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ANALYSIS OF GENETIC DIVERSITY AMONG LOCAL AND EXOTIC *PISUM SATIVUM* GENOTYPES THROUGH RAPD AND SSR MARKERS

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Abstract

Pea is an important temperate legume that is used as a vegetable and pulse crop. Study of genetic diversity in the crop using molecular markers helps to enhance the plant breeding efforts. The experiment was conducted to evaluate 88 pea germplasm accessions from the National Genebank of Pakistan that consisted of 61 landraces and 27 improved varieties from Pakistan and other countries. Random Amplified Polymorphic DNA (RAPD) analysis was conducted using 20 decamer oligonucleotide primers. Fifteen previously reported primers were used for Simple Sequence Repeat (SSR) analysis. Data was recorded for 100 polymorphic RAPD bands and 51 polymorphic SSR bands. Data analysis was conducted using statistical package NTSYSpc version 2.21q. Cluster analysis of germplasm using RAPD analysis revealed three major groups. First group included 11 landraces from Pakistan and one landrace from Afghanistan. This group was further divided into two sub-groups by SSR analysis. Accession of Afghanistan was separated from 11 accessions of Pakistan that remained in the same cluster. Clustering of 11 accessions from Pakistan and one accession from Afghanistan by RAPD analysis shows that this region has distinctive landraces as previously reported. Separation of landraces from Pakistan and Afghanistan by SSR shows that Pakistan has its indigenous landraces of *Pisum sativum* germplasm. Further studies should be conducted on pea landraces and wild relatives from Pakistan for their utilization in crop improvement.

Key words: Pea, Landrace, Variety, Genetic variation, Crop improvement.

Introduction

Pea (*Pisum sativum*) is an important temperate legume crop in the world (Holdsworth *et al.*, 2017). Green peas are used as vegetable, while dry peas are used as pulse crop. Green peas are third most widely grown vegetable crop after onions and tomatoes, whereas dry peas are fourth most important pulse crop after dry beans, chickpea and cowpea (Anon., 2019). Nutritional studies show that consumption of pea improves the intake of fiber, protein, vitamins and minerals (Mitchell *et al*., 2009). Genetic diversity in pea genetic resources is imperative for plant breeding and crop improvement. Study of genetic diversity in crop germplasm is conducted through study of polymorphism in morpho-biochemical and molecular markers (Shinwari *et al.,* 2013; Jan *et al.,* 2017; Jan *et al.,* 2019). Molecular markers developed using genome sequence data are being extensively used to characterize genetic diversity in plant genetic resources including landraces, modern cultivars, and crop wild relatives (Sadia *et al.,* 2018; Shinwari *et al.,* 2018; Akbar *et al.,* 2019). Information about genetic diversity discovered using molecular techniques helps the plant breeders to identify, monitor and select the desirable genotypes for developing improved varieties (Shah *et al.,* 2015; Smith *et al.*, 2015).

Random amplified polymorphic DNA (RAPD) and microsatellite or simple sequence repeats (SSRs) are PCR based molecular markers being widely used to study the genetic diversity in crop germplasm. Kole *et al.*, (2015) used RAPD makers to analyze variation in pea varieties and recorded significant polymorphism. Wu *et al.*, (2017) studied the genetic diversity in pea germplasm from USDA and National Genebank of China (NGC) using SSR markers. Significant genetic diversity was observed in germplasm from USDA and NGC. Spring and winter type pea accessions from northern and southern China showed distinct groups. Rana *et al.*, (2017) studied world pea germplasm collection of 151 accessions using 20 SSR primers. Three groups of accessions were observed on the basis of genetic relationships through cluster analysis. It was inferred that three gene pools co-existed in accessions belonging to different geographic regions. Tahir *et al.*, (2018) conducted experiment with RAPD markers to evaluate genotypes of peas grown in Iraq. The pattern of variation among genotypes was related to their origin.

National Genebank of Pakistan has a collection of local and exotic pea germplasm. It is necessary to evaluate germplasm for utilization in crop improvement. Studies are conducted to characterize the germplasm for morphological characters and seed storage proteins (Ghafoor *et al.*, 2005; Ghafoor & Arshad, 2008; Iqbal *et al.*, 2017; Nisar *et al.*, 2008). In order to understand the genetic diversity in local and exotic pea germplasm in National Genebank of Pakistan, it is necessary to study the variation using molecular markers. The objective of this study is to estimate the genetic diversity in pea germplasm from Pakistan and comparing it with the available world germplasm collection using the PCR based techniques of RAPD and SSR.

