UDC 575.633
https://doi.org/10.2298/GENSR2102473S
Original scientific article

GENETIC DIVERSITY STUDIES IN INDIAN GERMPLASM OF PEA (*Pisum sativum* L.) USING MORPHOLOGICAL AND MICROSATELLITE MARKERS

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Singh J., R.K. Dhall, Y. Vikal (2021). *Genetic diversity studies in Indian germplasm of pea (Pisum sativum L.) using morphological and microsatellite markers*. - Genetika, Vol 53, No.2, 473- 491.

Forty seven garden pea genotypes were characterized using thirty four pea specific microsatellite markers. Mean square values were highly significant for all the characters. Nearly 75% of the total variation was contributed by 3 traits *viz.* green pod yield per plant (43.18), plant height (20.91) and number of nodes per plant (10.27). 28 polymorphic SSR markers were used. Average PIC value was 0.55. NTSYS clustering based on UPGMA grouped the genotypes into two major groups I and II (dwarf and tall, respectively). The larger group consisted of 43 genotypes (subgroups I-VII) and smaller group had 3 genotypes (subgroup VIII). One genotype (PMR-19) separated from other genotypes. The dendrogram depicted overall similarity coefficient ranged from 0.59 to 0.89. The lowest genetic similarity (0.39) was observed between major group I and major group II. Maximum genetic similarity (0.89) was found between two pairs i.e. PB-89 and C-96 (first pair) and Angoori and GS-10 (second pair). Neighbor joining tree thus obtained highly corresponds to the clustering observed using NTSYS indicating that the analysis was reliable. The findings of the present study can be used to select diverse genotypes to be used as parents of crosses aimed for breeding improved pea cultivars.

Key words: pea, genetic variability, morphological traits, SSR markers.

INTRODUCTION

Garden pea (*Pisum sativum* L.) a diploid (2n=2x=14) cultivated species of family Fabaceae (Leguminosae) and is native of region between Mediterranean and Near East. It was cultivated since early Neolithic times and is believed to evolve from *P. sativum* ssp. *elatius* and

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P. sativum ssp. *humile* (ZOHARY and HOPF, 1973). However, the exclusive origin and primary source of diversity of the crop is not well known (DAVIES, 1976). It is one of the world's oldest crops, as it was first cultivated with cereals like barley and wheat 7000 years ago (MCPHEE, 2003). The Hindu-Kusch region in India, which includes southern slopes of Himalayan mountain range, is one of its secondary centre's of diversity (AMBROSE, 2008). It is one of the most important food legume for its versatile use as vegetable, pulse, and animal feed (CHOUDHURY *et al.*,2007).India is the second largest producer of pea in the world and accounts for twenty-one percent of the world production (SINGH and DHALL, 2018). Punjab is the fifth largest producer of pea in the country and accounts for 6.7 percent of India's production. It is the second important vegetable crop of Punjab and is grown in an area of 31.3 thousand hectares with an annual production of 315.87 thousand tons (DHALL, 2017).

Crop improvement depends largely on the availability of genetic variability in breeding material, their effective evaluation, and utilization. Molecular markers provide an accurate and powerful tool for analyzing the relationships among accessions based on the estimation of genetic similarity. Therefore, characterization of genotypes at genetic level, supplemented by phenotypic characters provides the first step towards efficient conservation, maintenance and utilization of existing genetic diversity in crop germplasm.SSR markers are widely used for characterization of pea germplasm especially due to the high level of polymorphism (KASHIA *et al.,* 1997; BURSTIN *et al.,* 2001; LORIDON *et al.,* 2005). In recent reports, there exist considerable diversity in pea for food and feed types (TAR'AN *et al.,* 2005), vegetable or dry seeded types (ELLIS *et al.*,1998) or between cultivated and wild relatives (BARANGER *et al.*,2004; NASIRI *et al.,* 2009) but cultivated pea varieties were grouped within a single cluster with subsets and estimated polymorphism level was lower than pea landraces points that only a part of the genetic variability available in traditional crop materials (MARTIN-SANZ *et al.*,2011).

The objectives of this study were to characterize the genetic diversity and structure of cultivated populations of garden pea, which are maintained at the field gene bank of Department of Vegetable Science, Punjab Agricultural University, Ludhiana for various horticultural traits and to identify the extent of duplication within the field gene bank. It was expected that the genetic diversity trees so developed would permit elimination of the duplicates and would create a core pea collection to be utilized by the breeders.

MATERIAL AND METHODS

Plant materials

Forty seven garden pea (*Pisum sativum* L.) germplasm comprising breeding lines, accessions, and cultivated varieties were selected from germplasm collection of two hundred lines on the basis of different agro-climatological zones of India or internationally for their use in the present investigation.

Cultural practices

Garden pea germplasm was sown at Vegetable Research Farm, Department of Vegetable Science, Punjab Agricultural University, Ludhiana (30°55' N & 75°54' E) for two years to know the morphological and genetic diversity among them. The genotypes were planted

in Randomized Complete Block Design (RCBD) having three replications for two years. Each genotype was planted on flat beds in plots comprising of a row of 3m length for each accession, at spacing of 45 cm between rows and 10 cm between plants. The number of plants per bed are 30. The soil was Gangetic alluvial with a sandy clay loam texture, pH 8.5, organic carbon 0.18%, available N: 240 kg ha-1, available P: 13.6 kg ha-1 and available K: 75 kg ha-1 at the time of initiation of the experiment. Recommended doses of inorganic N as urea (110 kg $ha^{-1}year^{-1}$), inorganic P as P_2O_5 (62 kg $ha^{-1}year^{-1}$) were applied.

