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Genetic Diversity Assessment in Pea (*Pisum sativum* L.) using Microsatellite Markers

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Abstract

The present study was conducted on genetic diversity analyses among 24 pea genotypes during 2017–2018 to assess the molecular diversity of pea genotypes using SSR markers. Out of 62, eleven markers were found to be polymorphic and the polymorphic information content (PIC) of the simple sequence repeat (SSR) markers ranged from 0.19 to as high as 0.64. Molecular profiling of these genotypes using 11 SSRs distributed throughout the genome generated 32 alleles with a mean of 2.91 alleles per locus. The genetic dissimilarity based on simple matching coefficient for 24 genotypes ranged from 0.00 to 0.91 with an average of 0.52. Cluster analyses grouped 24 genotypes into two major clusters with one outlier and supported by principal coordinate analysis (PCoA) in which genotypes were distributed across four quadrangles. Analysis of molecular variance (AMOVA) showed significant estimated value at degree of 1000 permutations. Percentage of variability was higher among individual (67%) than among populations (11%). Percentage of variability within individual was also higher (22%) than among populations (11%). Pop1 (I=0.707, He=0.446, and uHe=0.466) shows higher diversity than pop2 (I=0.630, He=0.381 and uHe=0.398). The percentage of polymorphic loci per population (PPL) ranged from 81.82% (pop2) to 90.91% (pop1) with an average of 86.36%. The present study demonstrates the utility of microsatellite markers for estimating molecular diversity as well as genotype identification in pea. This study also suggests a potential use of these markers in further association studies.

Keywords: *Peas*, genetic diversity, SSR markers, PIC

1. Introduction

The pea (*Pisum sativum* L.) is an economically and nutritionally important annual herbaceous legume belongs to the family *Fabaceae*. It is a cool season crop grown for both fresh green pods as vegetable and dried seeds as pulse throughout the world (Dahl et al., 2012; Panwar et al., 2018; Kripalini et al., 2019). It has high percentage of digestible protein along with carbohydrates and vitamins (Bastianelli et al., 1998; Singh et al., 2015; Das et al., 2019). It possesses antidiabetic, antifungal, antibacterial, anti-inflammatory, antioxidant, and antihypercholesterolemia activities and also shown anticancer property (Rungruangmaitree and Jiraungkoorskul, 2017). It is used as pulse or in soup, canned, processed or dehydrated and green shelled forms. In India it is grown as winter crop in the plains of north India and as summer crop in the hills (Kumar et al., 2016; Bijalwan

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et al., 2018). It occupies an area of about 540.48 thousand ha with annual production of 5422.14 thousand mt (Anonymous, 2018) in India and in Uttarakhand state, pea is grown on an area of 13.09 thousand ha with an annual production of 93.40 thousand mt (Anonymous, 2018). Irrigated wheat and other pulses are main competitor for pea areas in India (Govardhan et al., 2013).

Genetic improvement of the crop requires understanding of its genetic diversity. The knowledge of genetic divergence of the germplasm is one of the key factors in breeding programmes. In traditional breeding, genetic diversity and genotype identification is mainly based on morphological traits which are often affected by the environment. The potential of molecular markers, which are independent of environment effects, in evaluating genetic diversity and its successful implementation in crop improvement has been well recognized. Several molecular markers viz., restriction fragment length polymorphisms (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and SSR (simple sequence repeat) etc. have been used in the analyses of pea genetic diversity and linkage map construction (Lu et al., 1996; Loridon et al., 2005; Nasiri et al., 2008; Ahmad et al., 2010; Ahmad et al., 2012; Cieslarova et al., 2012; Sun et al., 2014; Handerson et al., 2014; Rana et al., 2017).

