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Analysis of genetic divergence in pea (*Pisum sativum* L.) using quantitative traits and RAPD markers

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Abstract

Genetic diversity among 14 tall and 14 dwarf cultivars/ elite lines of pea (*Pisum sativum* L.) was assessed based on 10 quantitative traits and 72 RAPD primers. Dendrogram based on quantitative traits revealed six clusters. In principal component analysis (PCA), the first three PCs together accounted for 61.48% of the total variation, and the grouping was consistent with that of UPGMA method. RAPD-based dendrogram showed three major clusters; cluster II was further divided into three subclusters. The first three PCs of RAPD data accounted for 29.28% of the total variation, and the grouping pattern was similar to that obtained by UPGMA. The tall genotypes in both the types of clustering indicated a lower level of diversity compared to the dwarf ones. The correlation estimated by Mantel test between the quantitative trait and RAPD matrices was non-significant (r = –0.26) for reason of targetting different genomic regions by RAPD markers the morphological traits. Cophenetic correlations which reflect the goodness of fit for a tree were 0.73 and 0.79 for quantitative traits based and RAPD based dendrogram, respectively.

Key words: Genetic diversity, *Pisum sativum*, quantitative trait, RAPD

Introduction

Analysis of genetic relationship among genotypes of crop species has diverse applications in crop breeding, such as assay of genetic variability among cultivars, identification of parental combinations for the maximum expression of heterosis, generation of the maximum genetic variability in segregating generations and introgression of desirable genes from diverse germplasms into elite breeding materials [1]. The reliability of a genetic marker and its usefulness in genetic diversity analysis are positively associated with the heritability and the level of polymorphism exhibited by the marker. DNA-based markers are considered to be the best tools for determining genetic relationships/ diversity as they are independent of environmental interaction [2]. RAPD technique has been extensively used for studying genetic diversity and phylogenetic relationships in several legumes including pea [3]. The present investigation was undertaken to study the level of genetic diversity present in cultivars/elite breeding lines of pea using RAPD and quantitative trait markers.

Materials and methods

Plant material

The plant material consisted of 28 cultivars/elite breeding lines of pea; of these, 14 lines were tall (average height, 157.0 cm) and 14 were dwarf (average height, 64.1 cm) (Table 1).

Quantitative characters

The 28 genotypes were grown in the crop season of first and second year in a randomized block design with five replications in a three row plot of row length 4 m, row spacing 30 cm and plant spacing of 10 cm. Recommended agronomic practices were followed to raise a good crop. Data on 10 quantitative characters were recorded on five randomly chosen plants from each replication (Table 1). The inoculation procedure and evaluation of rust reaction has been described earlier [4].

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Table 1. List of 28 cultivars of *P. sativum*, along with their source, pedigree, and quantitative traits recorded for the analysis of genetic diversity

