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Identification of field and gardenpea (*Pisum sativum*) varieties using RAPD and ISSR markers

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ABSTRACT Identification of seven varieties of garden and field pea (*Pisum sativum* L.) currently under seed multiplication chain in Himachal Pradesh was undertaken using 40 random decamer and 9 ISSR primers. A total of 251 amplicons including 111 RAPDs and 70 ISSRs bands were amplified. The ISSR markers exhibited better polymorphism and resolving power as compared to RAPDs. High pair-wise genetic similarity estimates ranging from 0.50 to 0.86 indicated narrow genetic base of the set of varieties. Clustering analysis based on pooled data grouped 7 varieties in 3 main groups. Four gardenpea varieties comprised one main group, whereas a field pea along with one gardenpea variety constituted other group. Field pea variety, Rachna, was most divergent from other varieties and did not group with any other varieties. All the 7 varieties were resolved completely using variety specific and/or other polymorphic markers amplified using single ISSR and RAPD primers, namely UBC 843 and OPJ 14, respectively. Four random decamer and 6 ISSR primers were identified as potential ones that could alone distinguish 5 or more varieties, out of a set of total 7 varieties. Study indicated possible use of variety-specific amplicons in developing diagnostic kits for identification of current set of pea varieties under cultivation in Himachal Pradesh.

Key words: ISSR, RAPD, polymorphism, *Pisum sativum*, biochemical markers, agro-morphological traits

The crop improvement programme in peas (*Pisum sativum* L.), has identified many varieties for cultivation in different regions of Himachal Pradesh. Out of which, 6-7 important varieties of garden and field pea are presently under seed multiplication chain in Himachal Pradesh. Improved seed production involves their multiplication at different stages and maintenance of genetic purity of seed through reliable identification of varieties during rogueing, monitoring and certification using suitable diagnostic traits and/or other reliable tools. Characterization of varieties can most easily be achieved using diagnostic phenological and/or agro-morphological traits and biochemical markers. However, these are limited in number and lack polymorphism. Agro-morphological traits are particularly most ambiguous due to the pronounced effect of environment on their expression. Recent advances in molecular biology have offered more powerful tools for varietal characterization including PCR-based markers. Among them, RAPD and ISSR are easy to amplify and show polymorphism randomly across whole of the genome.

In pea, various studies have successfully employed agro-morphological traits [1], total proteins [2, 3], isozymes [4, 5] and RAPDs [1, 5, 6, 7] for identification of cultivars. Recently, one study has proposed effective identification of pea varieties based on inter retrotransposon amplified polymorphism [8]. However, no study has been undertaken to characterize garden and field pea varieties in seed production chain in Himachal Pradesh using reliable and repeatable techniques like DNA markers. Therefore, present study was

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undertaken with an aim of screening of available markers to assess their suitability to reliably identify set of 7 pea varieties currently under seed production chain in Himachal Pradesh, and to identify variety-specific amplicons for their further use in development of diagnostic kits.

MATERIALS AND METHODS

Genetically pure seeds of 7 varieties of pea, used in seed production chain in Himachal Pradesh, including 5 varieties of garden pea (Lincoln, Arkel, Kinnouri, Palampriya and Azad Pea1) and 2 of field pea (Rachna and Swati), were used for characterization and identification using RAPD and ISSR markers (Table 1). Seeds were germinated and genomic DNA was isolated from young leaves of at least 5 plants as a composite sample following CTAB method [9]. The quantity and quality of DNA were estimated by gel electrophoresis using 0.8 per cent agarose. The stock DNA was diluted with double distilled autoclaved water to make a working solution of 20-25 ng/µl for PCR analysis.