Microsatellites, or SSRs are short tandem nucleotide repeats of 1–6 bp with repetition up to 100 times (Tautz and Schlotterer, 1994). SSRs are typically single locus, multi-allelic and codominant markers. They require low molecular weight sample DNA, easy to detect by PCR, relatively abundant, and located uniformly throughout genomes of eukaryotes (Powell et al., 1996). SSR loci are inherently unstable with high mutation rates, a phenomenon that is reported to be caused by DNA polymerase slippage and/or unequal recombination. Due to their high mutability, SSRs play a significant role as molecular markers for evolutionary and population genetic studies. SSRs have become popular for genetic diversity and mapping of various traits in many crops (Holton, 2003; Choudhary et al., 2016) because of several advantages compared to other molecular markers such as co-dominant nature, high reproducibility, high polymorphism, abundancy and richness, ease of manipulation, PCR-based and readily portable within a species; of these features, they are widely employed. With the above view point, the present study was undertaken with the objective to assess the molecular diversity of pea genotypes using SSR markers.

2. Materials and Methods

The investigation was undertaken at ICAR-Vivekananda Parvatiya Krishi Anusandhan Sansthan, Almora during November, 2017–June, 2018. The experimental material for the present investigation comprised of 24 garden pea genotypes including six adapted varieties (Arkel, AP-3, VM-7, VM-10, VM-11 and VM-12) and 18 advanced lines developed at ICAR-VPKAS, Almora. Simple Sequence Repeats (SSR) markers were identified from linkage maps of earlier study (Loridon et al. 2005; Sun et al. 2014) based on the polymorphism reported. Total genomic DNA was isolated from 5 g m^{-1} of fresh young leaf tissue following the cetyl tri-methyl ammonium bromide (CTAB) method (Murray and Thompson, 1980). DNA was precipitated with chilled iso-propanol and DNA pellet was rinsed with 70% ethanol for 10-15 min to remove excess CTAB. The pellet was dried at room temperature overnight and dissolved in TE buffer (pH=8). The purified DNA was quantified on 0.8% agarose gel along with a 100 bp DNA ladder. The total genomic DNA was diluted to 10 ng μl^{-1} for use in PCR analysis.

PCR reactions were carried out in a Veriti Thermal Cycler (Applied Biosystems®) using PCR microplates. The total PCR reaction volume was 10 µl, composed of 1.0 µl of 10 ng genomic DNA, 1.0 µl 10X PCR buffer (Tris with 15 mM $MgCl_2$, Conc. 10X), 0.8 µl deoxynucleotides (dNTPs), 1.0 µl primer, 0.2 µl Taq DNA polymerase (conc. 3 U μl^{-1}) and 6.0 µl sterile deionized water. PCR tubes containing the above components were capped and given a pulse spin to allow proper settling of reaction mixture. PCR amplification was performed with initial denaturation at 94°C for 4 min followed by 35 cycles of 94°C for 30s, annealing step for 45s with respective annealing temperatures (depending upon the primer), 72°C for 1 min, and a final extension at 72°C for 7 min before cooling at 4°C. Amplification products were resolved on 3.5% metaphor agarose gel and DNA bands were visualized by staining with ethidium bromide. Out of 62 markers, 31 were found to show amplification and of which 20 markers were found to be monomorphic. Therefore, 11 polymorphic markers were used for the genetic diversity assessment of garden pea genotypes.

The amplicons were scored as alleles for each of the marker loci. The alleles were scored manually and allele sizes (base pairs) were determined comparing with 100 bp DNA ladder. The allele size data from all the markers were used for statistical analysis. Total number of alleles, major allele frequency, gene diversity, heterozygosity and polymorphic information content (PIC) were estimated using PowerMarker V3.25 (Liu and Muse, 2005). Any allele appearing in only one genotype was considered as unique allele, whereas allele with a frequency of <0.05 was considered as rare allele. Genetic dissimilarity was calculated for pairwise comparison of genotypes using Jaccard's coefficient with 1000 bootstraps. Neighbour-Joining method implemented in DARwin6 was used for constructing the dendrogram (Perrier et al., 2006). Principal coordinate analysis (PCoA) was calculated to supplement the clustering pattern (Gower, 1966). SSR allelic composition for each genotype at every marker locus was determined by counting the number of alleles per locus and the allele frequencies and polymorphism information content (PIC) was determined using the formula,