Morphological traits

Five plants were selected randomly from each genotype, in each replication and were used for recording observations. For morphological characterization the genotypes were evaluated for twenty one morphological traits and three biochemical traits namely days to first flowering, days to 50% flowering, first blossom node, number of primary branches per plant, plant height (cm), days to first pod picking, days to last pod picking, number of nodes per plant, number of podding nodes per plant, number of pods per plant, number of pods per node (one/two/both), grain filling period (days), number of seeds per pod, number of locules per pod, 100 seed weight (g), pod weight (g), pod length (cm), pod width (cm), pod thickness (cm), green pod yield per plant (g), seed yield per plant (g), shelling percentage, crude protein (%), soluble protein (%) and total sugars (%). Crude protein content was estimated by Kjeldahl method of nitrogen estimation (MCKENZIE and WALLACE, 1954). Soluble protein content was estimated by Lowry's method (LOWRY *et al.*, 1951). Total sugar (%) content was estimated by DUBIOS *et al.* (1956).The mean values of five plants, for each replication, in 47 accessions were used for analysis of variance. The following procedure was adopted for the estimation of different statistical parameters.

Analysis of variance

Source of variation	Degree of freedom	Mean squares	Expected squares	Mean
Replications (r)	(r-1)	Mr	$\sigma^2e + g\sigma^2r$	
Genotypes (g)	(g-1)	Mg	$\sigma^2 + r\sigma^2g$	
Error	(r-1) (g-1)	Me	σ^2e	
Total	rg-1			

Where,

d.f = degree of freedom
S.S = Sum of squares
M.S = Mean squares
Mr = Mean sum of squares due to replication
Mg = Mean squares due to genotype
Me = Mean squares due to error
r = Number of replications
g = Number of treatments (varieties)

The mean square due to replication and genotypes were tested against error variance by 'F' test at (r-1), (r-1) (g-1) and (g-1), (r-1) (g-1) degree of freedom respectively at 5 and 1 percent levels of significance.

$$\text{Genotypic variance } (\sigma^2 g) = \frac{(Mg - Me)}{r}$$

$$\text{Phenotypic variance } (\sigma^2 p) = \sigma^2 g + \sigma^2 e$$

$$\text{Error variance } (\sigma^2 e) = Me$$

Morphological diversity analysis

Genetic diversity based on morphological traits was estimated by Numerical Taxonomic and Multivariate Analysis System (NTSYS-pc) version 2.02e (ROHLF, 1998) software programme. Dissimilarity matrix values based on Euclidian distances (Euclid Coefficient of dissimilarity) were calculated and dendrogram was constructed using UPGMA (Unweighted Pair Group Method using Arithmetic Averages) method of SAHN (SNEATH and SOKAL, 1973) programme available in NTSYS.

DNA extraction and quantification

Plant DNA was isolated following a modified DNA isolation method using 4% (w/v) CTAB (Cetyl Trimethyl Ammonium Bromide) (DOYLE and DOYLE, 1990). Quantity and quality of DNA samples were determined by gel electrophoresis.

SSR analysis

Thirty four SSR primer pairs were selected from previously conducted mapping and diversity studies in pea (ZONG *et al.,* 2008; TAR'AN *et al.,* 2005; NASIRI *et al.,* 2009) (Table 1). *In vitro* amplification using polymerase chain reaction (PCR) was performed in a 96 well microtiter plate in an M J Research PTC 200 or Eppendorf Master Cycler using 20 ng/µl of genomic DNA of each genotype in a final volume of 20 µl per reaction. The PCR mix consisted of 2.0 µl template DNA, 1.0 µl of each primer (5.0 µM), 0.5 µl dNTP (1 mM), 2.0 µl$MgCl_2$ (25 mM), and 0.2 µl Taq polymerase (5U/µl),4.0 µl PCR buffer (10 X) in a total reaction volume of 20 µl. The PCR conditions were: initial denaturation at 94°C for 3 min, followed by 38 cycles of denaturing at 94°C for 0.5 min, annealing at optimum temperature (48.1-59.7ºC) depending upon the primer sequence (Table 1)for 0.5 min and 72°C for 2 min. After the last cycle, samples were kept at 72°C for 5.5 min for final extension. The PCR products were resolved on 2.5% agarose (Amresco 30175 Solon Ind. PKWY, Solon, Ohio 44139) gel. The gel was run at 10 V/cm, visualized under UV light and photographed using UVP gel documentation system (Model GDS 7600).

The SSR allele sizes were determined by the position of bands relative to the DNA ladder. Total number of alleles was recorded for each microsatellite marker in all the genotypes under study by giving the number to amplified alleles as 1, 2, 3 and 4. The amplified bands were recorded as 1 (band present) and 0 (band absent) in a binary matrix. The lines that did not show any amplification were scored as null alleles and the amplification was repeated 2-3 times. If the band appeared in the negative control the whole PCR reaction experiment was repeated.

Table 1. Details of SSR markers employed in the present study

Locus	Sequence('5-3')	Length (bp)	T_m (ºC)
PSMPB14	F: GAGTGAGCTTTTTAGCTTGCAGCCT	25	59.7
	R: TGCTTGAGAACAGTGACTCGCA	22	
PSAA18	F: CTGTAGACCAAGCCCAAAAGAT	22	55.0
	R: TGAGACACTTTTGACAAGGAGG	22	
PSAA175	F: TTGAAGGAACACAATCAGCGAC	22	55.7
	R: TGCGCACCAAACTACCATAATC	22	
PSAC58	F: TCCGCAATTTGGTAACACTG	20	53.4
	R: CGTCCATTTCTTTTATGCTGAG	22	
PSAC75	F: CGCTCACCAAATGTAGATGATAA	23	52.8
	R: TCATGCATCAATGAAAGTGATAAA	24	
PSAA219	F: ATTTGTGCAATTGCAATTTCATT	23	51.1
	R: CGAAAACGCTTTGCATCCTA	20	
PSAD83	F: CACATGAGCGTGTGTATGGTAA	22	55.0
	R: GGGATAAGAAGAGGGAGCAAAT	22	
PSAD270	F: CTCATCTGATGCGTTGGATTAG	22	53.4
	R: AGGTTGGATTTGTTGTTTGTTG	21	
PSAA456	F: TGTAGAAGCATAAGAGCGGGTG	22	56.7
	R: TGCAACGCTCTTGTTGATGATT	22	
PSAB23	F: TCAGCCTTTATCCTCCGAACTA	22	55.1
	R: GAACCCTTGTGCAGAAGCATTA	22	
PSAB47	F: TCCACAATACCATCTAAATGCCA	23	54.2
	R: AATTTGTTCAGTTGAAATTTCGTTTC	26	
PSAD280	F: TGGTGCTCGTGATTAATTTCACATA	25	54.8
	R: ACTAAACAACCAACTGCCAAAACTG	25	
PSAB109	F: GAACCCTTGTGTAGAAGCATTTGTG	25	56.5
	R: GAGCTACTGTGAGTCTGATGCCATTAT	27	
PSAB141	F: ATCCCAATACTCCCACCAATGTT	23	56.3
	R: AGACTTAGGCTTCCCTTCTACGACTT	26	
AD134	F: TTTATTTTTCCATATATTACAGACCCG	27	51.6
	R: ACACCTTTATCTCCCGAAGACTTAG	25	
AA315	F: AGTGGGAAGTAAAAGGTGTAG	21	51.8
	R: TTTCACTAGATGATATTTCGTT	22	
PSGAPA1	F: GACATTGCCAATAACTGG	18	48.5
	R: GGTTCTGTTCTCAATACAAG	20	
AF016458	F: CACTCATAACATCAACTATCTTTC	24	49.5
	R: CGAATCTTGGCCATGAGAGTTGC	23	
AF004843	F: CCATTTCTGGTTATGAAACCG	21	51.8
	R: CTGTTCCTCATTTTCAGTGGG	21	