A total of 40 random decamer oligonucleotides of Operon series (Operon Technologies) and 9 ISSR markers were used for molecular analysis of genomic DNA. The PCR reactions were conducted in 12.5 µl of reaction mixture containing 1 µl (20-25 ng) of genomic DNA, 1.25 µl 10X PCR buffer, 0.75 µl of $MgCl_2$ (25mM), 1 µl dNTP's (Genome Diagnostics, Parwanoo, India), 0.6 µl primer, 0.1 µl of Taq DNA polymerase (Larova Gm and H Berlin, Germany) and 7.8 µl of sterilized distilled water. For ISSRs, 10 pmol of each primer was used, keeping other ingredients similar. The amplification was carried out in a Thermal Cycler (Applied Biosystems, Singapore) set at initial cycle of 94°C for 5 min. For RAPDs, further amplification was repeated 40 times consisting of each cycle of denaturing at 94°C for 1 min, annealing at 37°C for 1 min and extension at 72°C for 2 min.

Final extension of 5 min at 72°C was carried out before rapid cooling to 4°C. For ISSRs, the PCR programme was set at initial cycle of 95°C for 4 min, 52°C for 45 seconds and 72°C for 2 min. Further amplification was repeated 45 times consisting of denaturation at 95°C for 30 seconds, annealing at 52°C for 45 seconds and extension at 72°C for 2 min. Final extension of 10 min at 72°C was carried out before rapid cooling to 4°C. Amplified PCR products were separated on 1.4 and 2.0 per cent (w/v) agarose gel in 1X Tris–Acetate–EDTA (TAE) buffer containing ethidium bromide (0.5 ng/ml) for RAPDs and ISSRs, respectively. The DNA profile was visualized using the Gel Doc system (Bio-Rad).

The RAPD and ISSR profiles were scored for each band; presence and absence of each band of a particular molecular weight were scored to generate a binary data matrix with '1', indicating presence of a particular molecular weight band and '0' indicating its absence. The binary data of each type of marker and pooled over markers were used to generate similarity matrices using Jaccard coefficient in SIMQUAL programme of NTSYS-PC package [10]. Cluster analysis of genotypes based on similarity values obtained using Jaccard coefficients was done by Unweighted Pair Group Method with Arithmetic Averages (UPGMA) in SAHN programme of NT SYS-PC package for each set of molecular data separately as well as pooled together.

To know the association among data obtained using 2 types of markers, correlation/ correspondence between similarity matrices derived from them was worked out using MxComp programme of NTSYS-PC package and tested using Mantel's test. Per cent polymorphic bands were calculated as the number of polymorphic bands amplified by a primer to that of total number of bands produced by the primer. Number of varieties resolved was recorded primer-wise. Generated varietal fingerprint data were further used to identify variety-specific amplicons, most informative primers as well as a minimum set of primers in each type of molecular markers that can resolve and identify each variety reliably.

RESULTS AND DISCUSSION

Analysis of seven varieties of garden and field pea using 40 random decamer and 9 ISSR primers

produced a total of 251 amplicons including 181 RAPD and 70 ISSR bands (Table 1). Average number of bands amplified was higher for ISSRs (7.78 per primer) than RAPDs (4.52 amplicons per primer). A single band was amplified by primers OPA-17, whereas a maximum of 9 amplicons were recorded for primer OPJ-7. The ISSR primers amplified relatively higher number of bands per primer ranging from 3 (UBC 811) to 10 (UBC 843). Of the total 181 RAPD amplicons, 72 (39.8%) were monomorphic, whereas 109 (60.2%) were polymorphic in nature.

Of the 70, ISSR amplicons, 55 (78.6%) exhibited polymorphism. The variation in banding pattern indicated a high degree of polymorphism in pea varieties. The number of polymorphic bands ranged from 0 to 7 with an average of 2.72 polymorphic bands per RAPD primer. Primer, OPA-3 and OPD-20, generated no, polymorphic bands, whereas OPJ-7 produced highest number (7) of polymorphic bands. Seven primers, OPA-4, OPA-12, OPA-17, OPD-12, OPD-13, OPF-9 and OPQ-11, generated highly (80% and more) polymorphic bands.

The ISSR primers generated more polymorphic amplicons ranging from 1 (UBC 811) to 9 (UBC 810) with a mean of 6.11 polymorphic amplicons per primer as compared to RAPD primers. Tar'an *et al.* [1] reported highest polymorphism (51 %) from SSR analysis, whereas RAPDs and ISSRs showed almost equal polymorphism (44% and 43%, respectively) on a set of 65 pea genotypes.

