFP 4 are major contributors. Like three tall cultivars mentioned earlier, two dwarf cultivars (IPFD 99-13 and HUDP 15, where exotic S 143 is a common parent from John Innes Centre, U.K.) was also not included in any cluster because of the wide geographical distribution of one of their parents. Bootstrap analysis was performed to evaluate the degree of support for clusters within the dendrogram and it was observed that sub-clusters within

Table 3. Genetic similarity matrix of selected 24 fieldpea cultivars

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1.000																							
0.628	1.000																						
0.706	0.637	1.000																					
0.701	0.597	0.7747	1.000																				
0.671	0.617	0.669	0.701	1.000																			
0.564	0.575	0.644	0.630	0.652	1.000																		
0.571	0.581	0.583	0.571	0.582	0.576	1.000																	
0.623	0.549	0.714	0.679	0.641	0.627	0.576	1.000																
0.643	0.530	0.673	0.648	0.641	0.587	0.548	0.761	1.000															
0.701	0.634	0.728	0.724	0.736	0.671	0.563	0.649	0.669	1.000														
0.681	0.665	0.772	0.735	0.726	0.703	0.611	0.671	0.671	0.843	1.000													
0.501	0.487	0.544	0.576	0.588	0.563	0.579	0.547	0.536	0.552	0.573	1.000												
0.486	0.567	0.578	0.522	0.551	0.571	0.614	0.554	0.552	0.567	0.598	0.583	1.000											
0.509	0.505	0.557	0.528	0.565	0.540	0.458	0.516	0.576	0.600	0.631	0.536	0.534	1.000										
0.600	0.582	0.647	0.663	0.745	0.722	0.574	0.612	0.630	0.722	0.743	0.597	0.632	0.583	1.000									
0.566	0.516	0.578	0.566	0.586	0.561	0.577	0.677	0.698	0.576	0.580	0.565	0.563	0.492	0.632	1.000								
0.562	0.590	0.629	0.626	0.637	0.642	0.556	0.567	0.548	0.675	0.716	0.503	0.595	0.547	0.665	0.516	1.000							
0.500	0.529	0.556	0.561	0.538	0.584	0.530	0.593	0.556	0.563	0.575	0.511	0.541	0.466	0.540	0.576	0.555	1.000						
0.623	0.602	0.683	0.659	0.691	0.646	0.603	0.771	0.784	0.689	0.700	0.591	0.627	0.586	0.688	0.687	0.603	0.630	1.000					
0.566	0.637	0.621	0.591	0.576	0.606	0.667	0.624	0.587	0.628	0.630	0.582	0.644	0.511	0.646	0.634	0.568	0.601	0.710	1.000				
0.597	0.497	0.619	0.595	0.607	0.591	0.489	0.695	0.790	0.616	0.619	0.520	0.527	0.560	0.598	0.623	0.550	0.523	0.717	0.572	1.000			
0.523	0.569	0.571	0.550	0.561	0.545	0.624	0.573	0.519	0.560	0.573	0.508	0.565	0.433	0.554	0.601	0.519	0.596	0.618	0.732	0.503	1.000		
0.530	0.517	0.571	0.550	0.526	0.527	0.561	0.612	0.611	0.598	0.574	0.497	0.538	0.500	0.563	0.594	0.543	0.525	0.612	0.610	0.596	0.586	1.000	
0.573	0.497	0.531	0.537	0.531	0.532	0.531	0.615	0.605	0.547	0.569	0.503	0.525	0.506	0.593	0.598	0.505	0.547	0.645	0.632	0.610	0.571	0.544	1.000