Materials and Methods

Pea germplasm accessions were obtained from the Genebank at Plant Genetic Resources Program, National Agricultural Research Centre, Islamabad. These accessions included 34 local accessions collected from Pakistan including 25 landraces and 9 commercially cultivated varieties. There were 54 exotic accessions introduced from Afghanistan, Albania, Argentina, Brazil, Canada, China, Czech Republic, Denmark, Ethiopia, Germany, Greece, Guinea, India, Iran, Iraq, Italy, Ivory Coast, Japan, Lebanon, Mexico, Nepal, Netherlands, Norway, Paraguay, Peru, Sweden, Syria, Turkey, United Kingdom, USA, Russia and Venezuela. A total of 88 pea germplasm accessions were used in the experiment that consisted of 61 landraces and 27 improved varieties (Table 1).

Table 1. Local and exotic pea germplasm accessions used in present study.

Country	Number of accessions	Germplasm
Afghanistan	1	16581 (Landrace)
Albania	1	16710 (Landrace)
Argentina	1	03106 (Landrace)
Brazil	1	03093 (Landrace)
Canada	2	16552 (Landrace), 16577 (Improved cultivar)
China	1	16719 (Landrace)
Czech Republic	1	10238 (Improved cultivar)
Denmark	2	13207, 13218 (Improved cultivar)
Ethiopia	3	13255, 13253, 16639 (Landraces)
Germany	1	03170 (Improved cultivar)
Greece	1	16572 (Landrace)
Guinea	1	03195 (Landrace)
India	2	03097, 03109 (Landraces)
Iran	1	03105 (Landrace)
Iraq	1	03170 (Landrace)
Italy	1	16682 (Landrace)
Ivory Coast	1	03269 (Landrace)
Japan	4	31458, 31503, 31510, 37701 (Landraces)
Lebanon	1	03180 (Landrace)
Mexico	1	03270 (Improved cultivar)
Nepal	1	03135 (Landrace)
Netherlands	6	16635, 16724 (Landraces), 16488, 16489, 10312, 10293 (Improved cultivars)
Norway	1	03056 (Landrace)
Pakistan	34	03302, 03309, 03313, 03314, 03318, 10339, 10390, 10470, 10471, 10472, 10473, 10474, 10475, 10476, 10477, 10478, 10479, 10499, 10517, 10522, 10523, 10543, 10567, 10568, 10569, 10570 (Landraces), Climax, Dasan, DMR-20, DMR-4, DMR-7, Green feast, Melcon, Meteor, Rondo (Improved cultivars)
Paraguay	2	03107, 03108 (Improved cultivars)
Peru	2	03271 (Landrace), 03196 (Improved cultivar)
Sweden	2	16609, 16501 (Improved cultivars)
Syria	2	03168, 03169 (Landraces)
Turkey	3	03190, 16642 (Landraces) 16579 (Improved cultivars)
United Kingdom	3	16604, 16535 (Landraces) 02935 (Improved cultivars)
USA	2	16531, 16532 (Landraces)
Russia	1	13251 (Landrace)
Venezuela	1	03086 (Landrace)

Table 2. List of selected RAPD primers used for DNA fingerprinting of pea germplasm.

No.	Name	Sequence (5′-3′)	Polymorphic bands
P151	OPA-20	GTTGCGATCC	6
P152	OPB-01	GTTTCGCTCC	8
P163	OPB-12	CCTTGACGCA	4
P165	OPG-09	CTGACGTCAC	4
P168	OPH-05	AGTCGACGCC	7
P169	OPH-06	ACGCATCGGA	5
P197	SC10-38	GACCCCGGCA	6
P212	P4	GTTAGGTCGT	7
P282	UBC-106	CGTCTGCCCG	4
P285	UBC-155	CTGGCGGCTG	6
P286	UBC-110	TAGCCCGCTT	3
P291	UBC-183	CGTGATTGCT	2
P304	OPM-16	GTAACCAGCC	4
P306	OPN-20	GGTGCTCCGT	5
P309	OPP-04	GTGTCTCAGG	7
P311	OPQ-20	TCGCCCAGTC	2
P312	OPX-01	CTGGGCACGA	5
P313	OPAD-04	GTAGGCCTCA	3
P315	OPL-04	GACTGCACAC	8
P316	OPL-18	ACCACCCACC	4