S.No.	Accession	Source	Pedigree	Plant height (cm)	No. of primary branches	No. of pods/ plant	Pod length (cm)	No. of seeds/ pod	25-seed wt. (g)	Seed yield/ plant(g)	Days to flower	Days to maturity	No. of rust pustules/ leaf*
	Tall												
1.	HUP-2	BHU, Varanasi	(Alfanud x C-5064) S-143	144.4	2.0	15.4	5.7	4.6	5.1	13.8	87.3	124.8	5.8
2.	Rachna	CSAUA&T, Kanpur	T-163 x T-10	143.1	2.1	16.1	6.6	4.8	5.2	15.0	79.2	125.7	15.8
3.	DMR-42	IARI, New Delhi	DMR-37 x P-1463	170.1	1.8	16.4	6.2	3.9	4.9	12.6	85.2	132.2	9.2
4.	KPMR-551	CSAUA&T, Kanpur	Rachna x KFP-4	153.4	2.0	18.3	6.5	4.0	5.0	13.1	79.5	125.3	9.8
5.	KPMR-615	CSAUA&T, Kanpur	DMR-37 x P-1463	158.7	2.2	17.3	6.7	5.2	5.2	17.2	74.5	130.2	6.4
6.	KPMR-619	CSAUA&T, Kanpur	Thuuoout x Ufa-75	175.0	2.0	14.6	6.7	4.2	5.8	17.7	85.2	129.8	6.2
7.	IPF-99-25	IIPR, Kanpur	PDPD-8 x Pant P-5	172.8	1.5	16.7	5.9	3.9	4.7	12.2	76.2	126.0	15.7
8.	VL-40	Almora	T-163 x VL-1	146.7	2.2	14.0	6.0	4.6	5.7	12.1	79.2	124.8	19.5
9.	DMR-46	IARI, New Delhi	HFP-4 x P-1522	170.1	2.2	17.1	6.5	4.4	4.9	15.9	78.3	125.7	36.6
10.	KPMR-660	CSAUA&T, Kanpur	Rachna x KPMR-157	148.2	1.8	14.4	6.3	3.7	5.3	12.3	78.8	123.8	16.4
11.	IPF-1-17	IIPR, Kanpur	Rachna x Kiran	148.2	1.9	12.2	6.3	4.5	5.6	11.1	80.5	127.3	16.1
12.	IPF-1-22	IIPR, Kanpur	HUP-2 x DPF-62	153.4	1.6	10.1	6.4	5.4	4.9	14.6	80.2	126.0	6.0
13.	VL-41	Almora	VL-1 x P-388	141.8	1.9	15.0	6.3	4.6	4.7	12.2	76.0	123.7	34.9
14.	KPMR-662	CSAUA&T, Kanpur	Rachna x KPMR-65	172.2	1.8	13.4	6.3	4.3	5.7	14.9	78.0	123.0	38.7
	Dwarf												
15.	HFP-4	HAU, Hisar	T-163 x EC-190196	54.9	2.2	16.1	6.4	3.9	5.1	13.9	71.3	117.2	14.3
16.	HUDP-15	BHU, Varanasi	PG-3 (PG-3 x S-143) FC-1	70.4	2.0	15.4	7.1	5.3	5.1	16.6	73.5	122.8	0.2
17.	KPMR-144-1	CSAUA&T, Kanpur	Rachna x HFP-4	60.5	2.4	17.9	6.5	4.0	4.1	13.5	82.8	118.2	9.9
18.	DDR-49	IARI, New Delhi	Sel. From ET 2255-1	59.6	1.7	13.4	6.7	4.7	4.8	11.3	75.2	115.2	39.7
19.	KPMR-526	CSAUA&T, Kanpur	KPMR-156 x HFP-4	56.3	1.8	14.1	6.2	4.4	3.8	11.0	82.5	122.7	21.5
20.	LFP-283	PAU, Ludhiana	LFP-48 (HFP-4 x LFP-48)	69.4	1.7	14.5	7.2	5.2	5.4	13.9	83.5	114.5	32.1
21.	HUDP-16	BHU, Varanasi	BHUD-110 x FC-1	94.1	1.7	16.5	6.6	4.6	6.0	14.8	76.5	124.3	0.4
22.	Pant P-13	GBPUA&T, Pantnagar	HFP-4 x FC-1	55.5	2.2	10.4	6.2	4.2	5.6	11.3	72.7	122.0	1.8
23.	Pant P-14	GBPUA&T, Pantnagar	HFP-4 x Longitee	68.3	1.8	10.7	6.9	4.8	5.4	8.7	76.7	121.7	0.1
24.	Pant P-20	GBPUA&T, Pantnagar	HFP-4 x FC-1	70.7	1.5	8.5	6.1	4.8	5.1	7.7	79.7	124.2	0.7
25.	IPFD-1-9	IIPR, Kanpur	HFP-8909 x HUDP-7	67.3	1.7	12.6	6.4	4.1	5.1	11.1	82.7	126.0	2.0
26.	IPFD-1-10	IIPR, Kanpur	PDPD-8 x HUDP-7	69.6	2.4	16.5	6.4	4.4	5.4	9.7	73.7	123.3	29.7
27.	HFP-9811	HAU, Hisar	PH-1 x Bonnevillae	47.6	2.0	11.3	6.6	4.5	6.0	11.1	66.5	121.2	56.7
28.	HFP-9830	HAU, Hisar	HFP-4 x Arkel	53.7	2.0	14.5	6.8	4.1	4.8	14.1	71.5	123.8	193.5

*Observations were recorded on the third and fourth leaves above the one on which the first rust pustules were observed.