Note: Serial number (1 to 24) of the cultivars are same as given in Table 1

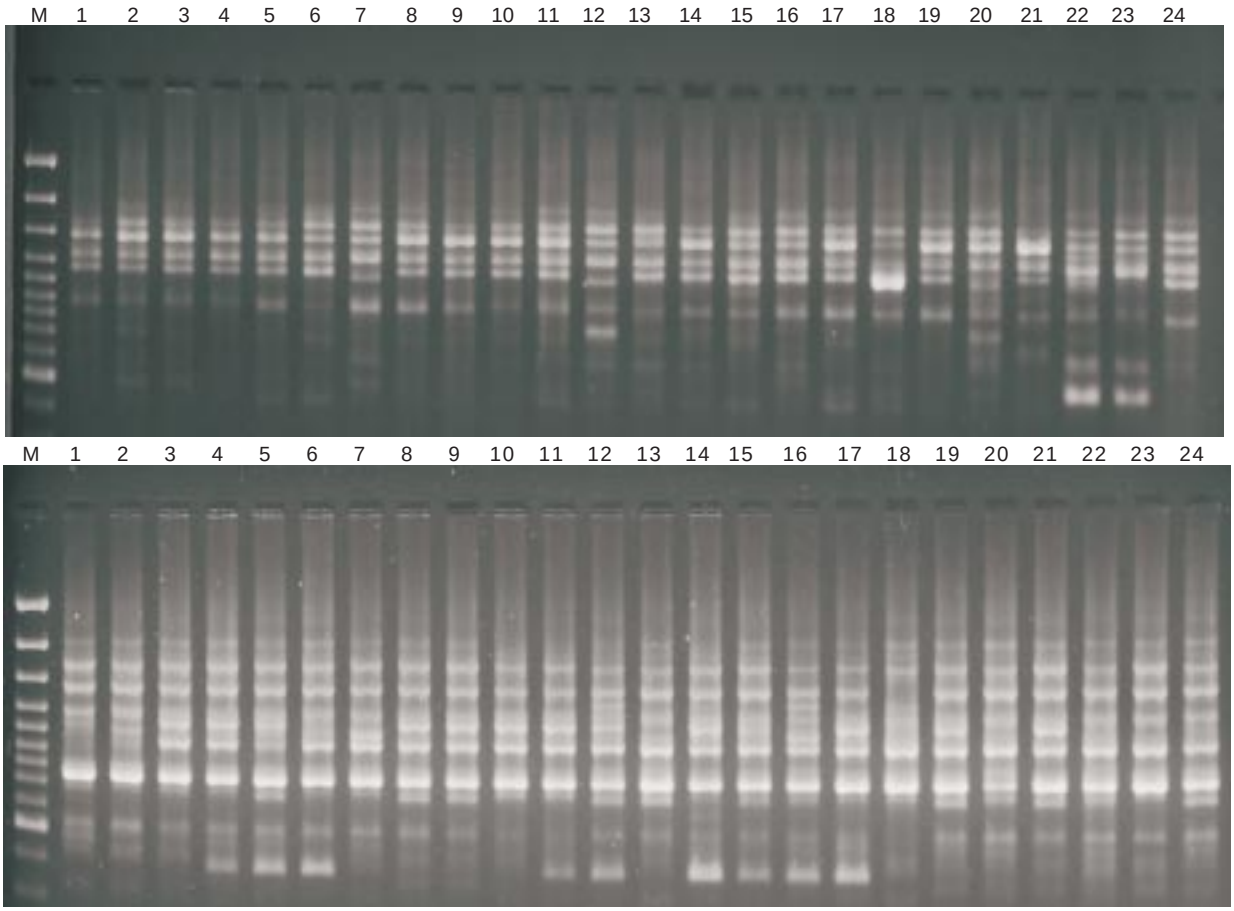

Fig. 1. RAPD profile of fieldpea cultivars obtained with primers OPP 01 (above) and OPBA 11 (below). Serial number of cultivars corresponds to Table 1. M=Standard DNA marker, 100bp DNA ladder plus

the major clusters were supported by high boots trap value and proved the robustness of the clusters formed in the dendrogram.