Standard CTAB method (Doyle & Doyle, 1987) was followed for isolation of genomic DNA. Seeds were sown and germinated to get seedlings. Young leaves were taken from two weeks old seedlings and approximately 0.2g of the sample was ground to a fine powder in liquid nitrogen using a chilled mortar and pestle and then transferred to sterile 1.5ml microcentrifuge tubes. DNA extraction buffer (2xCTAB with mercaptoethanol) was added to each tube prior to incubation in 55ºC water bath for 30 minutes. Subsequently 400µl of Chloroform-Isoamyl alcohol (24:1) was added to the tubes and contents were mixed by gentle inversion for 20 minutes. Samples were centrifuged at 12000rpm for 5 minutes and upper aqueous phase was transferred into new tubes with 400µl of ice chilled Iso-propanol and centrifuged at 12000 rpm for five minutes. Supernatant was transferred to new 1.5ml tubes and added equal volume of precipitation buffer and centrifuged at 12000rpm for 1 minute to get nucleic acid at bottom. Then 100µl of HS-TE buffer and 1µl of RNase was added and the mixture was left overnight at room temperature. An equal volume of isopropanol was added to the tubes, mixed gently and kept for 15 minutes. Later supernatant was discarded. Finally DNA pellet was washed by 70% ethanol, air dried and resuspended in 50µl of 0.1xTE buffer.

After extraction of DNA it was quantified by running along with standards in 0.8% agarose gel electrophoresis with 0.5xTBE buffer at 50 volts for 20 minutes. Gel was stained with ethidium bromide and observed under the UV light. By comparison with standards the DNA quantity of each sample was determined. Using 0.1xTE buffer, 5ng/µl concentration of each DNA sample was used in the experiment.

In present study 20 RAPD primers were used. The sequence information of primers is given in Table 2. PCR solution for each DNA sample with all primers was made in a total volume of 20µl containing 1xPCR buffer, 1.5mM $MgCl_2$, 0.2mM dNTPs, 0.4µM RAPD primer and 1unit/rxn Gold Taq DNA polymerase. DNA thermal cycler was programmed for 45 cycles of 93.5ºC denaturing temperature for 1 minute, 36ºC annealing temperature for 2 minutes and 72ºC extension temperature for 3 minutes with the fastest possible thermal transitions. Electrophoresis was performed in 1.5% agarose gel. Then the gel was stained with ethidum bromide and visualized and photographed under UV light.

Large numbers of SSR markers have been identified using the sequence information of *Pisum sativum* (Burstin *et al.*, 2001; Loridon *et al*., 2005; Gong *et al.*, 2010). Primer sequences of 15 SSR markers used in the experiment are given in Table 3. PCR solution for each DNA sample with all primers was made by using 1xPCR buffer, 1.5mM $MgCl_2$, 0.2mM dNTPs, 20pmoles/rxn each SSR primer and 1unit/rsx Gold Taq DNA polymerase. Then it was subjected to 38 thermal cycles, each cycle with 94ºC denaturing temperature for 45 seconds, 55ºC annealing temperature for 1 minute and 30 seconds and 72ºC extension temperature for 3 minutes. Electrophoresis was performed in 3% agarose gel. Then the gel was stained with ethidum bromide and visualized and photographed under UV light.

Data matrices were created from photographs of gels by assigning 1 to visible bands and 0 to absent bands. Computer statistical package NTSYS pc version 2.21q (Exeter Software, Setauket, NY) was used for data analysis. Estimates of similarity were calculated using the Dice coefficient (Dice, 1945; Nei & Li, 1979). Following formula was used:

$$\text{Similarity (F)} = 2Nab/ (Na + Nb)$$

where

Na = number of bands present in accession ‘a’

Nb = number bands present in accession ‘b’ and

Nab = number of bands present in both accession ‘a’ and ‘b’.