DNA isolation

The genomic DNAs of all the 28 pea genotypes were isolated using Qiagen DNeasy Plant Mini Kit (Qiagen, Germany). The PCR was performed for 40 cycles in a reaction volume of 25 µl containing 10 mM Tris-HCl, pH 9.0, 2 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, 200 µM of each dNTP, 4 µM of primer, 1.25 Units of *Taq* DNA polymerase and 30 ng of genomic DNA. A total of 72 decamer primers were selected for final RAPD-PCR analysis from 150 primers (GC content 50-80%) that were initially screened with two genotypes to select primers producing distinct scoreable bands. The PCR amplification was carried out in a thermal cycler (Techne, Touchgene Gradient, UK). Amplified products were separated on 1.5% agarose gels having 0.5 µg ml^{-1} of the ethidium bromide at 75V for 3 h. The gels were documented under a UV light source in a gel documentation system (AlphaImager, Alpha Innotech Corporation, San Leandro, CA). RAPD bands were scored as present or absent [4].

Statistical analyses

For quantitative traits, analysis of variance (ANOVA) was performed according to a three factor randomized block design using model III of SPSS programme (http: //www.spss.com). The other statistical analyses for the quantitative traits were conducted using NTSYSpc version 2.02i [5]. Standardization of data for each trait was done separately prior to cluster analysis for minimizing effects of scales of measurement. Standardization was done by dividing the deviation of mean for a line from the mean for the 28 lines with the standard deviation for the given trait using the STAND module of NTSYSpc software. The standardized data were used for generating the distance matrix. Dendrogram was generated based on the average taxonomic distance matrix using unweighted pair group method with arithmetic averages (UPGMA). Intra- and inter-cluster distances were calculated by taking average of the genetic distance of the genotypes falling in that cluster. Quantitative traits of the genotypes grouped in various clusters were averaged separately and the yield traits and the genetic distances between clusters were correlated.

The RAPD products were scored '1' for its presence, and '0' for its absence for each primer-genotype combination. Data was entered into a binary data matrix from which Jaccard's coefficient of similarity [6] was measured using SIMQUAL module of NTSYSpc. Pair-wise similarity matrix was used as an input for UPGMA-based dendrogram construction using SAHN module of NTSYSpc.

Principal component analysis (PCA) was also done to check the results of UPGMA based clustering. The correlation matrix was calculated from the standardized data matrix using SIMINT module of NTSYSpc. The first two and the first three PCs were used for 2-dimensional and 3-dimensional plotting, respectively, against each other using module PROJ and MXPLOT of NTSYSpc. PCA analysis for RAPD data was carried out in the same manner as that for the quantitative data, but the standardization step was not used in this case.

The optimum number of clusters was determined by multivariate analysis of variance (MANOVA) using SPSS software. First, the cut points on dendrograms were chosen. Then the clusters obtained at each cut points were taken as treatments and the genotypes falling within that group were considered as replications for that treatment. The analysis was repeated with each cut point with all markers selected for cluster analysis. The cut point that yielded highest F value was considered for the optimum number of cluster determination [7].

Mantel test was done using the MXCOMP module of NTSYSpc to compare the Jaccard's similarity values from RAPD analyses and the average taxonomic distance values from the quantitative traits. The efficiency of clustering algorithm was compared through estimation of the cophenetic correlation coefficients (MXCOMP module of NTSYSpc) and was used to test the goodness of fit for the dendrograms.