Thorough analysis of the cluster I shows highest genetic similarity between morphologically same cultivars DMR 7 and Ambika (DMR 22 is one of the parents) and rest of the tall cultivars included in the cluster had T 163 as one of the common parent (directly or indirectly). In the second cluster (II), all the dwarf genotypes have HFP 4 as a common parent. HFP 4 itself has been grouped in the third cluster (III), where it has been observed that the dwarf genotypes with either T 163 or HFP 4 as a parent has been included. Interestingly, T 163 itself is one of the parents of HFP 4. Among the parents, T 163 was found to be most widely used because of its broad adaptability. Other frequently used direct or indirect ancestors were EC 109196 and T 10 and they were used for incorporation of different desired characters like dwarfism, disease resistance etc.

Identification of cultivars

Analysis of the polymorphic RAPD primers has led to generation of few genotype specific bands, which were confirmed by reproducing the result by keeping the constant experimental condition. It has been observed that many of the primers have amplified specific bands either unique to genotypes or specific to few genotypes. Keeping this genotype specific amplification pattern in mind, a diagrammatic mode of DNA fingerprint comprising of genotype specific bands generated by specific primers has been generated (Fig. 3), which could be used as a reference for cultivar specificity and could be of great use for concerned breeders, variety registration authority or seed production units etc. Conversion of the genotype specific amplification products into specific PCR primers like CAPS could be more useful in varietal identification and detection of duplicates.