Pair-wise genetic similarity (gs) estimates among 7 varieties based on the pooled data of 251 amplicons from both types of markers showed similarity ranging from 0.50 (Swati and Palam Priya/Kinnouri) to 0.86 (Kinnouri and Palam Priya). This clearly indicated high genetic similarity among current set of varieties and importance of identification of variety-specific amplicons as diagnostic markers for validation of their genetic purity under seed production programmes.

High level of genetic similarities (gs=0.50-0.86) among important cultivars of field and gardenpea also depicted their narrow genetic base (Table 2). This might be due to immense selection pressure exerted for economical traits during their breeding and/or utilization of common parents in regional pea breeding programmes. Reduced diversity/genetic base have been demonstrated for European pea breeding programmes [11, 12]. Although number of ISSR markers used in the present study was low compared to RAPD, the data generated showed considerable agreement among 2 sets as showed by positive correspondence among them validated by Mantel t-test.

Dendrograms generated based on similarities of each type of marker as well as pooled data over markers invariably classified 7 varieties into 3 main clusters (Fig. 1a-c). Field pea varieties, Rachna and Swati, showed least similarity with other cultivars and did not group with any other cultivars as indicated by dendrograms derived from ISSR and RAPD data. On the other hand, based on each set of data, gardenpea varieties Palam Priya and Kinnouri, showed high genetic similarities (gs>0.76) and clustered together.

Based on pooled data which can be considered more informative, 4 gardenpea varieties, Azad Pea 1, Palam Priya, Kinnouri and Arkel, clustered together in largest group, whereas field pea variety, Swati and gardenpea variety, Lincoln comprised second group. Field pea variety, Rachna, did not show much similarity with any other variety and alone constituted third group.

The number of varieties resolved using each RAPD and ISSR primer ranged from 0 to 7. Overall, average number of varieties resolved by each primer was higher for ISSR markers (4.56) than RAPD primers (1.80). Resolving power is a very useful parameter for molecular diagnosis of any species from mixed population [13]. Of the 40 random decamer and 9 ISSR primers screened, 4 decamer primers (OPA-12, OPA-18, OPF-3 and OPJ-14) and 6 ISSR primers (UBC 825, UBC 835, UBC 840, UBC 843, UBC 844 and UBC 845) showed high resolving power by distinguishing 5 or more varieties, out of the present set of 7 pea varieties. RAPD and ISSR primers, namely OPJ-14 and UBC 843, respectively, resolved complete set of 7 pea varieties employing single

Table 1. Level of detected polymorphism and resolving power of 40 random decamer and 9 ISSR primers used in characterization of 7 varieties of *Pisum sativum*

Primer	Sequences (5'-3')	Total bands amplified	Polymorphic bands	Polymorphic bands (%)	No. of varieties resolved
		RAPDs			
OPA-1	CAG GCC CTT C	6	2	33.3	0
OPA-2	TGC CGA GCT G	5	1	20.0	0
OPA-3	AGT CAG CCA C	2	0	00.0	0
OPA-4	AAT CGG GCT G	5	4	80.0	4
OPA-7	GAA ACG GGT G	2	1	50.0	0
OPA-10	GTG ATC GCA G	4	2	50.0	0
OPA-12	TCG GCG ATA G	6	5	83.3	5
OPA-13	CAG CAC CCA C	7	2	28.6	1
OPA-15	TTC GCA ACC C	4	2	50.0	0
OPA-17	GAC CGC TTG T	1	1	100.0	0
OPA-18	AGG TGA CCG T	6	4	66.7	5
OPA-19	CAA AGC TCG G	6	4	66.7	2
OPD-1	ACC GCG AAG G	2	1	50.0	1
OPD-3	GTC GCC GTC A	5	2	40.0	1
OPD-4	TCT GGT GAG G	4	3	75.0	1
OPD-5	TGA GCG GAC A	3	1	33.3	1
OPD-8	GTG TGC CCC A	4	3	75.0	2
OPD-10	GGT CTA CAC C	5	3	60.0	3
OPD-11	AGC GCC ATT G	5	2	40.0	2
OPD-12	CAC CGT ATC C	6	5	83.3	1
OPD-13	GGG GTG ACG A	4	4	100.0	1
OPD-15	CAT CCG TGC T	4	2	50.0	0
OPD-18	GAG AGC CAA C	4	3	75.0	0
OPD-20	ACC CGG TCA C	2	0	0.00	0
OPF-2	GAG GAT CCC T	2	1	50.0	1
OPF-3	CCT GAT CAC C	4	3	75.0	5

Table 1 contd ...