$$\mathrm{PIC} = \left(1 - \sum_{i=1}^{k} \hat{p}_i^2\right) \frac{2n}{2n-1} - \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} 2\hat{p}_i^2 \hat{p}_j^2$$

where *pi* was the estimated allele frequencies of *k* alleles (*i*=1 to *k*) and *n* was the number of individuals. Analysis of Molecular Variance (AMOVA) was carried out using GenAlEx 6.5 (Peakall and Smouse, 2012). From AMOVA, the fixation index (Fst) and Nm (haploid number of migrants) within the population were obtained from GenAlEx v6.503 (Peakall and Smouse, 2012). Fst measures the amount of genetic variance that can be explained by population structure based on Wright's F-statistics (Wright, 1965), whereas Nm=[(1/Fst) − 1]/4. An Fst value of 0 indicates no differentiation between the subpopulations whereas a value of 1 indicates complete differentiation (Bird et al., 2017). In addition, genetic indices such as number of loci with private allele, number of different alleles (Na), number of effective alleles (Ne), Shannon's information index (I), observed heterozygosity (Ho) and expected heterozygosity (He) were also calculated using GenAlEx v6.503 (Peakall and Smouse, 2012).

3. Results and Discussion

A set of 11 polymorphic SSR markers was used for genetic diversity assessment of 24 garden pea genotypes. The marker statistics of the polymorphic markers are presented in Table 2. A total of 32 alleles were detected with a mean of 2.91 and a range of 2 to 6 alleles per locus. The allele size ranged from 90 to 500 bp, thereby revealing a high level of genetic diversity. The average major allele frequency was found to be 0.66, with a range of 0.50 (AA-53, AB-72 and AD-270) to 0.88 (AA-122) (Table 1). The highest gene diversity observed was 0.68 (AA-53), whereas the lowest was 0.22 (AA-122) with an average of 0.46. The polymorphic information content (PIC) of the SSR markers ranged from 0.19 to as high as 0.64. AA-53 amplified as many as 6 alleles with a PIC of 0.64 whereas AA-122 amplified only 2 alleles with a PIC as low as 0.19. Handerson et al. (2014) reported PIC values ranging from 0.105 to 0.560 per locus in pea. Higher average PIC value was reported by Ahmad et al. (2012), Cieslarova et al. (2012) and Rana et al. (2017).

Table 1: Primer details and summary statistics of genotyping assay in 24 genotypes

Marker	MAF	NAA	GD	EH	PIC
A-5	0.63	4	0.55	0.00	0.50
AA-53	0.50	6	0.68	1.00	0.64
AA-122	0.88	2	0.22	0.00	0.19
AA-317	0.64	3	0.49	0.14	0.41
AB-28	0.79	2	0.33	0.00	0.28
AB-68	0.60	2	0.48	0.13	0.36
AB-72	0.50	2	0.50	0.00	0.38
AB-122	0.67	3	0.49	0.00	0.42
AC-76	0.79	2	0.33	0.00	0.28
AD-270	0.50	4	0.64	0.00	0.58
AD-73	0.79	2	0.33	0.00	0.28
Mean	0.66	2.91	0.46	0.12	0.39

MAF: Major allele frequency; NAA: No. of alleles amplified; GD: Gene diversity; EH: Expected Heterozygosity (Hu)

Molecular marker-based estimation of heterozygosity among the inbred lines has been found to be a useful tool to assess the cycle of inbred development besides understanding the purity of the seed lot. SSR markers being co-dominant in nature can differentiate heterozygotes from the homozygotes. The expected heterozygosity for the marker loci ranged from 0.00 to 1.00. The mean heterozygosity detected by all SSR loci was 0.12, thereby suggesting that majority of the loci (except few loci) have attained homozygosity and genotypes are fixed. However, some loci such viz., AA-53 (1.00), AA-317 (0.14) and AB-68 (0.13) showed relatively high heterozygosity (Table 1). Heterozygosity in some of these loci has also been reported earlier (Rana et al., 2017). Yang et al. (2014) observed number of alleles ranged from two to five, the heterozygosity from 0 to 0.96 in grasspea (*Lathyrus sativus* L.) using SSRs. Marker, AA-53 produced unique allele (145bp) whereas three rare alleles were found namely A-5 (350bp), AA-53 (145bp) and AA-317 (300bp). The markers that generated unique alleles may prove useful as they often associate with trait expression and can be used in fingerprinting of the inbreds and varieties as well as to identify the purity of the hybrid (Nepolean et al., 2013; Sivaranjani et al., 2014; Choudhary et al., 2016; Ram et al., 2016).