AA430902	F: CTGGAATTCTTGCGGTTTAAC	21	52.2
	R: CGTTTTGGTTACGATCGAGCAT	22	
PSMPA6	F: CTTAAGAGAGATTAAATGGACAA	23	48.1
	R: CCAACTCATAATAAAGATTCAAA	23	
PSMPA9	F: GTGCAGAAGCATTTGTTCAGAT	22	54.0
	R: CCCACATATATTTGGTTGGTCA	22	
PSMPB16	F: GCATTTGTGCAGTTTCAATTTCG	23	54.1
	R: CCAATTACGGACAATGTTTGATCA	24	
PSMPD21	F: TATTCTCCTCCAAAATTTCCTT	22	49.6
	R: GTCAAAATTAGCCAAATTCCTC	22	
PSMPD23	F: ATGGTTGTCCCAGGATAGATAA	22	52.9
	R: GAAAACATTGGAGAGTGGAGTA	22	
PSMPSAA5	F: TGCCAATCCTGAGGTATTAACACC	24	56.9
	R: CATTTTTGCAGTTGCAATTTCGT	23	
PSMPSAA205	F: TACGCAATCATAGAGTTTGGAA	22	51.8
	R: AATCAAGTCAATGAAACAAGCA	22	
PSMPSAA278	F: CCAAGAAAGGCTTATCAACAGG	22	53.8
	R: TGCTTGTGTCAAGTGATCAGTG	22	
PSMPSAD141	F: AATTTGAAAGAGGCGGATGTG	21	53.7
	R: ACTTCTCTCCAACATCCAACGA	22	
PSMPSAD237	F: AGATCATTTGGTGTCATCAGTG	22	52.8
	R: TGTTTAATACAACGTGCTCCTC	22	
PSMPSAA473	F: CAATCGATCAGACAGTCCCCTA	22	55.8
	R: AAGCTCACCTGGTTATGTCCCT	22	
PSMPC20	F: GAGTTCTCCGTAATAGAAGGCT	22	53.7
	R: CACTCTGTTCTGCTTCATCATC	22	
PSMPAA67	F: CCCATGTGAAATTCTCTTGAAGA	23	53.0
	R: GCATTTCACTTGATGAAATTTCG	23	
PSMPSAD135	F: TGGCATTAGATTCTCCAGCACA	22	56.4
	R: TGAGGAGGTGAACGTAAAAGCA	22	

Statistical analysis

The data was also subjected to Unweighted Pair Group Method with Arithmetic Mean (UPGMA) analysis to generate Neighbour Joining (NJ) tree dendogram using the software DARwin 5.0 (PERRIER and JACQUEMOUD-COLLET, 2006). Factorial correspondence analysis (FCA) was performed using the same software. PIC value for each primer was calculated using the formula given by NEI (1983) to determine the extent of polymorphism shown by each SSR marker.

RESULTS

Variability studies using morphological traits

Garden pea genotypes were examined to assess the amount of variability present among different genotypes in respect to a number of morphological and biochemical traits. The treatment mean squares were highly significant for all the studied traits.

Contribution of different traits towards genetic divergence

The contribution of the characters towards the genetic divergence is presented in Table 2. Out of the twenty four traits evaluated, green pod yield per plant contributed the maximum (43.18%) towards the observed diversity, followed by plant height (20.19%) and number of nodes per plant (10.27%). Days to first flowering (1.76%), primary branches per plant (1.30%), 100 seed weight (2.02%) and crude protein (1.09%) contributed very little while, total sugars (5.03%), grain filling period (4.11%) and seed yield per plant (4.20%) moderately contributed to overall genetic diversity. First blossom node, total pods per plant, number of seeds per pod, number of locules per pod, pod weight, pod length, pod width and pod thickness had no significant contribution towards the divergence.

Table 2. Per cent contribution of the traits towards genetic divergence

Source	Times ranked First	Contribution (%)
Days to first flowering	19	1.76
Days to 50% flowering	29	2.68
First blossom node	0	0.00
No. of primary branches per plant	15	1.30
Plant height (cm)	226	20.91
Days to first pod picking	2	0.19
Days to last pod picking	23	2.13
Number of nodes per plant	111	10.27
Number of podding nodes per plant	1	0.09
Number of pods per plant	0	0.00
Grain filling period (days)	48	4.11
Number of seeds per pod	0	0.00
Number of locules per pod	0	0.00
100 seed weight (g)	23	2.02
Pod weight (g)	0	0.00
Pod length (cm)	0	0.00
Pod width (cm)	0	0.00
Pod thickness (cm)	0	0.00
Green pod yield per plant (g)	505	43.18
Seed yield per plant (g)	13	4.20
Shelling percentage	4	0.37
Crude proteins (%)	11	1.09
Soluble proteins (%)	1	0.08
Total sugars (%)	50	5.03

Cluster analysis based on morphological data

Dendrogram of forty seven genotypes was constructed based on twenty seven morphological and biochemical traits using UPGMA method in order to study genetic diversity (Figure 1).