Results and discussion

Genetic diversity based on quantitative characters

Analysis of variance revealed significant genotypic differences, but non-significant replication and year differences; therefore, average of the five replications was used for further analyses. Taxonomic distances estimated from quantitative traits ranged from 0.50 to 2.30 (data not shown). Cluster analysis was done using the taxonomic distance matrix, and a dendrogram was constructed (Fig. 1). The optimal number of clusters was determined by MANOVA. The highest F value for Pillai's Trace (3.152) was obtained with the cut-point at 1.35 of taxonomic distance scale. At this cut-point, the 28 genotypes were grouped into six clusters. Cluster I consisted of a single genotype, HFP 9830, which was the most divergent from the rest of genotypes. Cluster II had two genotypes, while six genotypes formed cluster

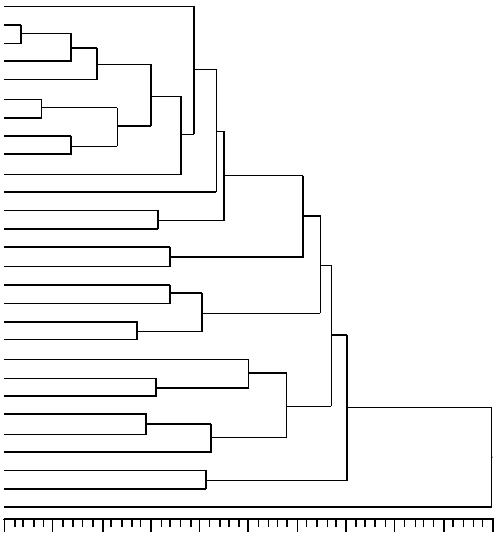

Fig. 1. Dendrogram of 28 pea genotypes based on average taxonomic distance of quantitative characters. The arrow on distance scale indicates the optimum cut-point for cluster differentiation

III. Clusters IV, V and VI consisted of four, two and 13 genotypes, respectively. Thus 12 of the 14 tall genotypes were included in cluster VI, while the tall genotype KPMR 615 was present in clusters V and IPF 1-22 occurred in cluster III. The dwarf line HUDP 16 grouped in cluster VI with the tall genotypes. Earlier workers have reported the grouping of tall and dwarf pea genotypes in different clusters [8, 9].

The intra- and intercluster distances indicated presence of less variability within the tall group than that within the dwarf group (Table 2). Most of the tall and dwarf lines evaluated in this study were elite lines developed by various pea breeders and were being evaluated for release as commercial varieties. Clusters II and V showed the high inter-cluster distance (2.02);

Table 2. Inter- and intra-cluster taxonomic distance values in the six clusters based on 10 quantitative characters.

Cluster	I	II	III	IV	V	VI
I	0.00	1.97	2.03	1.75	2.02	1.99
II		1.07	1.55	1.53	1.74	1.45
III			1.15	1.54	1.59	1.43
IV				1.03	1.57	1.40
V					0.96	1.37
VI						0.96

these clusters also complemented each other for the different yield traits. Cluster means of 10 quantitative characters (Table not shown) revealed that genotypes included in cluster V showed maximum number of pods/ plant (16.35), maximum pod length (6.91cm), highest number of seeds/plant (5.27g), and highest seed yield/ plant (16.94g). Therefore, crosses between genotypes of clusters II and V may be expected to generate a wide range of variation for the yield and other traits. Such segregants will have longer grain filling duration and may be expected to be superior yielding than the parents.

PCA, based on the standardized mean values of quantitative characters, was used to examine the clustering based on UPGMA. PC1, PC2, and PC3 accounted for 26.07%, 19.10% and 16.30% of the variation, respectively and the cumulative variation of these thee PCs was 61.48%. PCA did not reveal any distinct clusters, but the dwarf and tall genotypes were concentrated in separate areas of the 2-D and 3-D plots (Fig. 2&3). This is consistent with the pattern from the UPGMA. Plant height, days to maturity and days to flower were highly associated with PC 1, whereas seed yield/plant, number of pods/plant, number of primary branches and pod length were highly correlated with PC2 (data not shown). Number of seeds/ pod, 25-seed weight and pod length were the main contributors to PC3.