The genetic dissimilarity calculated based on Jaccard's coefficient for 24 genotypes ranged from 0.00 to 0.91 with an average of 0.52 (Table 2). The genotypes VRP-6 and VP 1332 were found to be the most diverse showing maximum dissimilarity (0.91) whereas the genotypes VP 1018, VP 1228, VP 1218 and VP 1219 were found to be the most similar (0.00).

Cluster analyses grouped 24 genotypes into two major clusters, some of them having sub-clusters (Figure 1). Cluster 1 emerged as largest cluster possessing five released varieties (Arkel, AP-3, VM-7, VM-10 and VM-11) and seven advanced lines (developed at ICAR-VPKAS, Almora). Cluster 2 also have two sub-clusters with a total of 11 lines including one released variety (VM-12) and 10 advanced lines, along with as VP-907 as outlier. The result of cluster analysis was further supported by principal coordinate analysis (PCoA) in which genotypes were distributed across four quadrangles. The result of molecular data-based cluster analysis also correlates to good extent with dendrogram derived on the basis of morphological, yield and other quality traits e.g., except for genotypes namely VP 1228, VP 1332, VP 1511, VM-10 and VM-11 (Figure 1 and 2). However, each group has more subgroups on basis of morphological based clustering as compared to molecular data, being more affected by the environment. Hence, molecular markers (being environment neutral) generate precise grouping of genotypes. Furthermore, more similarity in morphological and molecular data-based grouping can be

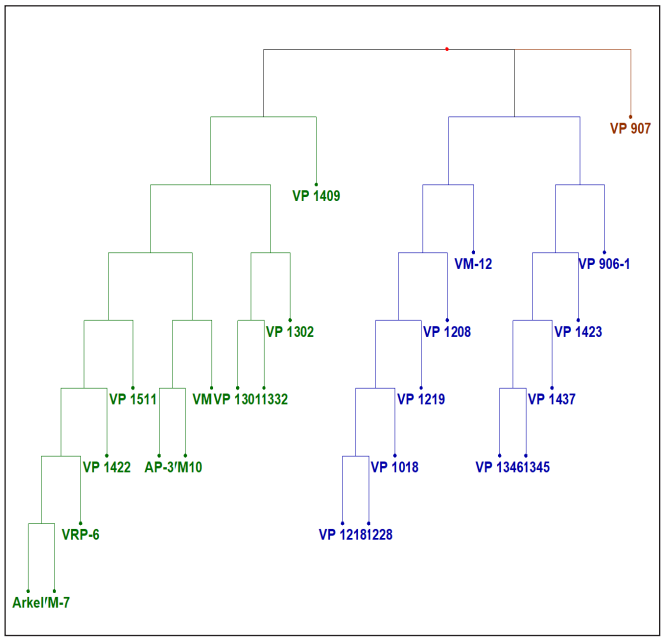

Figure 1: Cluster analysis (based on molecular data) depicting genetic relationships among 24 pea genotypes