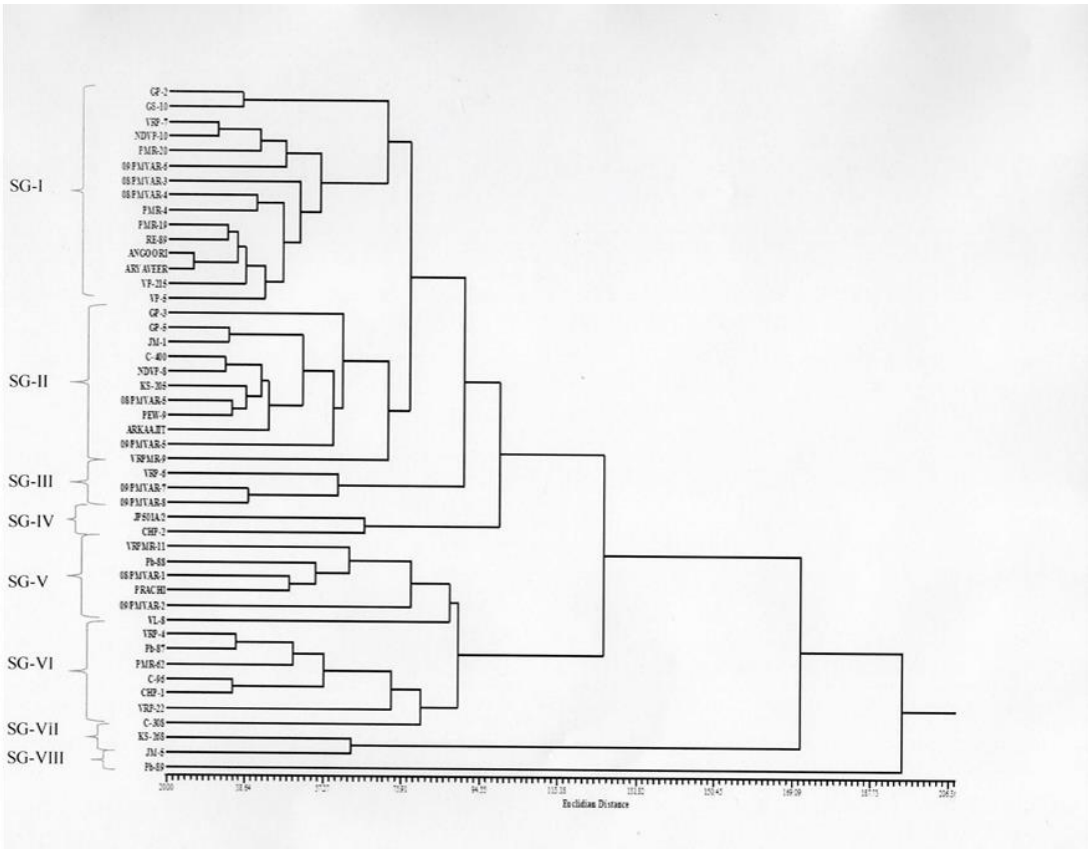

Figure 1. Dendrogram showing Euclidean Distance of 47 pea genotypes using NTSYS based on morphological data

Genetic dissimilarity values or euclidean distance (ED) between genotypes ranged from 25.68 to 195.17. The forty seven garden pea genotypes were divided into three major groups. Group I was further divided into six subgroups i.e. SG-I, SG-II, SG-III, SG-IV, SG-V and SG-VI (Figure 1). SG-I further consisted of two groups with two genotypes (GP-2 and GS-10) in one group and thirteen other genotypes in second group (Figure 1). Compared to whole dendrogram minimum euclidean distance (25.98) was observed in this subgroup between Angoori and Aryaveer. SG-II consisted of eleven genotypes (GP-3, GP-5, JM-1, C-400, NDVP-8, KS-205,

08/PMVAR-5, PEW-9, Arka Ajit, 09/PMVAR-5 and VRPMR-9). The genotypes GP-3 and VRPMR-9 were diverged from other members of SG-II by forming single lineages at Euclidean distance of 61.5 and 73 respectively. The subgroup SG-III consisted of VRP-6, 09/PMVAR-7 and 09/PMVAR-8 and diverged from other two subgroups (I and II) at a euclidean distance of 91 while SG-IV consisted of two genotypes only. In SG-V, there were six genotypes (VRPMR-11, PB-88, 08/PMVAR-1, Prachi, 09/PMVAR-2 and VL-8) while in subgroup SG-VI, there were seven genotypes (VRP-4, PB-87, PMR-62, C-96, CHP-I, VRP-22 and C-308). The subgroups SG-V and SG-VI diverged from other groups *viz.* SG-I, SG-II, SG-III and SG-IV at a euclidean distance of 124.34. All these subgroups of major group I diverged from another major group i.e. II (SG-VII) at a distance of 171. The only genotype i.e. PB-89 diverged (ED=195.17) from other seven subgroups (major group I and II) as single lineage and formed the major group III (SG-VIII). Based on clustering analysis using morphological data, no specific distinction could be made between genotypes except for SG-VII which differed from other groups for having two out of three tall type genotypes (KS-268 and JM-5), while the third tall type genotype i.e. JP501A/2 was found in SG-IV in pairing with CHP-II.

Characterization of pea germplasm using SSR markers

A total of 34 SSR markers were analyzed on 47 pea genotypes for assessing the genetic diversity. Altogether 28 primer combinations revealed 103 polymorphic patterns all revealing atleast one difference among 47 genotypes (Table 3). Presence of alleles ranged from two to eight alleles per locus. The highest numbers of alleles were amplified by primers PSMPB16 (8) followed by PSMPSAD 135 (7), PSAB 47 (6), PSAB 109 (6) and AD 134 (6) while the lowest number of alleles i.e. two, were amplified by PSMPB 14, PSAB 23, PSAB 141, PSGAPA 1, PSMPA 6, PSMPSAA 205, PSMPSAA 278, PSMPSAD 141, PSMPC 20 and PSMPAA 67 while primer PSAA175, AA315, PSMPD21, AF016458, AF004843 and AA430902 were found to be monomorphic. The PIC values provide an estimate of the discriminating power of a marker by taking into account not only the number of alleles at a locus but also relative frequencies of those alleles in the genotypes. In present study, PIC value ranged from 0.04 (PSAB 23) to 0.85 (PSMPB16) with an average PIC value of 0.55 across 47 pea genotypes (Table 3).