Genetic diversity based on RAPD profile

From among the 150 primers initially screened with two genotype, 72 primers were used for RAPD-PCR analysis

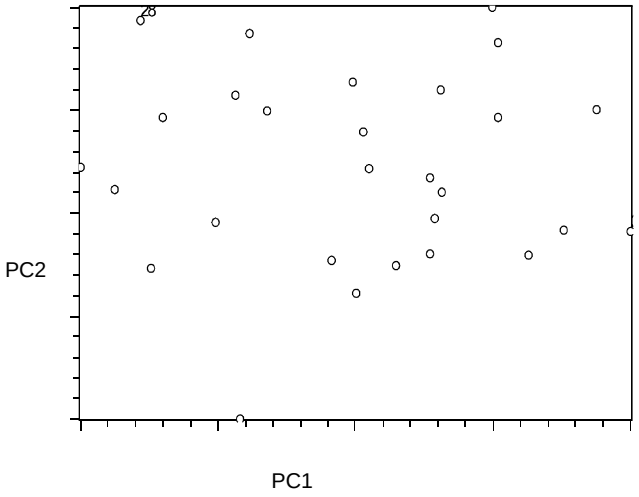

Fig. 2. A 2-dimensional plot of principal component 1 and 2 based on ten quantitative characters of pea genotypes

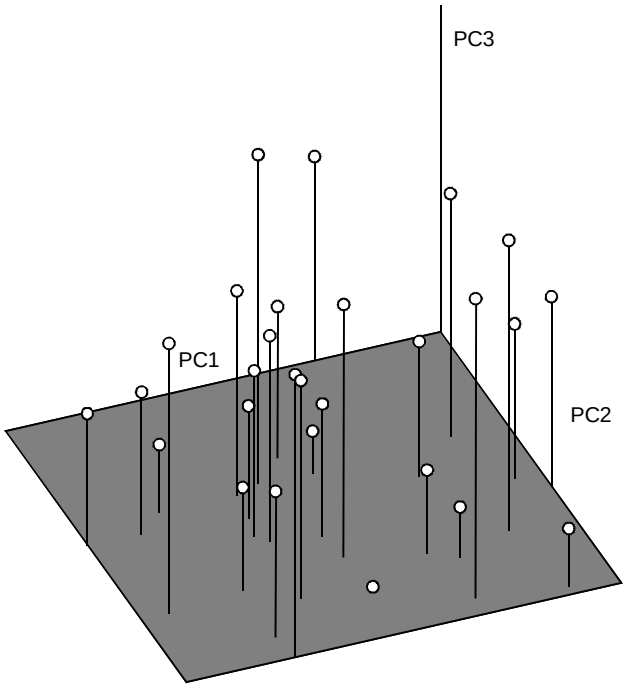

Fig. 3. A 3-dimensional plot of principal component 1, 2 and 3 based on ten quantitative characters of pea genotypes

that produced reproducible bands. Fifty three primers (73.6%) generated. 524 fragments, of which 282 amplicons (53.8%) were polymorphic. The number of polymorphic bands per primer ranged from 1 to 10, the average being 5.3. These findings are within the range observed in earlier studies with pea [3, 10, 11]. Primers OPI11, HU11 (TGCTCACGAA, Fig. 4), OPW01, HU12 (TGCTC AGCAG), OPK06, OPQ20, OPW02 and OPZ03 were the most informative primers as 75% or more of the amplicons were polymorphic. The primers having G+C content of 60% yielded the highest average number (4.1) of polymorphic bands per primer, while those having 50% G+C content amplified the lowest (2.7). The primers with 70% and 80% G+C contents gave intermediate values (3.7 and 3.0 bands per primer, respectively). These findings are same as reported earlier [12]. However, other reports on pea [3] states that primers with 50% G+C content produces the highest number (24.5 bands) of bands.