Genetic relationship among germplasm accessions was estimated on the basis of similarity coefficients. Cluster analysis was carried out by SAHN using the unweighted pair group method with arithmetic mean (UPGMA) (Sneath & Sokal, 1973) to construct the dendrogram.

Table 3. List of selected SSR primers used for DNA fingerprinting of pea germplasm.

No.	Name		Sequence(5′-3′)	Polymorphic bands
1.	PA6	Forward	CTT AAG AGA GAT TAA ATGGAC AA	4
		Reverse	CCA ACT CAT AAT AAA GAT TCA AA	
2.	PA7	Forward	CTT GAA ATA CTA AGG CAC CAT A	3
		Reverse	GTC AAC ACT CTT TGT TTT ACC A	
3.	PA8	Forward	ACA AAG TTA GGT CAT CTA TCC A	2
		Reverse	CAT GCG ACG TTG TTA GTT A	
4.	PA9	Forward	GTG CAG AAG CAT TTG TTC AGA T	4
		Reverse	CCC ACA TAT ATT TGG TTGGTC A	
5.	PB14	Forward	GAG TGA GCT TTT TAG CTT GCA GCC T	4
		Reverse	TGC TTG AGA ACA GTG ACT CGC A	
6.	PB16	Forward	GCA TTT GTG CAG TTT CAA TTT CG	3
		Reverse	CCA ATT ACGGAC AAT GTT TGA TCA	
7.	PC20	Forward	GAG TTC TCC GTA ATA GAA GGC T	4
		Reverse	CAC TCT GTT CTG CTT CAT CAT C	
8.	PD24	Forward	CTT ATA GCG CAC ACA AGT GGT T	3
		Reverse	ATC TCA TCG CTA GGT GCT ACA A	
9.	AA31	Forward	CAT TTG TGT AGT TGT AAT TTC ATT	6
		Reverse	TGA GAA TGG ATV ATG TTA TGA T	
10.	AA67	Forward	CCC ATG TGA AAT TCT CTT GAA GA	2
		Reverse	GCA TTT CAC TTG ATG AAA TTT CG	
11.	AD99	Forward	TGG CGG AGA AAC TTT AGA TGT G	3
		Reverse	AAA TAT GGA GTT TTC GCA GGT G	
12.	AD100	Forward	TAC ACC CAA GAC GAC AAG CCT	1
		Reverse	GGA GCT TCC GCT TGA TTC TCT	
13.	AD134	Forward	TTT ATT TTT CCA TAT ATT ACA GAC CCG	5
		Reverse	ACA CCT TTA TCT CCC GAA GAC TTA G	
14.	AD141	Forward	AAT TTG AAA GAGGCGGAT GTG	3
		Reverse	ACT TCT CTC CAA CAT CCA ACG A	
15.	AD144	Forward	AGG CGT CAG CAG ATT GAC TAT T	4
		Reverse	TGC CAT CGG AAA CAA CTC TAA A	

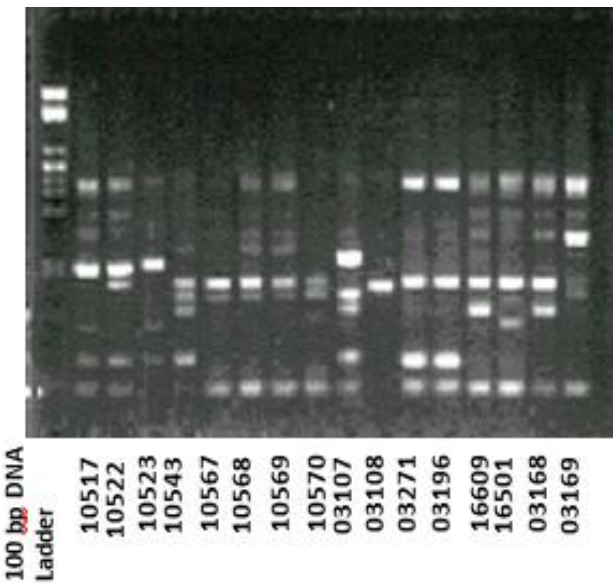

Fig. 1. RAPD analysis of pea germplasm using primer p151.