Genetic similarity matrix was used to construct a dendrogram based on Unweighted Pair Group Method with Arithmetic Average (UPGMA). On the basis of average linkage genetic similarity coefficient between 47 genotypes ranged from 0.59 to 0.89 as depicted in dendrogram (Figure 2). Genetic similarity (GS) index of similarity between pair of lines ranged from 0.59 (between JP501A/2 and GP-3) to 0.89 (between PB-89 and C-96 and between Angoori and GS-10). The minimum value for GS was observed between tall (JP501A/2) and dwarf type (GP-3) genotypes while maximum GS value was found between two pairs of dwarf type genotypes i.e. PB-89 and C-96, Angoori and GS-10. The high similarity observed between the genotypes PB-89 and C-96 was expected since these lines were derived from the same germplasm. The second highest GS was found between GS-10 and VRP-22 (0.86) followed by Pb-87 and PEW-9 (0.83) and between VRPMR-9 and VRPMR-11 (0.83). The second lowest GS was found between JP501A/2 and Aryaveer (0.42) followed by GP-5 and PB-87 (0.43), JM-5 and PB-87 (0.45), JP501A/2 and VRP-7 (0.47), PMR-4 and VRP-7 (0.48) and between JP501A/2 and VRPMR-9 (0.49).

Table 3.Level of polymorphism and PIC for 28 SSR markers

Primer	Chromosome No.	Number of Alleles Amplified		Percent of	PIC
		Total	Polymorphic	Polymorphism	Value
PSMPB14	7	2	1	50	0.14
PSAA18	2,5	5	5	100	0.70
PSAC58	5	4	4	100	0.70
PSAC75	1	3	3	100	0.61
PSAA219	4	5	5	100	0.75
PSAD83	2	3	3	100	0.66
PSAD270	3	4	4	100	0.71
PSAA456	7	3	3	100	0.33
PSAB23	5	2	2	100	0.04
PSAB47	1	6	6	100	0.71
PSAD280	5	5	5	100	0.71
PSAB109	2	6	6	100	0.77
PSAB141	3	2	2	100	0.24
AD134	2,3,7	6	6	100	0.78
PSGAPA1	5	2	2	100	0.43
PSMPA6	3	2	2	100	0.12
PSMPA9	4	3	3	100	0.54
PSMPB16	7	8	7	87.5	0.85
PSMPD23	2	3	3	100	0.64
PSMPSAA5	3	5	5	100	0.76
PSMPSAA205	2	2	2	100	0.34
PSMPSAA278	3	2	2	100	0.45
PSMPSAD141	3,6	2	2	100	0.50
PSMPSAD237	7	5	5	100	0.73
PSMPSAA473	2	4	4	100	0.75
PSMPC20	1	2	2	100	0.08
PSMPAA67	1	2	2	100	0.47
PSMPSAD135	6	7	7	100	0.78
TOTAL		105	103	98.09	

Genetic similarity matrix was used to construct a dendrogram based on Unweighted Pair Group Method with Arithmetic Average (UPGMA). On the basis of average linkage genetic similarity coefficient between 47 genotypes ranged from 0.59 to 0.89 as depicted in dendrogram (Figure 2).The 47 pea genotypes studied in the present investigation constituted two major groups i.e. G-I and G-II. G-I consisted of 43 genotypes (subgroups I-VII) and G-II consisting of three genotypes (subgroup VIII) comprising tall type material in the germplasm. One genotype (PMR-19) formed separate lineage from both the major groups i.e. G-I and G-II at similarity coefficient of 0.59. Major groups (I and II) diverged at a similarity coefficient of 0.60 (Figure2). Seven subgroups were distinguished from two major groups by truncating the dendrogram at

similarity coefficient of 0.66. The major group consisted of subgroups I, II, III, IV, V and VI as shown in Figure 2. The subgroup I comprised of ten genotypes GP-2, VRP-22, Angoori, GS-10, PB-89, C-96, 09/PMVAR-8, 08/PMVAR-4, JM-1 and CHP-I among which Angoori and GS-10 along with PB-89 and C-96 showed highest similarity coefficient of 0.89 between themselves. The genotypes Angoori and GS-10 along with GP-2 and VRP-22 showed a similarity coefficient of 0.79 with C-96 and PB-89. Subgroup II also consisted of four pea genotypes, *viz*. VRP-7, KS-205, 09/PMVAR-5 and 09/PMVAR-7. In subgroup II, VRP-7 and KS-205 with 09/PMVAR-5 and 09/PMVAR-9 showed 0.67 similarity coefficient while, between themselves similarity coefficient was found to be 0.77 and 0.78 respectively. Sub-group III consisted of GP-3, GP-5 and Aryaveer while subgroup IV consisted of VRP-4 and NDVP-10 which showed a 0.74 similarity coefficient.VRP-6, PMR-62, PB-88, RE-89, PMR-4, 08/PMVAR-2, C-308, C-400, PMR-20 and Arka Ajit were presented in sub group V. Subgroup VI consisted of three genotypes viz. VRPMR-9, VRPMR-11 and Prachi out of which VRPMR-9 and VRPMR-11 showed 0.84 similarity coefficientbetween themselves while they diverged at a similarity coefficient of 0.73 from Prachi. Subgroup VII consisted of 11 genotypes, out of which 09/PMVAR-2 and CHP- II showed a similarity coefficient of 0.77 with 09/PMVAR-6 while 08/PMVAR-5 and 08/PMVAR-3 showed a similarity coefficient of 0.79 with VP-5. The genotype VL-8 formed separate lineage and joined with latter three at 0.74 similarity coefficient. All these seven genotypes diverged from rest four (PB-87, PEW-9, NDVP-8 and VP-215) at similarity coefficient of 0.69. Major group G-II or subgroup VIII comprising of three genotypes (JP 501A/2, KS-268 and JM-5) being cultivated were separated from all other genotypes might be due presence of unique plant height i.e. tall type varieties while PMR-19 formed separate lineage and joined with major groups G-I and G-II at similarity coefficient of 0.59.The dendrogram obtained through bootstrap procedure clustered the genotypes into two groups mainly on the basis of tall and dwarf type cultivars but the least similarity between tall (JP501A/2) and dwarf type (GP-3) in present study was found to be 0.39.