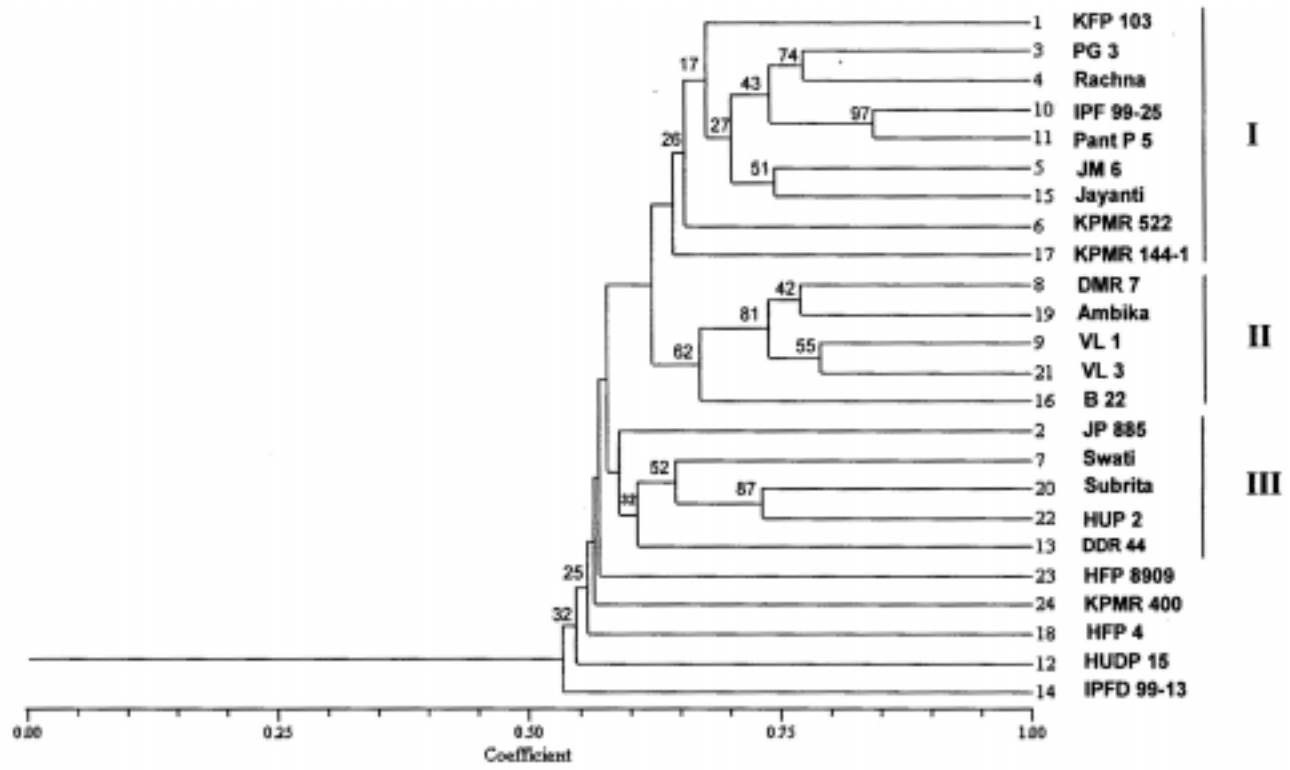

Fig. 2. UPGMA dendrogram depicting patterns of genetic diversity estimated by highly informative 24 RAPD primers among 24 cultivars of fieldpea. Scale on bottom is Jaccard's coefficient of similarity. Major clusters are indicated as I, II and III on right margin. Numbers at branch point indicate support for cultivars clustered to the right of the number, values are per cent of bootstrap samples that exhibited the cluster (no number at branch indicates support less than 10%)

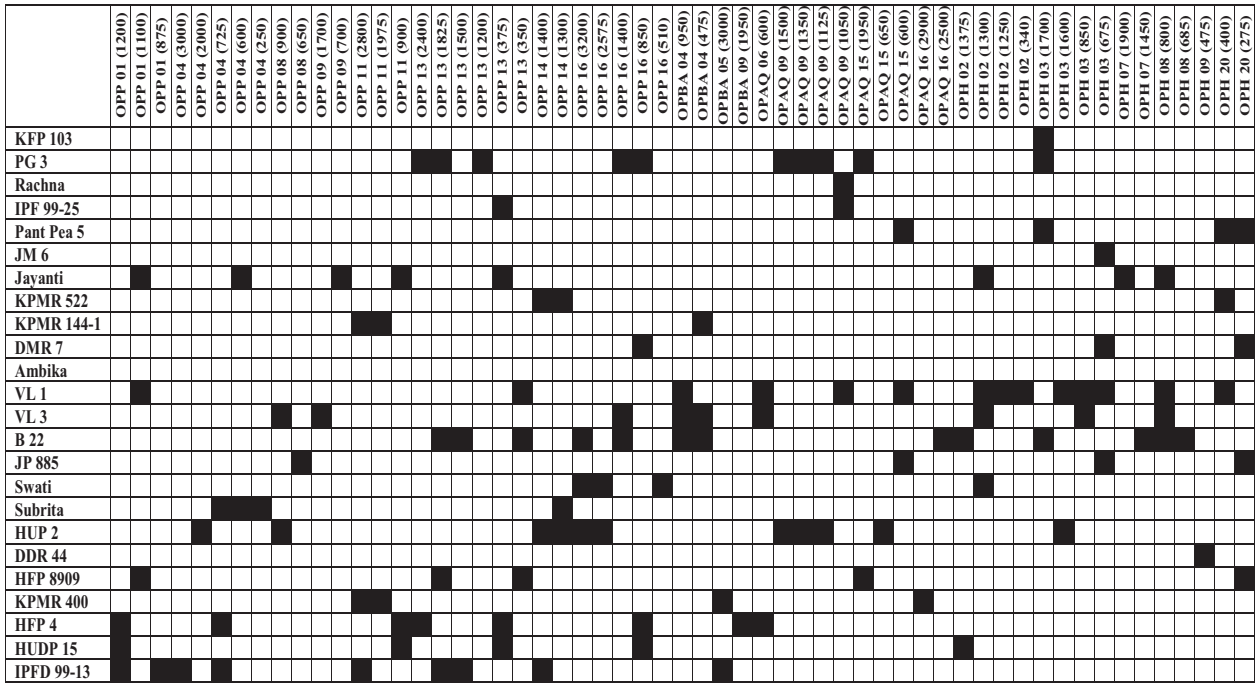

Fig. 3. Diagrammatic presentation of genotype specific DNA fingerprint of selected fieldpea cultivars. Rows correspond to cultivars and columns represent the amplified product with respect of a marker across the cultivars

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