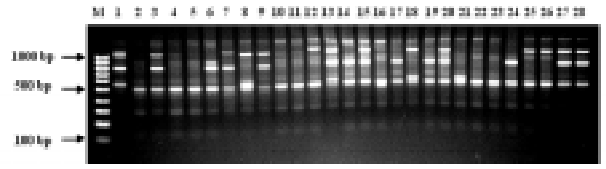

Fig. 4. Ethidium bromide stained electrophoretic pattern of randomly amplified polymorphic DNA of pea genotypes with primers HU11. M is marker lane run with 100bp DNA ladder and lanes 1-28 are genotypes of *Pisum sativum*

Genetic similarities of RAPD markers were measured by using Jaccard's similarity coefficient which ranged from 0.29 to 0.88 (table not shown). The range of Jaccard's similarity coefficient decreased from 0.29-0.88 to 0.67-0.96 when both polymorphic and monomorphic bands were considered. These findings are in agreement with some [13], while in disagreement with others [14]. Therefore, the monomorphic bands were excluded from analysis. A dendrogram (Fig. 5) based on Jaccard's similarity coefficient was constructed using UPGMA. The highest F value of Pillai's Trace (4.059) was obtained for the cut-point at 0.43 of the similarity coefficient scale. At this cut-point, the 28 genotypes were grouped into three major clusters. Cluster I consisted of two genotypes, and cluster II had the maximum number (25) of genotypes; the latter could be divided into sub-clusters IIA (a single genotype), IIB (15 genotypes) and IIC (nine genotypes). HUP-2 was the lone genotype in cluster III.

The intra- and inter-cluster distance is given in Table 3. Cluster III contained a single tall genotype, HUP-2, which gave exceptionally different banding patterns from the other genotypes, indicating that it is genetically different from the others. Most of the tall genotypes occurred in cluster IIC, while four of the tall lines showed more similarity with the dwarf genotypes

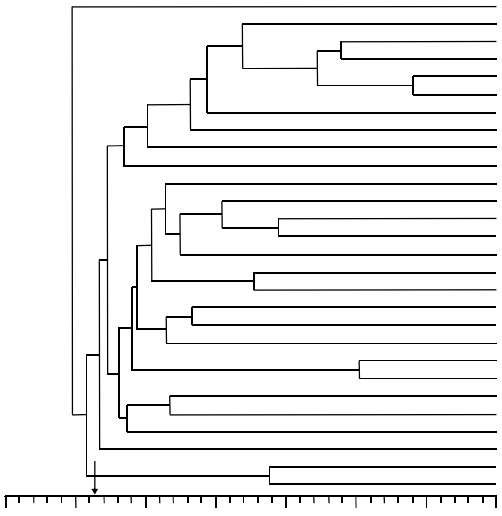

Fig. 5. Dendrogram of pea genotypes based on Jaccard's similarity coefficients of RAPD markers. The arrow on similarity scale indicates the optimum cut-point for cluster differentiation

Table 3. Inter- and intra-cluster distance values between the three clusters based on 282 RAPD markers.

Cluster	I	IIA	IIB	IIC	III
I	0.32	0.57	0.58	0.60	0.63
IIA		0.00	0.56	0.57	0.66
IIB			0.49	0.56	0.59
IIC				0.40	0.62
III					0.00

placed in cluster IIB (Fig. 5). In comparison, the dwarf genotypes tended to cluster in different groups. The results of clustering indicate that the tall genotypes included in this study possessed a relatively narrower genetic diversity than did the dwarf genotypes.

PCA for the RAPD markers revealed that the PC1, PC2, and PC3 accounted for 12.9%, 8.7%, and 7.7% of the variation, respectively. Together, the first three PCs accounted for 29.28%of the total variation. The 2-dimensional (Fig. 6) and 3-dimensional plots (Fig. 7) prepared using the first two and the first three PCs, respectively, grouped the genotypes into six clusters, except that the genotypes KPMR 619 and DMR 46, and DDR 49 were not included in any of the clusters. The genotypes of cluster I, II, III and IV in PCA form the cluster IIB including HUP-2 which was most distant in UPGMA dendrogram. The genotypes of cluster IIC in UPGMA form the cluster V in PCA except KPMR-619 and DMR-46 that are most distinct in PCA, and therefore, not included in cluster. Again, UPGMA based cluster I and cluster VI of PCA included same two genotypes HFP-9830 and HFP-9811. After comparing