SSR analysis showed that there exists a moderate genetic diversity among the germplasm analyzed as indicated by their grouping all together at 0.59 similarity coefficient and GS estimates from pair-wise comparisons among 47 genotypes ranges from 0.39 to 0.89. All the 47 selected genotypes were analyzed for dissimilarity coefficient using computer software Darwin 5 which is more robust and gives significance levels for tree construction. Boot strap was carried out at 3000. Neighbor joining and UPGMA method were used to generate a tree, pictorially presented in Figure 3. Tree obtained for 47 genotypes corresponded to the clustering observed using NTSYS except for genotypes of one pair of SG-II (PMR-62 and VRP-6) which diverged from each other and one pair of SG-I (09/PMVAR-8 and 08/PMVAR-4) and four genotypes of SG-VII (PEW-9, PB-87, NDVP-8 and VP-215) which got shifted from clusters obtained on the basis of similarity coefficient (but remain paired). The two systems generated very similar or identical topologies which conferred robustness to the results and indicated that the analysis was reliable. Maximum dissimilarity coefficient was shown between the genotypes JP501A/2 and GP-3 (0.75), while minimum dissimilarity coefficient (0.19) was found between two pairs one between PB-89 and C-96 and second between (Angoori and GS-10). The unrooted tree grouped the genotypes into four major groups (designated as A-I, A-II, A-III and A-IV).GP-2, VRP-22, Angoori, GS-10, PB-89, C-96, JM-1, PMR-19 and CHP-I formed the group A-I

similar to that of SG-I of similarity coefficient analysis except for 09/PMVAR-8 and 08/PMVAR-4 which diverged to group A-IV and entry of PMR-19 which formed separate lineage in NTSYS analysis. JM-1, PMR-19 and CHP-I were divergent within group A-I. Genotypes of SG-III, SG-IV and SG-V of similarity coefficient analysis formed single group A-II in dissimilarity Coefficient analysis but these subgroups remain diverged within group A-II with similar pairing pattern except for genotypes PMR-62 and VRP-6. Major group A-III comprised of all the genotypes of SG-VI, SG-VII and SG-IV of NTSYS analysis except fourgenotypes of SG-VII diverged to group A-IV. Major group A-IV comprised of 10 genotypes (four from SG-II, two from SG-I and four from SG-VII) but all the genotypes remain diverged as per NTSYS analysis. The variation between two systems viz. NTSYS version 2.2e and DARwin 5 is might be due to differences in the method of clustering by two systems i.e. by computing similarity and dissimilarity coefficients respectively.

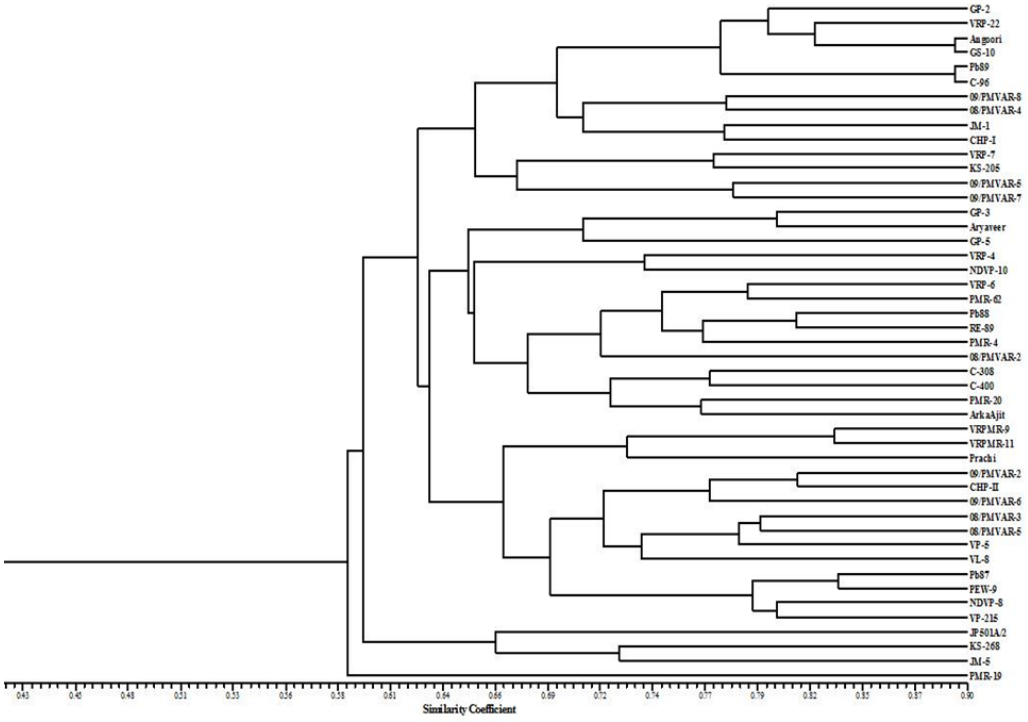

Figure 2. Dendrogram of 47 garden pea genotypes showing cluster analysis based on genetic similarity data