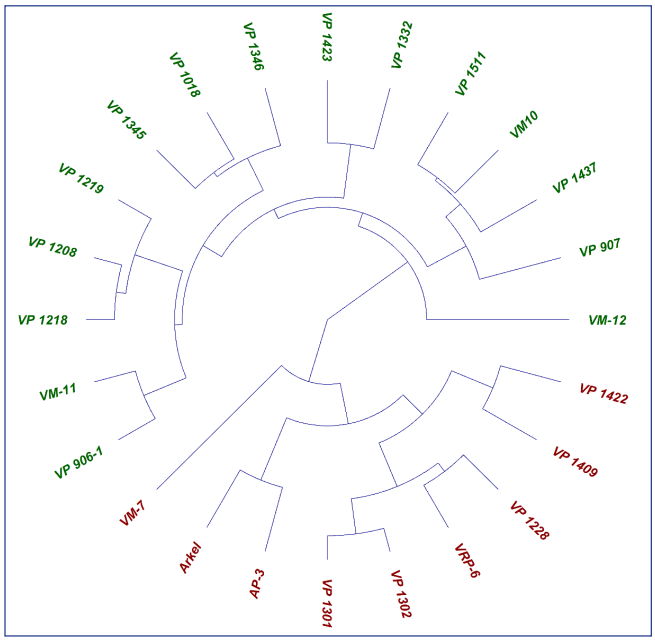

Figure 2: Dendrogram based on similarity of morphological traits and yield parameters among 24 garden pea accessions

Table 2: Dissimilarity matrix of pea genotypes based on pairwise comparison of genotypes

	1	2	3	4	5	6	7	8	9	10	11	12
2	0.71											
3	0.71	0.59										
4	0.71	0.67	0.59									
5	0.17	0.71	0.71	0.71								
6	0.43	0.74	0.67	0.80	0.31							
7	0.78	0.40	0.67	0.80	0.78	0.59						
8	0.53	0.86	0.74	0.67	0.53	0.67	0.80					
9	0.78	0.74	0.67	0.40	0.78	0.80	0.67	0.40				
10	0.78	0.67	0.59	0.40	0.78	0.80	0.59	0.50	0.15			
11	0.78	0.74	0.67	0.40	0.78	0.80	0.67	0.40	0.00	0.15		
12	0.78	0.74	0.67	0.40	0.78	0.80	0.67	0.40	0.00	0.15	0.00	
13	0.78	0.74	0.67	0.40	0.78	0.80	0.67	0.40	0.00	0.15	0.00	0.00
14	0.78	0.81	0.61	0.53	0.78	0.86	0.86	0.53	0.44	0.53	0.44	0.44
15	0.78	0.74	0.67	0.59	0.84	0.91	0.86	0.74	0.67	0.67	0.67	0.67
16	0.63	0.68	0.68	0.61	0.71	0.86	0.81	0.61	0.68	0.68	0.68	0.68
17	0.71	0.74	0.74	0.67	0.78	0.80	0.74	0.59	0.59	0.59	0.59	0.59
18	0.63	0.80	0.59	0.40	0.53	0.59	0.80	0.50	0.40	0.40	0.40	0.40
19	0.43	0.86	0.74	0.74	0.31	0.50	0.86	0.50	0.74	0.74	0.74	0.74
20	0.72	0.75	0.61	0.61	0.72	0.75	0.68	0.53	0.33	0.44	0.33	0.33
21	0.72	0.70	0.56	0.56	0.72	0.76	0.70	0.56	0.38	0.47	0.38	0.38
22	0.63	0.78	0.53	0.53	0.53	0.63	0.84	0.71	0.63	0.63	0.63	0.63
23	0.71	0.74	0.50	0.59	0.71	0.80	0.80	0.59	0.50	0.59	0.50	0.50
24	0.72	0.75	0.61	0.61	0.72	0.75	0.68	0.53	0.33	0.44	0.33	0.33

Table 2: Continue...

	13	14	15	16	17	18	19	20	21	22	23
2											
3											
4											
5											
6											
7											
8											
9											
10											
11											
12											
13											
14	0.44										
15	0.67	0.44									
16	0.68	0.56	0.33								
17	0.59	0.68	0.40	0.33							
18	0.40	0.53	0.59	0.61	0.50						
19	0.74	0.75	0.80	0.75	0.74	0.50					
20	0.33	0.47	0.68	0.76	0.68	0.53	0.53				
21	0.38	0.41	0.63	0.71	0.70	0.56	0.56	0.07			
22	0.63	0.53	0.63	0.71	0.71	0.31	0.31	0.36	0.36		
23	0.50	0.33	0.59	0.68	0.74	0.59	0.59	0.21	0.14	0.31	
24	0.33	0.47	0.68	0.76	0.68	0.53	0.53	0.00	0.07	0.36	0.21