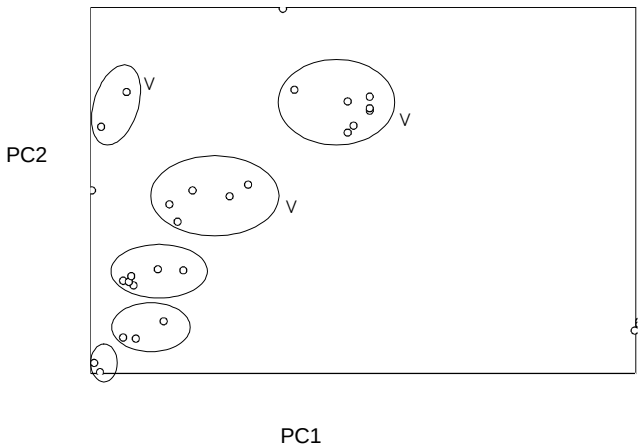

Fig. 6. A 2-dimensional plot of principal component 1 and 2 based on RAPD markers of pea genotypes

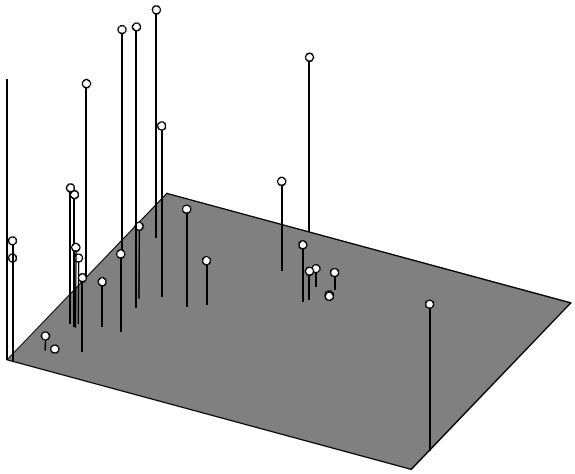

Fig. 7. A 3-dimensional plot of principal components 1, 2 and 3 based on RAPD markers of pea genotypes

the clusters it is evident that for RAPD markers the results of UPGMA clustering and PCA are mostly congruent. These observations agree with those of earlier studies on genetic diversity based on molecular marker data in pea using UPGMA and PCA [11].

The correlation estimated by Mantel test between the quantitative trait and RAPD matrices was non-significant (r = –0.26). The reason for poor agreement is that the morphological traits are controlled by only a subset of the genomic regions, while RAPD markers come from the entire genome and are likely to be unrelated to the morphological traits [15]. In addition, the expression of phenotypic traits is markedly influenced by the environment. The cophenetic correlation obtained based on quantitative trait dendrogram was less than 0.8 (r = 0.73), which is interpreted as poor fit. Dendrogram based on Jaccard's coefficient produced higher (r = 0.79) cophenetic correlation, which is nearly in the range of good fit. A low cophenetic correlation indicates that some distortion might have occurred.

In the present study, genetic divergence among tall and dwarf genotypes of *P. sativum* was evaluated using ten quantitative characters and 282 RAPD markers. Both the sets of markers classified most of the pea genotypes into tall and dwarf groups; the tall group showed a relatively narrower genetic diversity than the dwarf group, which is contrary to the findings from previous studies based on only quantitative characters [8]. This discrepancy may be due to the differences in pea genotypes included in the present and earlier studies. The present study included elite

breeding lines being generated from the pea breeding programmes of the country. It appears that there is a case for deliberately enhancing the genetic diversity at least in the elite tall breeding materials of pea.

The RAPD profiles of all the primers that generated sharp, intense and easily scoreable polymorphic bands were surveyed to obtain a set of a minimum number of primers that could distinguish all the 28 genotypes from each other. A minimum of three primers, *viz.*, OPI11, OPW01 and HU11, or HU11, OPQ20 and OPI11, were needed to separate all the 28 lines of pea. Earlier workers have also reported that a set of small number of RAPD primers was able to successfully identify the *P. sativum* [3, 10].

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