Results

The number of polymorphic amplification products generated by each RAPD primer varied from 4 (OPA-15) to 16 (OPB-17) with an average of 8.3 bands/primer. A total of 186 (89.4%) polymorphic bands were observed ranging from 2 to 15 fragments/primer. The primer OPA-17 and OPB-17 gave the highest number of polymorphic fragments (15), while the minimum number of polymorphic bands (2) using OPA-05 primer. The average number of polymorphic fragments per primer among the 88 peas genotypes was 7.4.

RAPD amplification of DNA with 20 primers generated 100 polymorphic bands (Fig. 1). Genetic similarities of RAPD profiles were estimated via Dice coefficient of similarity. The highest genetic similarity (0.982) was found between varieties Green Feast and Melcon, while accession 03314 from Pakistan and accession 03269 from Ivory Coast showed the lowest genetic similarity (0.538) (Fig. 2).

Genetic similarity data were processed by cluster analysis (UPGMA). Cluster analysis of germplasm revealed three major clusters. First cluster included 11 accessions (03302, 10476, 10477, 10478, 10569, 10570, 10479, 03309, 03313, 03314 and 03318) from Pakistan and one accession (16581) from Afghanistan. The second cluster included one accession from China, while all other accessions from Albania, Argentina, Brazil, Canada, China, Czech Republic, Denmark, Ethiopia, Germany, Greece, Guinea, India, Iran, Iraq, Italy, Ivory Coast, Japan, Lebanon, Mexico, Nepal, Netherlands, Norway, Paraguay, Peru, Sweden, Syria, Turkey, United Kingdom, USA, Russia and Venezuela were placed in third cluster (Fig. 2). Germplasm accessions in the third cluster are further sub-divided into sub-clusters. It was observed that landraces from Pakistan including 10522, 10523, 10543, 10499, 10471, 10517 and 10472 are in the same group with varieties Meteor, Climax, Green feast and Melcon, while 10473 grouped with Rondo. Other accessions from Pakistan formed sub-groups with exotic accessions. Two accessions of Pakistan including 10339 and 10390 made a distinct sub-group from other local and exotic accessions.

Data analysis was also performed for 51 polymorphic bands generated by SSR analysis (Fig. 1). Though cluster analysis on the basis of genetic similarity revealed variation in the germplasm, no geographical correlation was observed for SSR markers. Then SSR data of 12 accessions from first cluster of RAPD analysis was subjected to cluster analysis. It was observed that SSR analysis of first cluster from RAPD analysis divided it into two parts (Fig. 3). One accession from Afghanistan was separated from 11 accessions from Pakistan that remained in the same cluster.

Discussion

Pea is reported to be domesticated together with wheat and barley in Near East (Zohary & Hopf, 1973). Pakistan and Afghanistan are considered to be in Central Asia according to the geographical classification of Vavilov on the basis of origin of cultivated plants. Central Asia is center of diversity for pea (Vavilov, 1992). A distinctive landrace of the pea in Central Asia is known as 'Afghanistan' type. This landrace is found in foothills and higher slopes of Afghanistan, Pakistan, Iran and Nepal (Weeden, 2007). Afghanistan type peas are reported to cluster distinctly from most other pea accessions (Holdsworth *et al.*, 2017).

The results of this experiment agree with past studies. When cluster analysis of RAPD and SSR data is compared with the geographical origin of germplasm accessions, it was observed that although most of accessions from different countries are scattered in the clusters, one RAPD cluster contains 11 landraces from Pakistan and one landrace from Afghanistan. Further classification of germplasm by SSR analysis separated the accession from Afghanistan and only eleven landraces from Pakistan are left in one cluster. Clustering together of landraces from Pakistan first by RAPD and then by SSR analysis indicates that Pakistan has its own set of indigenous landraces of peas as reported by Weeden (2007). Accession 16719 from China was placed in a different cluster from rest of germplasm. It shows the genetic diversity of Chinese germplasm as reported by Kole *et al.*, (2015). Some landraces from Pakistan group together with improved cultivars or exotic germplasm, while some make separate sub-groups. It indicates the genetic diversity in the peas germplasm from Pakistan. Further studies should be conducted to explore the genetic diversity in landraces and wild relatives of *Pisum sativum* from Pakistan in comparison with other Central Asian countries. It may help to assist pea breeding efforts to develop cultivars adapted to this region.

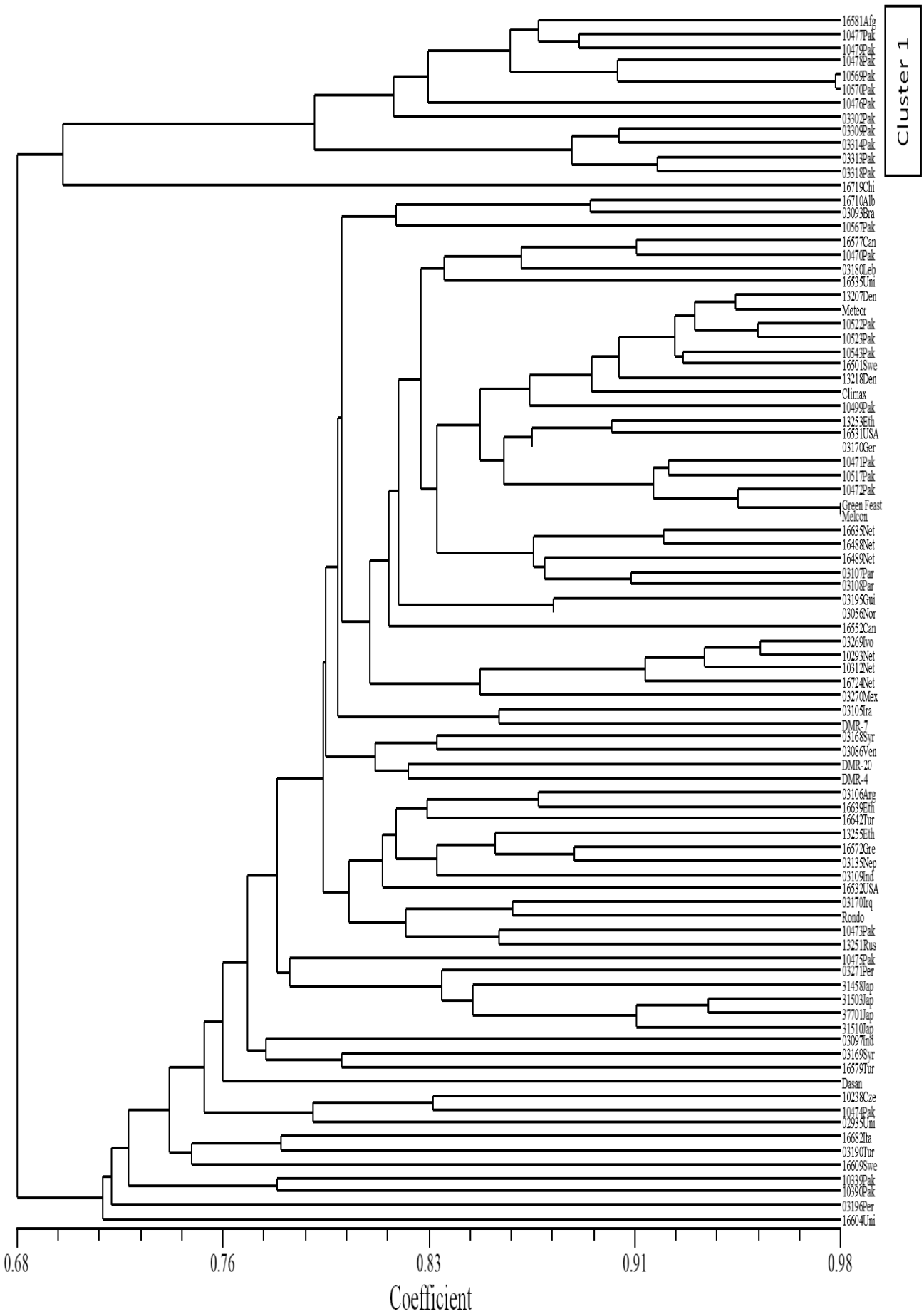

Fig. 2. Cluster analysis of pea germplasm on the basis of RAPD markers.