1: VM-7; 2: VM-10; 3: VM-11; 4: VM-12; 5: Arkel; 6: VRP-6; 7: AP-3; 8: VP 907; 9: VP 1018; 10: VP 1208; 11: VP 1228; 12: VP 1218; 13: VP 1219; 14: VP 906-1; 15: VP 1332; 16: VP 1301; 17: VP 1302; 18: VP 1409; 19: VP 1422; 20: VP 1345; 21: VP 1437; 22: VP 1511; 23: VP 1423; 24: VP 1346; Source: ICAR-VPKAS, Almora

attributed to limited molecular markers in present study.

Analysis of molecular variance (AMOVA) was carried out to assess variability among and within suggested groups. Results of analysis (Table 3) showed significant estimated value at degree of 1000 permutations. Percentage of variability was higher among individual (67%) than among populations (11%). Percentage of variability within individual was also higher (22%) than among populations (11%). Similar results were obtained in *Camelina Sativa* and Pearl millet by Luo et al., 2019 and Chandra et al., 2020, respectively. The genetic diversity indices were calculated to check for the allelic patterns across populations (Table 4). The grand mean value of different alleles (Na) and number of effective alleles (Ne) of the two subpopulations were 2.409 and 1.913, respectively (Table 4), and the mean value for the overall population in Shannon's index (I), diversity index (h), unbiased diversity index (uh) was 0.669, 0.414 and 0.432, respectively. Pop1 (I=0.707, He=0.446, and uHe=0.466) shows higher diversity than pop2 (I=0.630, He=0.381 and uHe=0.398). The percentage of polymorphic loci per population (PPL) ranged from 81.82% (pop2) to 90.91% (pop1) with an average of 86.36%.

Table 3: Analysis of molecular variance (AMOVA) of the genetic variation among and within two subpopulations of 24 garden pea accessions using 11 SSR markers

Source	df	SS	MS	Est. Var.	%
AP	1	11.479	11.479	0.295	11%
AI	22	96.833	4.402	1.888	67%
WI	24	15.000	0.625	0.625	22%
Total	47	123.313		2.808	100%
Fst	0.105				
Nm	2.131				

AP: Among pops; AI: Among individuals; WI: Within individuals

Molecular markers are useful in assessment of genetic diversity of germplasm and to predict progeny performance

Table 4: Mean of different genetic parameters including number of samples (N), number of different alleles (Na), number of effective alleles (Ne), Shannon's index (I), diversity index (h), unbiased diversity index (uh), and percentage of polymorphic loci (PPL) in each of the two subpopulations

Pop	N	Na	Ne	I	Ho	He	uHe	F	PPL (%)
Pop1	11.82	2.45	1.98	0.71	0.09	0.45	0.47	0.85	90.91
Pop2	11.91	2.36	1.84	0.63	0.14	0.38	0.39	0.69	81.82
Total	11.86	2.41	1.91	0.67	0.12	0.42	0.43	0.78	86.36

(Gopalakrishnan et al., 2012). Microsatellite markers are reliable markers for assessment of germplasm genetic owing to its co-dominant nature and reproducibility across laboratories. Out of the 31 markers being amplified (scorable and robust fragments), 11 markers were found to be polymorphic resulting in a substantiate polymorphism percentage of 34.4%. The percent polymorphism for a diploid species such as pea is higher, establishing the robustness of the SSR markers identified for use in the present study.

4. Conclusion

We assessed 11 SSR markers that showed significant polymorphism across 24 pea accessions and also explored the cluster analysis which grouped 24 genotypes into two major clusters with one outlier and supported by principal coordinate analysis (PCoA). These polymorphic markers may be used for genotyping pea populations. The identified diverse genotypes can be tested for various traits and mapping populations can be developed from extreme phenotypic combinations.

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