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AGRO-BIOCHEMICAL BASED CHARACTERIZATION AND MOLECULAR PROFILING OF LINSEED (*Linum usitattissimum* L.) CULTIVARS

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ABSTRACT

Nineteen commercial cultivars of flax (*Linum usitatissimum* L.) were subjected to genetic diversity analysis, fingerprinting and identification of informative marker allele(s) for seed purity assessment based on agro-morphological and biochemical quality traits and inter simple sequence repeat (ISSR) marker. A total of 74 reproducible bands ranged from 120 to 1200 bp were amplified out of which 68 were polymorphic among the cultivars. Dendrogram constructed showed no clear grouping of cultivars with regard to the parentage/pedigree record and breeding centres. The 2D plot obtained with PCA analysis clearly supported the results obtained by cluster analysis. Further, ISSR profiling helped in the identification of 15 genotypes by cultivar specific bands. The findings of this study would provide a useful guide for selecting specific cultivar with a desirable agronomic trait with distinct genetic background for linseed breeding program in India.

Key words: Agro-biochemical characterization, fatty acid, fingerprinting, ISSR markers, linseed

INTRODUCTION

Flax or linseed (*Linum usitatissimum* L.) is native to the region extending from the eastern Mediterranean to India and is the first domesticated species in human history. The genus *Linum* is the type genus for the flax family, Linaceae DC. (Dumort) comprising 22 genera and about 300 species distributed worldwide (Mc Dill*et al*., 2009). The Latin species name *usitatissimum* means most useful, pointing to the several traditional uses of the plant and their importance for human life. Linseed is used for both for seed and fibre purposes. One hundred grams of ground flax seed supplies about 450 kcal, 41 g fat, 28 g fiber and 20 g protein (Yari *et al.*, 2016).

Earlier linseed characterization was limited to morphological and isoenzymes to estimate the amount of diversity and relatedness in a set of genotypes but these have certain limitations. With the advancement of molecular techniques, a number of molecular markers, such as RFLP, cpDNA-specific probes, M13 Probe, RFLP, SSRs, RAPD, ISSR, AFLP, etc., have been developed and are currently used in marker assisted breeding for various purposes. To date, DNA variation patterns within species and among closely related taxa in *Linum* species have been characterized by random amplified polymorphic DNA (RAPD) (Diederichsen and Fu, 2006) inter-simple sequence repeat (ISSR) (Wiesnerova and Wiesner, 2004) and simple sequence repeat (SSR) (Cloutier *et al.*, 2009). Taken together, these studies have shown that cultivated flax has low genetic diversity as compared to the wild relatives or some other crops, possibly resulting from a domestication bottleneck.

ISSR is a PCR based molecular marker which has successfully been used to estimate the extent of genetic diversity at inter- and intra-specific level in a wide range of crop species. In a study on *Rhamnus persicifolia*, among the 10 primers used, any two were sufficient to detect the genetic diversity within and among six population-representatives of species-distribution range (Gianluigi Bacchetta *et al.*, 2011). Genetic diversity and genetic differentiation within and among six populations of *Sinocalycanthus chinensis* revealed high genetic diversity at species level (Zexin and Junmin, 2007). ISSR markers are frequently used for varietal diagnostic purposes in many crop species. The use of such highly informative primers lowers the cost, time and labour for diversity analysis. