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"Identification of Pea (*Pisum Sativum* L.) Varieties Using RAPD Markers"

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Abstract: *The present study was conducted to identify polymorphism among eight notified varieties of pea (Azad Pea 1, Azad Pea 2, Azad Pea 3, Azad Pea 4, Azad Pea 5, Arkel, Lincoln & Bonneville) for identification of varieties and diversity studies. Twelve random decamer primers were used to amplify genomic DNA of the said 8 varieties of pea and the banding pattern of all genotypes against each primer were compared to check for polymorphism followed by cluster analysis using UPGMA method. Fourteen unique bands were recognized out of all the polymorphic bands. One unique band was obtained for Azad Pea 1 from OPD-18, 4 unique bands for Azad Pea 2 from OPA-10, OPU-16, OPE-16, A-07, one band for Azad Pea 4 from OPD-07, 2 unique bands for Arkel from OPU-16 and OPN-06, 4 unique bands for Bonneville using OPA-02, OPD-07, OPY-15, OPN-06 and 2 unique bands for Lincoln from OPC-02. Cluster analysis of pea genotypes based on molecular data showed that a significant genetic variation was present among the various pea genotypes used. Bonneville was most divergent of all the genotypes used.*

1. INTRODUCTION

Pea (*Pisum sativum* L.) or Garden Pea is an annual herbaceous plant, with a life cycle of one year. It is a member of cool season legume family. It is widely cultivated in northern temperate regions. Besides their use as fresh or canned vegetable, peas are also grown for animal fodder, and for consumption as a split dry pulse or *dal*.

Despite the agricultural importance of pea, pea breeding is constrained by a large genome size of 4.3 Gbp [1], lack of genomic resources, and rich repetitive DNA, approximately75-97%of the pea gnome [2, 3].

There are number of varieties of peas available in India which are notified at national and state level. Identification and characterization of cultivars is a crucial factor to fulfill various objectives in seed production and these include verifying the identity of a variety, establishing the distinctness of a new variety, determining the varietal and hybrid purity, varietal description and documentation, varietal registration and certificate and to assure farmers for getting correct variety. Cultivars can be identified using various morphological characters also but they have their own demerits like easily influenced by environment which may lead to false identification of a variety and can take longer time therefore more time consuming. The modern technique for doing the same is with the help of molecular markers which are more rapid and reliable.

Differentiation among different varieties can be done on both phenotypic and genotypic basis. Phenotypic differentiation is possible only when the plant is fully mature and is done on the basis of visual characters only. Whereas, identification of varieties using different molecular markers can be done at any stage of growth and thus, may aid in varietal identification at any stage of growth . DNA molecular marker systems are particular segments of DNA which represent differences that are dispersed throughout the genome. Molecular markers may or may not be related with the expression of phenotypic traits, the DNA molecular marker technology which are based on sequence variation of specific genomic regions, provide powerful tools for variety or cultivar identification and seed quality control in various crops with the advantages of time-saving, less labor consumption and more efficiency [4; 5; 6; and 7].

Generally, molecular markers have proven to be useful tools for characterizing genetic

diversity in agricultural crops like tomato etc. Researchers have studied genetic variation in tomato landrace and cultivar collections using various molecular techniques, including restricted fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD) and simple sequence repeats (SSR) [8, 9,10,11,12].

Random Amplified Polymorphic DNA (RAPD) is based on *in vitro* amplification of randomly selected oligonucleotide sequences. Amplification takes place by simultaneous primer extension of complementary strands of DNA; the primers use the plant DNA as a template for PCR amplification. RAPD is very useful in the study of biodiversity, hybridization, marker assisted selection, gene mapping and genetic map construction.

It is a PCR based method which employs single primers of arbitrary sequence with 10 nucleotides (10–mer) to amplify unknown fragments from the template DNA. These are dominant type of markers. RAPDs are quick and easy to assay. In RAPDs only small amount of template DNA is needed and no prior knowledge of DNA fragment is needed. RAPDs are cost effective and are able to differentiate between genetically distinct individuals.

2. REVIEW OF LITERATURE

Pea (*Pisum sativum* L.) is a self-pollinated, cool-season annual and diploid crop having 14 chromosomes (2n=14, n=7) with a genome size of about ~5000Mbp [13]. It was the original model organism used in Mendel's discovery (1866) of laws of inheritance making it the foundation of modern genetics. However the subsequent progress in pea genome has lagged behind many other plant species largely as a consequence of genome size and low economic significance.

According to Semagn *et al.,* (2006) several molecular markers (molecular tools) are being adopted now a days for varietal identification because these techniques are reliable, unambiguous in nature and easy to adopt [14]. The underlying principle of molecular markers is that an easy to observe trait (marker) is tightly linked to a more difficult to observe trait. Consequently by selecting the already detected marker (trait) breeders indirectly locate or select for the desirable trait. These variations can be used as molecular markers to study the genetic makeup of a plant species and used to detect DNA based polymorphism [15].

RAPD's are the first PCR based molecular markers that are implied for assessing genetic variation and evolution of phylogenetic relationships. RAPD's are regarded as universal markers which get bound to many regions of genome and detect polymorphism of several chromosome regions.

These are preferred due to requirement of very small amount of DNA, easy to use and there is no need to use radioactive probes. RAPD technique has extensively been used by various researchers to detect DNA based polymorphism in Pea species.

The work done by various researchers is as under-

Samec *et al*. (1995) tested polymorphism among 6 economically important pea cultivars and found relatively high level of DNA polymorphism using polyacrylamide/urea gels and silver staining. In addition 59 polymorphisms were found using 13 arbitrarily designed primers producing 313 amplification products. Each tested cultivar was clearly distinguished from others. He found that in RAPD reactions involving 42 *Pisum sativum* genotypes representing 8 decamer primers generated 199 polymorphic products and each genotype was clearly identified and differentiated from others [16].

Cheghamirza *et al*., (2002) used RAPD method for identifying and mapping new molecular markers. In pea RAPD analysis of various cultivars and lines of pea was carried out using 10- mer random primers [17]. The presence of multiple polymorphisms between cultivars and lines was revealed, at least 1 fragment from any given primer was present in DNA of one form of pea and absent in the DNA of other form or cultivar.

Ahmad *et al*., (2010) used RAPD primers to characterise *Pisum sativum* limes at molecular level to obtain results to be utilised for selecting better parents for breeding programmes. They found that in 5 pea cultivars a total of 16 bands were scored corresponding to an average of 4 bands per primer with 6 bands showing polymorphism (37.5%). One out of 4 primers gave 75% polymorphism [18].

Tahir *et al*. (2015) studied 20 RAPD primers to estimate genetic diversity in 10 pea cultivars and found that 2 unique polymorphic bands were present a total of 89 fragments from 20 primers [19]. They concluded that analysis using RAPD markers provide more information about genetic diversity among pea cultivars whereas results of SDS PAGE showed low variation among the same pea cultivars.

3. MATERIALS AND METHOD

The present study on **"Identification of pea (*Pisum sativum* L.) varieties using RAPD markers"** was carried out at School of Biotechnology, Sher-e-Kashmir University of Agriculture Sciences and Technology of Jammu, Main Campus Chatha, Jammu.

Plant Material: A total of 8 varieties of pea (Table 1) were used in the present study for varietal identification. Seeds of these 8 varieties were grown in pots and young leaf tissue was collected for DNA extraction and further study.

Table 1: List of varieties used in the study

S. No.	Variety	Code	Source of Procurement
1.	Azad P-1	A1	CSAUT, Kanpur
2.	Azad P-2	A2	CSAUT, Kanpur
3.	Azad P-3	A3	CSAUT, Kanpur
4.	Azad P-4	A4	CSAUT, Kanpur
5.	Azad p-5	A5	CSAUT, Kanpur
6.	Arkel	A6	CSK, HPKV, Palampur
7.	Bonneville	B1	CSK, HPKV, Palampur
8.	Lincoln	L1	CSK, HPKV, Palampur

Genomic DNA Extraction and Purification

Procedure

The young leaf samples (two grams) were crushed into fine powder in liquid nitrogen using autoclaved pestle and mortar. The ground tissue was transferred to centrifuge tube containing 790 µl extraction buffer, (preheated to 65° C). The tubes containing grounded tissue were placed in water bath (with gentle intermittent shaking) for 1 hour at 65° C. In the next step equal volume of chloroform: isoamyl alcohol (CIA) mixture (24:1) was added to tissue extract and the contents were mixed by shaking gently. Then the tubes were centrifuged for 10 minutes at 13,000 rpm at 4°C.The supernatant was transferred to fresh centrifuge tubes carefully and 10ml of chilled isoproponal was added to each tube and mixed by inverting and stored at 4° overnight. The content was centrifuged again for 10 minutes at 13,000 rpm at 4° C. The supernatant was discarded. To this 80% ethanol was added and tubes were tapped for 4-5 times. Tubes were centrifuged at 8000rpm for 5 minutes at 4°C. The supernatant was discarded and tubes were air dried for 40-45 minutes till the smell of ethanol evaded and then incubated for drying purpose.50µl of distilled water was added along with 1 µl of RNase and incubated for 1 hr at 37ºC.The DNA was dissolved in TBE buffer and stored at -20° C.

DNA Quantification and Quality check:

Quantity and quality of DNA was estimated to check the concentration of DNA and contamination present in it. It was done using NanoSpec and Agarose Gel Electrophoresis.

- DNA samples were quantified using NanoSpec. Ratio between 1.8 and 2.0 shows the presence of pure DNA. The value less than 1.8 indicates the presence of protein contaminants and greater value than 1.8 indicates the presence of RNA. DNA samples were diluted using sterilized Milli Q water, to have final concentration of 50ng/µl.
- Quantity and quality (shearing if any) of DNA of all genotypes was checked by loading 2µl of DNA of each genotype mixed with 6µl of loading dye (6x) and 2µl of ddH_2O into separate wells on 0.8% agarose gel. It was prepared by melting 0.8 g agarose in 100 ml TBE (Tris, Borate EDTA, 1x) buffer in a microwave for 2 minutes. It was allowed to cool for a couple of minutes and then stained with ethidium bromide and stirred for some time. The gel material was then poured in the casting tray with combs in it and allowed to solidify for 20–25 minutes at room temperature. After the gel got solidified, combs were removed and the DNA sample was loaded into each well. The electrophoresis was carried out at 95V for 1 hour. It was then viewed under gel documentation system.

RAPD-PCR amplification

Requirements: Random primers, Template DNA: Crude genomic DNA extracts from young leaf samples of 8 varieties of peas were selected, dNTPs: The four individual dNTPs *viz*., dATP, dGTP, dCTP and dTTP were obtained, *Taq* DNA polymerase: Taq DNA polymerase (3U/µl) and 10 X *Taq* assay buffer were obtained, Nuclease free water and thermal cycler etc.

RAPD PRIMERS

A total of 12 random primers were used in the study. List of these primers along with their sequences are given below:

Table 2: List of primers used in the study

Sr. No.	Primers Name	Sequence (5'-3')
1.	OPA-02	TGCCGAGCTG
2.	OPA-07	GAAACGGGTG
3.	OPA-10	GTGATCGCAG
4.	OPB-10	CTGCTGGGAC

5.	OPC-02	GTGAGGCGTC
6.	OPC-09	CTCACCGTCC
7.	OPD-07	TTGGCACGGG
8.	OPD-18	TTGGCACGGG
9.	OPE-16	GGTGACTGTG
10.	OPN-06	GAGACGCACA
11.	OPU-16	CTGCGCTGGA
12.	OPY-15	AGTCGCCCTT

Table 3: Reagents with their working and stock concentrations of components used in a single PCR reaction.

S. No.	Reagents	Concentration (Stock)	Final Concentration in PCR reaction
1.	Template DNA	50ng/µl	150ng/µl
2.	Taq polymerase	5U/ µl	2.5U/µl
3	Buffer +$Mgcl_2$	25mM	2.5Mm
4	dNTPs	2.5mM	0.2mM
5	Primer	5pmole	0.6pmole
6	Sterile water		

Master mix required for a set reactions was prepared fresh, from the original stocks. The master mix was distributed tubes containing each of the template DNA from different genotypes and mixture was given a short spin to mix the contents.

PCR Amplification Program

PCR tubes containing master mix and DNA template were thoroughly mixed and subjected to the thermal profile given in Table 4. The amplification reaction was carried out in a gradient mastercycler.

Table 4: Thermal profiles used for DNA amplification

Steps	Cycles	Temperature	Duration
Initial Denaturation	1	94^0C	5 min
Denaturation	42	94^0C	1 min
Annealing		37^0C	1 min
Extension		72^0C	1 min
Final Extension	1	72^0C	7 min

Separation Of Amplification Products By Agarose Gel Electrophoresis

Requirements: Electrophoretic unit: Gel casting trough, gel preparation combS, power pack, UV transilluminator , Agarose, Bromophenol blue Ethidium bromide**,**10X TBE – pH-8.0**,**Working solution (1 X TBE)

Procedure: 1.5 g of agarose was weighed and added to a conical flask containing 100ml of 1 X TBE buffer. The agarose was melted by heating the solution on an electric heater and the solution was stirred to ensure even mixing and complete dissolution of agarose. The solution was then cooled to about 40-45^0 C. Two to three drops of ethidium bromide (0.5 µg ml-1) was added. The solution was poured into the pre levelled gel casting platform after inserting the comb in the trough. While pouring, sufficient care was taken for not allowing the air bubbles to trap in the gel. The gel was allowed to solidify and the comb was removed after placing the solidified gel into the electrophoretic apparatus containing sufficient buffer (1 X TBE) so as to cover the well completely. The amplified products (20µl) to be analysed were carefully loaded along with the marker into the sample wells, after adding 2-3 µl of loading dye (Bromophenol blue) with the help of a micropipette. Electrophoresis was carried out at 50-55 volts, until the tracking dye migrated to the end of the gel. Ethidium bromide stained DNA bands were viewed under transilluminator and photographed for documentation.

Data Analysis

PCR bands were detected in the gel and their sizes were estimated using 1kb standard marker. The banding patterns of all genotypes against each primer were compared (see fig: 1-12). Variable bands were used to score for polymorphism for identification of varieties and binomial data matrix was generated which was further used subjected to statistical softwares

Finally the cluster analysis was done, to determine genetic diversity. The RAPD data was scored by the presence and absence of bands. Presence of band was denotes as '1' and absence as '0' and missing as '9'. Binary data matrix was then constructed and used to construct a dendrogram based on UPGMA (unweighted pair group method of arithmetic averages) using NTSYSpc software (see fig: 13).

4. RESULTS AND DISCUSSION

• Quantity & Quality Check

The genomic DNA from all the 8 varieties of pea was isolated and quantity and quality of DNA was estimated to check the concentration of DNA. Intensity of intact bands was used to estimate the quality of DNA. Quality of DNA of all 8 varieties was good. DNA samples were also quantified using Nanospec. Ratio of OD at 260/280nm ranged between 1.8 to 2.0. DNA was finally diluted to a concentration of 50ng/µl for use in the PCR reaction

• PCR Amplification

Amplification of DNA was carried out according to thermal profile presented in Table 5. Separation of DNA amplification product was done using horizontal 1.4% agarose gel electrophoresis.2 µl of loading dye was added to tubes to get a final concentration of 1X. All the 13 gels were then subjected to gel-doc and photographed.

• Scoring Of Bands

The banding pattern of all genotypes against each primer were compared and used to generate results (Figure 1-13). Varietal identification was done on the basis of polymorphic bands (presence in one and absence in other varieties).

A total of 450 bands were produced (Figure 1-12). Based on primer sequence and variation in specific varieties results obtained are as follows:

- ✓ Variety Azad-P1 (A1) could be differentiated using primer OPD-18 from other 7 varieties by the presence of a unique band of size 900bp.
- ✓ Variety Azad-P2 (A2) could be differentiated from other 7 varieties by detecting the presence of unique band(s) of size 1100bp, 2400bp, 1400bp,750bp using primers OPA-10, OPU-16, OPE-16, OPA-07, respectively.
- ✓ Variety Azad-P4 (A4) could be differentiated from other 7 varieties on the basis of presence of a unique band of size 1400bp using primer OPD-07.
- ✓ Variety Azad-P6 (A6) could be differentiated by the presence of a unique band of size 1700bp using primer OPU-16 and another unique band of band size 350bp using primer OPN-06.
- ✓ Variety Bonneville (B1) could be differentiated from other 7 varieties by the presence of unique bands of size 500bp using primer OPN-06, 600bp using primer OPA-02,1000bp using primer D-07 and 3500bp using primer OPY-15.
- ✓ Variety Lincoln (L1) could be differentiated from other 7 varieties by the presence of 2 unique bands of size 350bp and 2100bp using primer OPC-02.

Table 5: RAPD BANDING PROFILE OF 12 PRIMERS

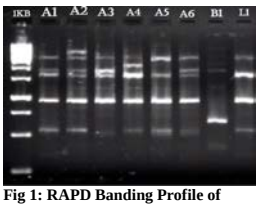

Fig 1: RAPD Banding Profile of Primer OPA-02

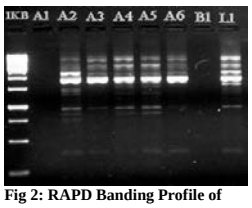

Fig 2: RAPD Banding Profile of Primer OPA-07

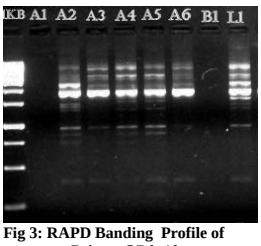

Fig 3: RAPD Banding Profile of Primer OPA-10

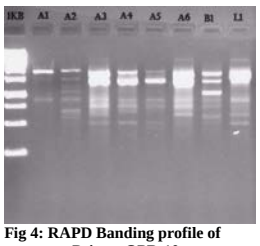

Fig 4: RAPD Banding profile of Primer OPB-10

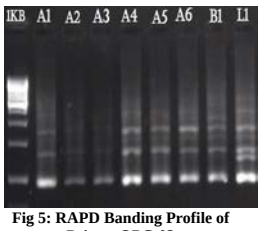

Fig 5: RAPD Banding Profile of Primer OPC-02

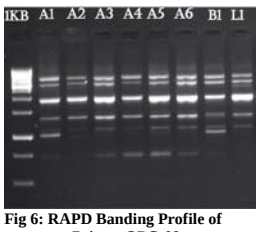

Fig 6: RAPD Banding Profile of Primer OPC-09

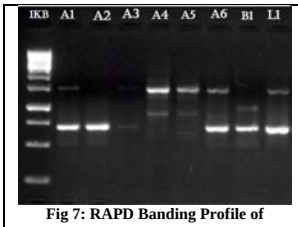

Fig 7: RAPD Banding Profile of Pimer OPD-07

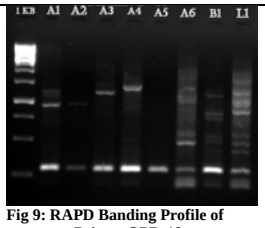

Fig 9: RAPD Banding Profile of Primer OPD-18

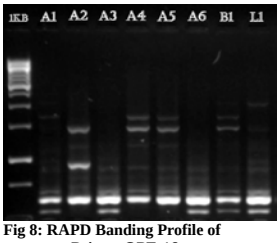

Fig 8: RAPD Banding Profile of Primer OPE-16

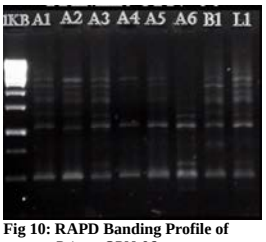

Fig 10: RAPD Banding Profile of Primer OPN-06

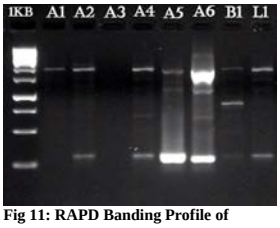

Fig 11: RAPD Banding Profile of Primer OPU-16

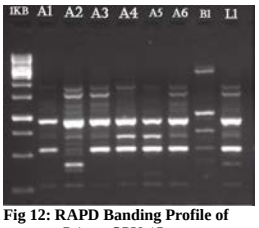

Fig 12: RAPD Banding Profile of Primer OPY-15

Based on primer sequence and variation in specific fourteen unique bands were recognised out of all the polymorphic bands. One unique band was obtained for Azad P1(A1) from OPD-18, Four unique bands for Azad P2 (A2) from OPA-10, OPU-16, OPE-16, A-07, one band for Azad P4 (A4) from OPD-07, Two unique bands for Arkel (A6) from OPU-16 and OPN-06.Four unique bands for Bonneville (B1) using OPA-02, OPD-07, OPY-15, OPN-06, Two unique bands for Lincoln (L1) from OPC-02.

- ## Cluster Analysis

The eight genotypes of peas were clustered in three major clusters. The cluster I was having Azad P1 (A1) and Azad P2 (A2). The cluster II was having Azad P3 (A3), Arkel (A6), Lincoln (L1), Azad P4 (A4) and Azad P5 (A5). Clustar was simplifolious having Bonneville (B1) only (see fig: 13)

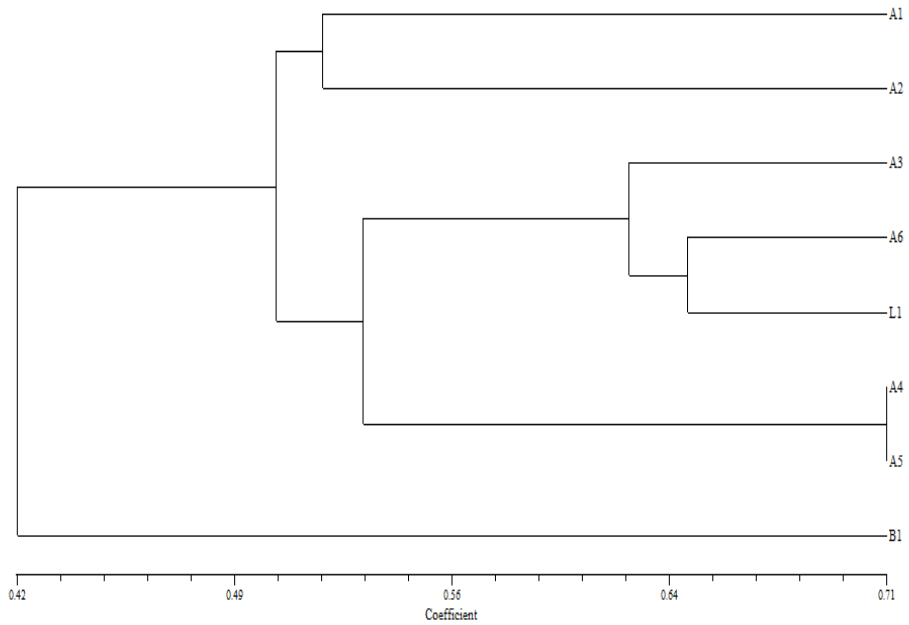

Fig 13: Cluster analysis of pea genotypes based on molecular data

5. SUMMARY AND CONCLUSIONS

Pea is widely cultivated in northern temperate regions and is used as fresh or canned vegetable. Peas are also grown for animal fodder, and for consumption as a split dry pulse or *dal*. There are number of varieties of peas available in India which are notified at national and state level. Identification and characterization of cultivars is a crucial factor to fulfill various objectives in seed production and these include verifying the identity of a variety, establishing the distinctness of a new variety, determining the varietal and hybrid purity, varietal registration and certification and to assure farmers for getting correct variety. Therefore, identification of varieties is a very crucial factor. The modern technique for doing the same is with the help of molecular markers which are more rapid and reliable.

The conclusions of the present study are:

Majority of the primers used in this study establish a major difference among different varieties of pea. These primers can also be utilized in diversity analysis studies and Marker Assisted Selection. The variability present in the germplasm used in the study is evident from the dendrogram analysis based on UPGMA. This diversity analysis has made us capable of harnessing the potential of such diverse genotypes in heterosis breeding and crop improvement programs.

6. Acknowledgement

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