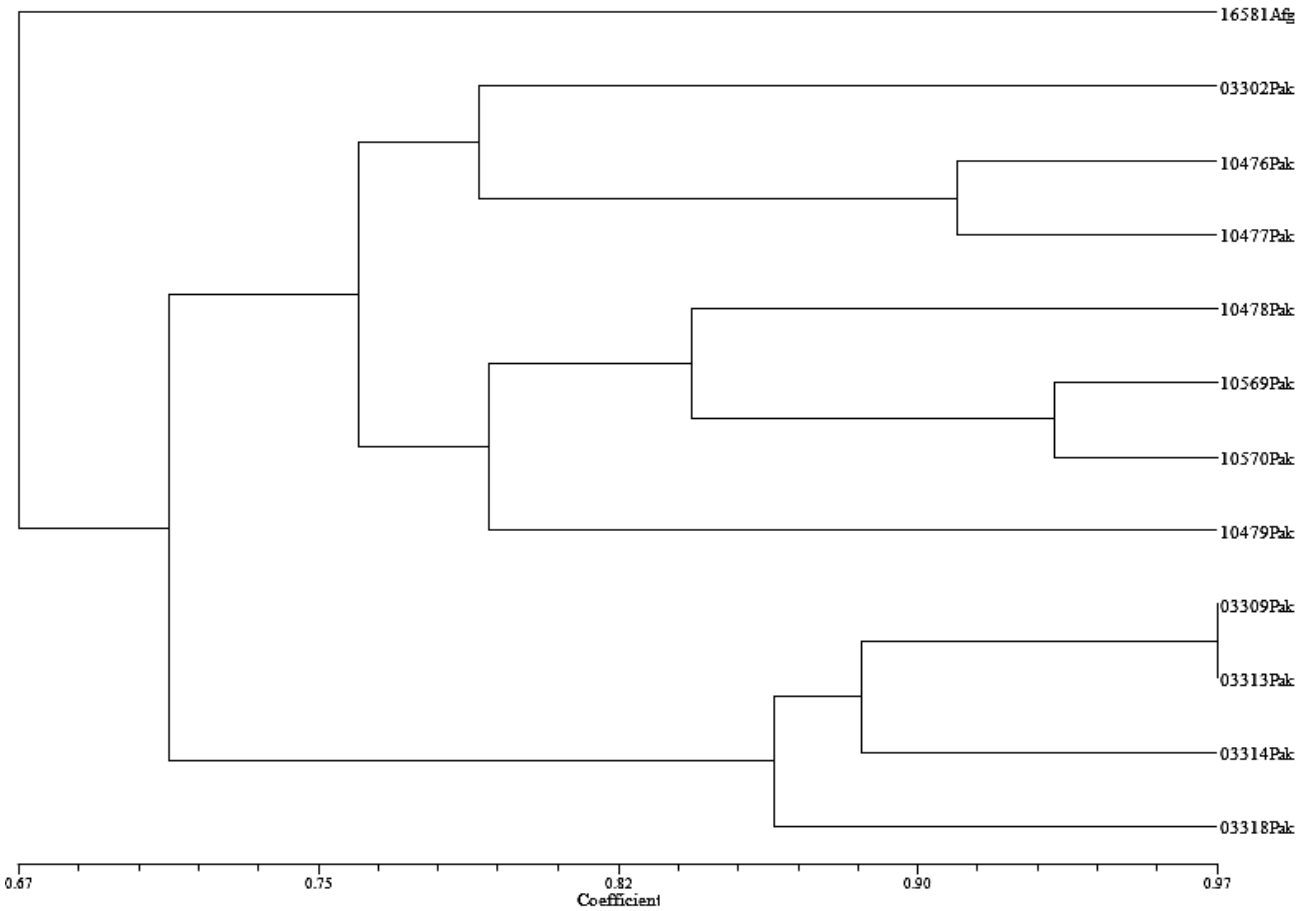

Fig. 3. Cluster analysis of pea germplasm on the basis of SSR markers.

Conclusion

Considerable genetic diversity was observed in the germplasm for RAPD and SSR markers. Peas germplasm collected from Pakistan shows significant variation. Eleven accessions from Pakistan made a distinct group similar to Afghanistan type peas. It indicates that Pakistan has its indigenous landraces of *Pisum sativum* germplasm. Collection, conservation and evaluation of peas genetic resources from Pakistan should be conducted for their utilization in crop improvement. Further studies on peas germplasm from Pakistan should be conducted using SSR or SNP markers.

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