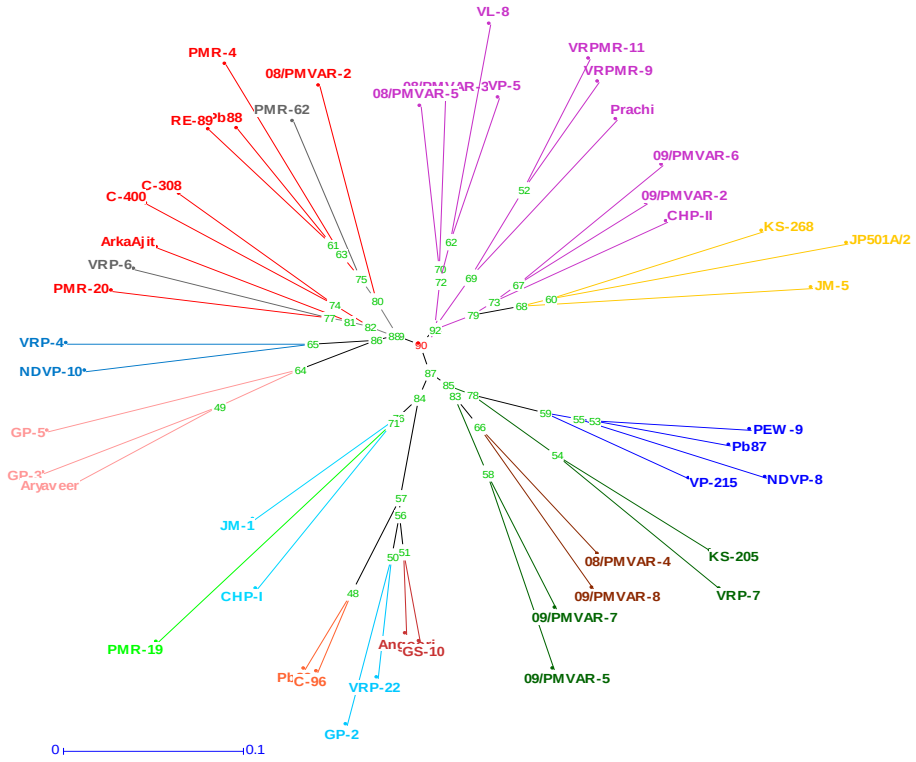

Figure 3. Dendrogram showing clustering of 47 pea genotypes based on molecular data using Darwin 5 software

Identification of variety specific markers

Seven SSR primers were able to distinguish one to three varieties on the basis of amplification of particular allele or presence of particular allele in selected genotypes (Table 4). For example PSMPC 20 marker was able to distinguish PMR-19 and NDVP-8 from rest of varieties by the presence of first allele. Similarly PSAB 23 distinguishes JP501A/2 due to second allele and PSMPA 6 was able to distinguish VRPMR-9, 08/PMVAR-1 and NDVP-8 from rest of varieties due presence of its first allele. The reproducibility of these specific markers was confirmed by three repetitions for PCR amplification and gel electrophoresis. The high discriminating power of the SSR loci suggested that a selection of the most robust SSR markers may be able to differentiate various genotypes.

Table 4. Variety specific SSR markers

Primer Name	Frequency of presence of allele	Allele Specific to varieties	Varieties
PSMPC 20	2/47	1	PMR-19, NDVP-8
PSAB 23	1/47	1	JP501A/2
PSMPA 6	3/47	1	VRPMR-9, 08/PMVAR-1, NDVP-8
AD 134	3/47	1	JP501A/2, PMR-19, CHP-II
PSAB 47	2/47	1	KS-205, VP-5
PSAA 18	2/47	1	VRP-6, NDVP-10
PSAA 456	2/47	1	VRPMR-11, VL-8

DISCUSSION

Cluster analysis based on morphological data

Morphological characteristics are used for cultivar identification however; it is known that morphological characteristics can be affected by different environmental conditions. Broadly the results were in association with study reported by KUMAR *et al.,* (1998) for clustering of sixty two pea genotypes on the basis of tall and dwarf plant types by comparing morphological traits. The present studies were also supported by the findings of SAXENA *et al.,* (1985) and KENANI *et al.,* (2003) for genetic diversity being independent of geographic distribution of evaluated genotypes.

The formation of large number of clusters with variable number of entries in each cluster is indicative of diversity. The genotypes from different countries or agro-ecological zones were found to scattered in different clusters. This suggests that a pattern of clustering of accessions was independent of their geographic origin. No parallelism was found between genetic and geographic diversity. SAXENA *et al.,* (1985) also reported that geographic origin did not correspond to the patterns of genetic diversity in *P. sativum.* This mixed grouping of genotypes from different origins in same cluster could be due to extensive utilization few donor species to generate pea genotypes across world or due to unidirectional selection pressure plasticized by the breeders in tailoring the promising cultivars. Lack of association between geographic and genetic divergence was also reported by HATAM and AMANULLAH (2001) and KENANI *et al.,* (2003) whereas in contrast NISAR *et al.,*(2008) could established clear cut separation of clusters comprising 246 accessions of pea on the basis of their geographical distribution.

Characterization of pea germplasm using SSR markers

The results for range of number of alleles per locus (2-8) is in close association to earlier reports by NASIRI *et al.*(2009) who also reported 2-8 number of alleles per locus in their analysis using 20 SSR primer pairs for establishing genetic diversity patterns in pea. The average number of alleles per primer pair in present study (3.75) is in agreement with previous studies reported by BURSTIN *et al.* (2001), TAR'AN *et al.* (2005), LORIDON *et al.* (2005) and SMYKAL *et al.* (2008b) who respectively found 3.6, 4.0, 3.8 and 3.8 as average number of alleles per locus while NASIRI *et al.* (2009) reported 3.30 as average number of alleles for cultivated varieties. The results for highest PIC value (0.85) and average PIC (0.55) value are in agreement with previous studies of NASIRI *et al.* (2009) who reported a PIC value as high as 0.839 and LORIDON *et al.* (2005), HAGHNAZARI *et al.* (2005) and SMYKAL *et al.* (2008b) reported average PIC value of

0.62, 0.53 and 0.52 respectively. The higher number of alleles amplified does not depict higher PIC values. The PIC value of a primer vary with the crop and the set of the genotypes used. Lower PIC value may be the result of closely related genotypes and higher PIC values may be the result of diverse genotypes. Marker loci with an average number of alleles running at equal frequencies have the highest PIC values (SENIOR *et al.*, 1998). SMITH *et al.* (1997) had found that the slightly higher average PIC value (0.62) probably resulted from their use of acrylamide gels for allele detection. Hence, the second reason could be due to differences in medium for resolving the amplified products i.e. agarose vs. polyacrylamide gels. In the present study, the probable reason for lower PIC value may be the closely related genotypes. The dendrogram obtained through bootstrap procedure clustered the genotypes into two groups mainly on the basis of tall and dwarf type cultivars but the least similarity between tall (JP501A/2) and dwarf type (GP-3).