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OPF-5	CCG AAT TCC C	4	2	50.0	1
OPF-9	CCA AGC TTC C	7	6	85.7	4
OPJ-5	CTC CAT GGG G	3	2	66.7	0
OPJ-7	CCT CTC G ACA	9	7	77.8	3
OPJ-11	ACT CCT GCG A	3	2	66.7	2
OPJ-13	CCA CAC TAC C	7	5	71.4	4
OPJ-14	CAC CCG GAT G	7	5	71.4	7
OPJ-19	GGA CAC CAC T	6	4	66.7	3
OPJ-20	AAG CGG CCT C	6	2	33.3	1
OPQ-6	GAG CGC CTT G	7	4	57.1	3
OPQ-9	GGC TAA CCG A	2	1	50.0	0
OPQ-11	TCT CCG CAA C	5	4	80.0	4
OPQ-14	GGA CGC TTC A	3	1	33.3	1
OPQ-20	TCG CCC AGT C	4	3	75.0	1
Range (total)		**1-9 (181)**	**0-7 (109)**	**0-85.7**	**0-7**
Mean ± SE		**4.52 ± 0.29**	**2.72 ± 0.26**	**58.0 ± 3.73**	**1.75 ± 0.29**
		ISSRs			
UBC 810	(GA)8T	9	9	100.0	4
UBC 811	(GA)8C	3	1	33.3	0
UBC 825	(AC)8T	9	6	66.6	5
UBC 835	(AG)8YC	8	8	100.0	5
UBC 840	(GA)8YT	9	8	88.8	6
UBC 841	(GA)8YC	6	3	50.0	2
UBC 843	(CT)8RG	10	8	80.0	7
UBC 844	(CT)8RC	9	7	77.7	6
UBC 845	(CT)8RG	7	5	71.4	6
Range (total)		**3-10 (70)**	**1-9 (55)**	**33.3-100**	**0-7**
Mean ± SE		**7.78 ± 0.72**	**6.11 ± 0.89**	**74.2 ± 7.37**	**4.55 ± 0.75**

primer at a time. Besides, many other primers showed potential in resolving the present set of varieties, when considered in combination with one or more other primers. For example, 3 ISSR markers UBC 810, 811 and 835 together resolved all 7 varieties unambiguously (Fig. 2). Variety-

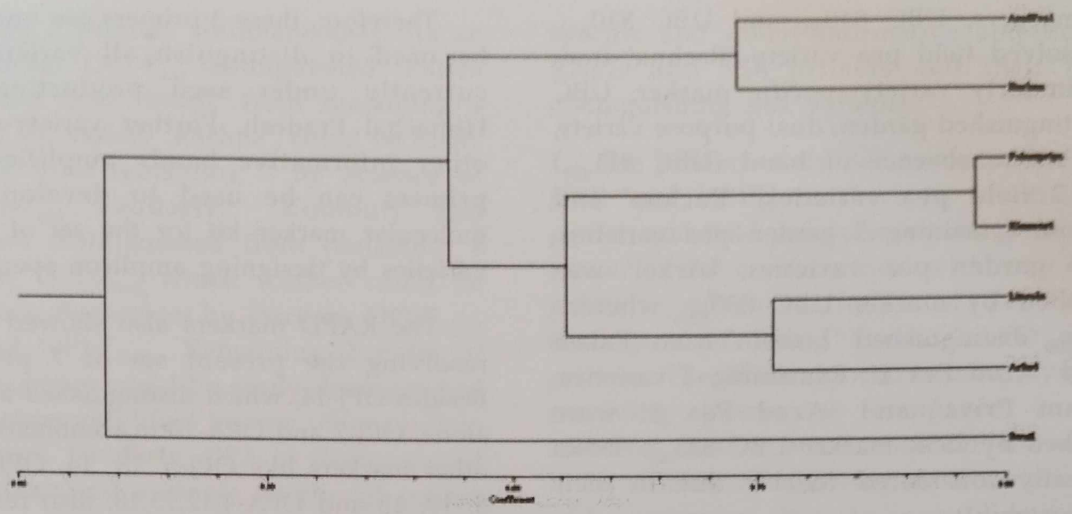

Fig. 1a. ISSR markers

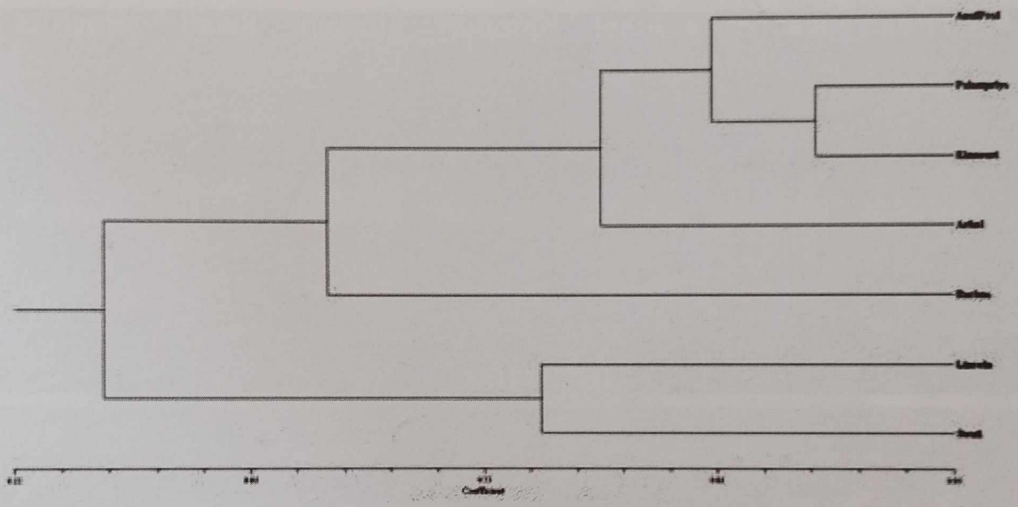

Fig. 1b. RAPD markers

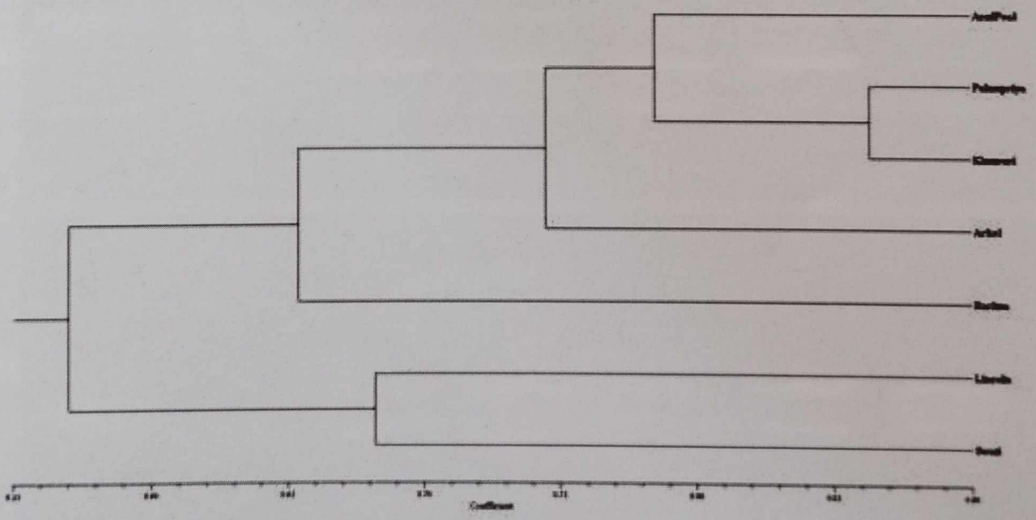

Fig. 1c. Pooled over markers

Fig. 1a-c. Dendrograms depicting genetic relationship among 7 varieties of pea based on Jaccard similarity coefficient calculated from ISSR, RAPD and pooled data

specific markers, UBC 810_{994} and UBC 810_{850}, clearly resolved field pea variety 'Rachna' from others. Similarly variety-specific marker, UBC 810_{732}, distinguished garden/dual purpose variety, 'Kinnouri'. The absence of band (UBC 811_{979}) resolved 2 field pea varieties, 'Rachna' and 'Swati' from remaining 5 garden pea varieties. Among 5 garden pea varieties, 'Arkel' was distinguished by marker UBC 835_{855}, whereas UBC 835_{1709} distinguished 'Lincoln' from 'Palam Priya' and 'Azad Pea 1'. Remaining 2 varieties, *viz.* 'Palam Priya' and 'Azad Pea 1' were distinguished by ISSR marker UBC 835_{967}. ISSRs are generally considered reliable due to their better repeatability.

Therefore, these 3 primers can unambiguously be used to distinguish all varieties of peas currently under seed production chain in Himachal Pradesh. Further variety-specific and other informative bands amplified by these primers can be used to develop diagnostic molecular marker kit for the set of present pea varieties by designing amplicon specific primers.

The RAPD markers also showed potential in resolving the present set of 7 pea varieties. Besides OPJ-14, which distinguished all 7 varieties alone, OPJ-7 and OPA-18 in combination with any other markers like OPJ-5, 13, 14, OPF-3, 5, OPD-8, 10, 15 and OPA-4,12,15,18, also resolved these

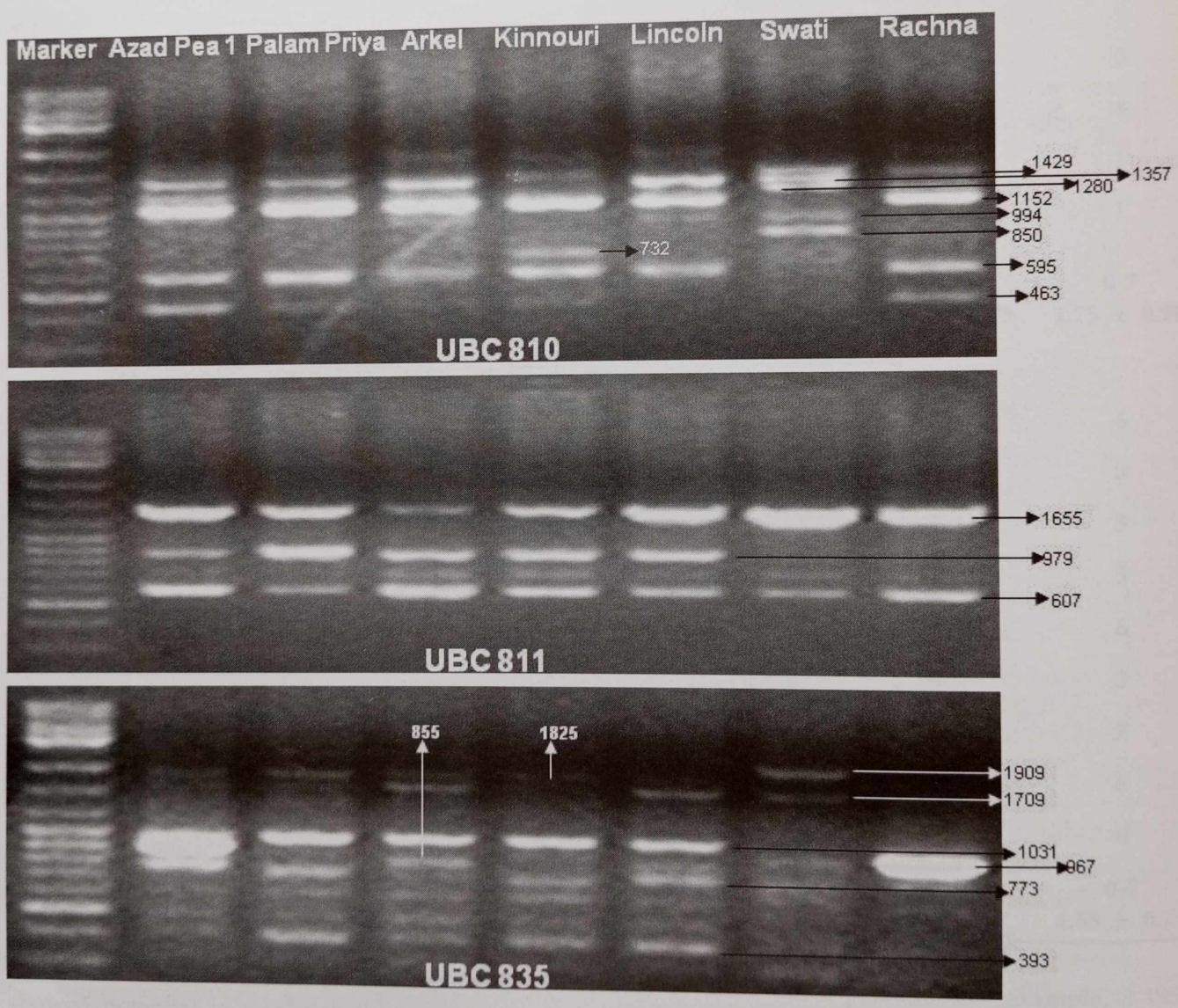

Fig. 2. ISSR profiles distinguishing 7 varieties of pea, employing 3 different primers arrows indicate sizes of amplicons in bp

varieties using 2 primers simultaneously. As an example, marker OPJ-7_{411} distinguished 'Palam Priya', 'Rachna' and 'Swati' from remaining 4 varieties (Fig. 3). Variety 'Rachna', could be easily distinguished from remaining 2 by absence of marker OPJ-$7_{464.}$ Similarly, 'Kinnouri' and 'Lincoln' were distinguished from remaining 4 using marker OPJ-$7_{860,}$ which further could be resolved among themselves by markers, OPJ-7_{1571}, OPJ-7_{1272} and OPJ-7_{1080}. Remaining 2 pairs of unresolved varieties could be easily distinguished by using markers amplified by any other random decamer primer. Similarly, OPA-18 distinguished 5 varieties alone and a single additional primer used with it could resolve whole set of 7 pea varieties of Himachal Pradesh. Earlier Tar'an *et al.* [1] have also reported amplification of variety-specific and other informative amplicons using RAPD and SSR primers and their utility in distinguishing pea varieties.

The present study demonstrated the utility of ISSR and RAPD markers in identification of garden and field pea varieties using single primer alone or in combination with one or other primers. Seven varieties of pea could be distinguished using single RAPD and ISSR primers. The ISSRs showed better polymorphism, resolving power as compared to RAPDs. However, RAPD primer like OPJ-14 was found to be efficient in resolving all 7 varieties of field and gardenpea. Variety-specific and/or other informative amplicons identified in the present study can be further used to develop diagnostic

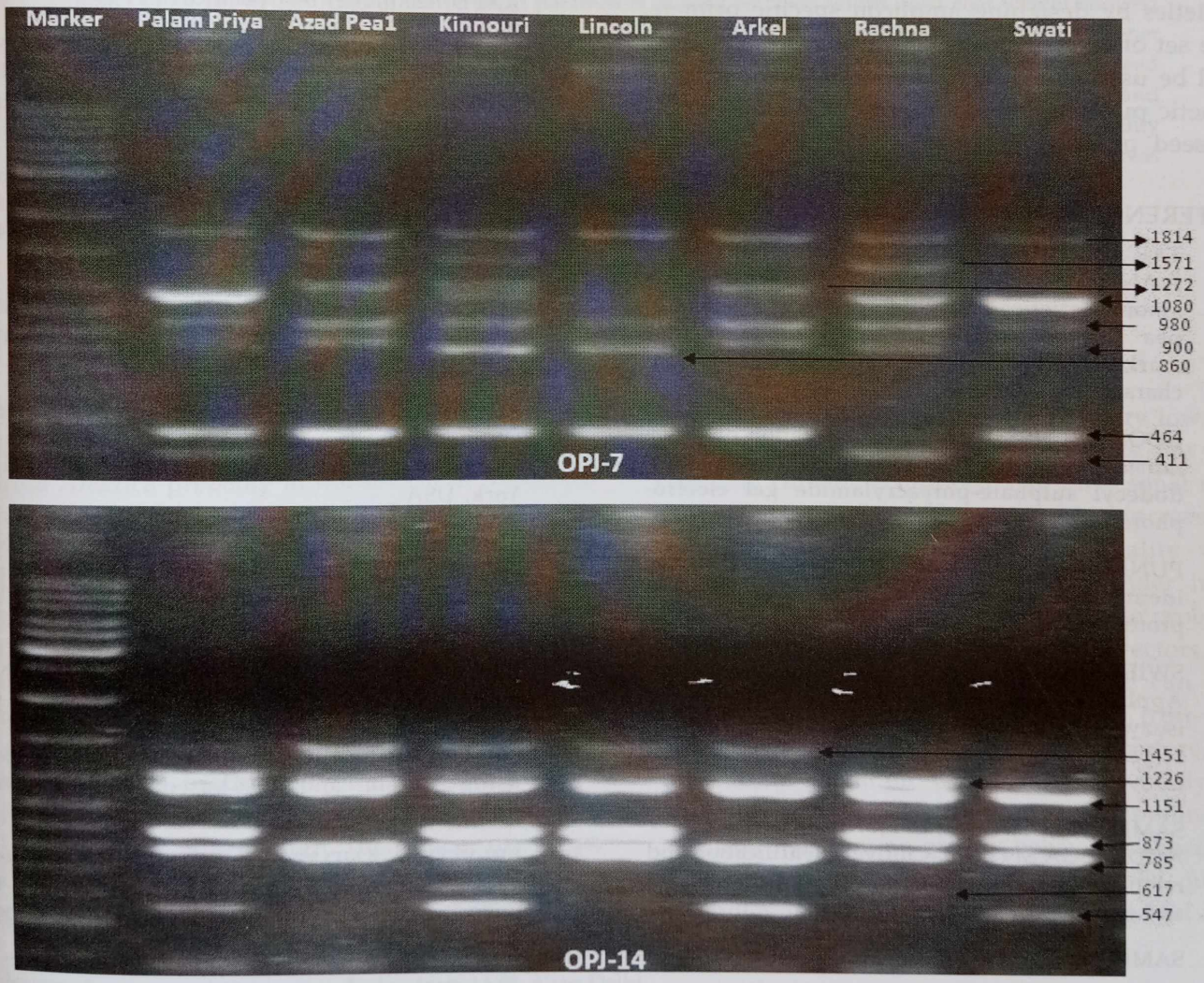

Fig. 3. RAPD markers distinguishing pea varieties using primers OPJ-7 and OPJ-14 arrows indicate sizes of amplicons in bp

Table 2. Genetic similarities among 7 varieties of pea based on pooled data of ISSR and RAPD markers using Jaccard coefficient and correspondence among ISSR and RAPD based similarities

Variety	Azad Pea 1	Palam Priya	Kinnouri	Lincoln	Arkel	Rachna	Swati
Azad Pea 1	-						
Palam Priya	0.794	-					
Kinnouri	0.775	0.863	-				
Lincoln	0.577	0.619	0.603	-			
Arkel	0.709	0.762	0.762	0.625	-		
Rachna	0.676	0.673	0.641	0.606	0.625	-	
Swati	0.520	0.509	0.509	0.681	0.606	0.563	-
Matrix correlation (r) ISSR vs RAPD			0.517*				

*Significant at P=0.021

kits for identification of current set of pea varieties by designing amplicon specific primers. The set of markers identified in the present study will be useful for identification and validation of genetic purity of these varieties at various stages of seed production.

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