SSR analysis showed that there exists a moderate genetic diversity among the germplasm analyzed as indicated by their grouping all together at 0.59 similarity coefficient and GS estimates from pair-wise comparisons among 47 genotypes ranges from 0.39 to 0.89. These results are in agreement with CUPIC *et al.* (2009) who also reported altogether similarity coefficient of 0.59, while the results for highest similarity coefficient (0.89) was in agreement with SARIKAMIS *et al.* (2010) who reported highest similarity coefficient of 0.90 while analyzing genetic diversity in 30 genotypes from Turkey. The overall genetic similarity range (0.59-0.89) is in agreement with previous study for genetic similarity range (0.59-0.87) reported by CHOUDHURY *et al.* (2007). Also, current results for genetic similarity range of 0.79 to 0.87 for most of pairs of genotypes is in agreement with previous reports by SIMIONIUC *et al.* (2002) and TAR'AN *et al.* (2005), who found high genetic similarity values (ranging from 0.84 to 0.94) between pairs of pea genotypes.

The relation among the cultivated varieties, as revealed by molecular markers were not significantly correlated with those based on morphological or biochemical characters for all the groups or pairs of genotypes, suggesting that two systems give different estimates of genetic relations among the varieties. These results may have arisen because the diversity at the molecular level, which is a priori neutral, may not reflect the diversity at the morphological or physiological level, as described by KARHU *et al.* (1996). Similar conclusions were also made by TAR'AN *et al.* (2005), SMYKAL *et al.* (2008a) and ZONG *et al.* (2009). In contrast, ESPOSITO *et al.* (2007) by using SRAP primer pairs for divergence studies in pea concluded that relationships revealed by molecular markers were significantly correlated with those based on the agronomic traits, suggesting that the two systems give similar estimates of genetic relations among the varieties.

Molecular studies showed that genotypes of diverse origin or geographical region were clustered in same group and genotypes collected or originated from same place were grouped into different and diverse clusters. In general, it was found that there is no association between the geographical region and clustering of genotypes. This may be due to free exchange of material from one place to another. Previous data on a Spanish collection of 120 pea landraces indicated that the groups formed on the basis of ISSR markers were not related to agro-climatic regions within Spain (LAZARO and AGUINAGALDE, 2006). SARIKAMIS *et al.* (2010) and MARTIN-

SANZ *et al.* (2011) were also unable to correlate the geographical origin of tested lines with the cluster relationship.

Identification of variety specific markers

Seven SSR primers were able to distinguish one to three varieties on the basis of amplification of particular allele or presence of particular allele in selected genotypes. The information revealed in cluster analysis may be useful in designing a breeding program. The selection of genotypes with higher genetic diversity level should be considered in pea breeding programs. Genetic diversity of the existing pea germplasm could be broadened by the inclusion of exotic parental material in breeding programs for desirable allele combinations which results in genetic gain.

CONCLUSIONS

Forty seven garden pea genotypes of different maturity groups collected from Indo-Gangetic plains of India were characterized using 34 pea specific microsatellite markers. Using molecular markers data, the garden pea germplasm was clearly subdivided into tall and dwarf plant types indicating that for assessing the genetic diversity, molecular markers were more reliable. The cluster analysis based on SSR markers revealed that grouping was not as per pedigree records or breeding center. Also, the relation among the cultivated varieties, as revealed by molecular markers were not significantly correlated with those based on morphological or biochemical characters, suggesting that two systems give different estimates of genetic relations among the varieties.

ACKNOWLEDGEMENTS

We gratefully acknowledge the Punjab Agricultural University, Ludhiana for providing financial support for conducting the research.

Received, February29th, 2020
Accepted December 18th, 2020

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PROUČAVANJE GENETIČKOG DIVERZITETA GERMPLAZME GRAŠKA (*Pisum sativum* L.) U INDIJI MORFOLOŠKIM I MIKROSATELITSKIM MARKERIMA

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Izvod

Četrdeset sedam genotipova baštenskog graška okarakterisano je pomoću trideset četiri mikrosatelitska markera specifična za grašak. Srednje vrednosti kvadrata bile su izuzetno značajne za sve osobine. Gotovo 75% od ukupne varijacije doprinele su 3 osobine, prinos zelene mahune po biljci (43, 18), visina biljke (20, 91) i broj nodusa po biljci (10, 27). Korišćeno je 28 polimorfnih SSR markera. Prosečna PIC vrednost bila je 0, 55. NTSIS klasterisanje zasnovano na UPGMA grupisalo je genotipove u dve glavne grupe I i II (patuljaste i visoke). Veću grupu činilo je 43 genotipa (podgrupe I-VII), a manja grupa imala je 3 genotipa (podgrupa VIII). Jedan genotip (PMR-19) se odvojio od ostalih genotipova. Dendrogram koji prikazuje ukupni koeficijent sličnosti kretao se u rasponu od 0, 59 do 0, 89. Najmanja genetska sličnost (0, 39) primećena je između glavne grupe I i glavne grupe II. Maksimalna genetska sličnost (0, 89) pronađena je između dva para, tj. PB-89 i C-96 (prvi par) i Angoori i GS-10 (drugi par). Tako dobijen klaster u velikoj meri odgovara klasterizaciji pomoću NTSIS-a, što ukazuje da je analiza bila pouzdana. Nalazi ove studije mogu se koristiti za odabir različitih genotipova koji će se koristiti kao roditelji ukrštanja namenjenih oplemenjivanju graška.

Primljeno 29.II.2020.
Odobreno 18. XII. 2020.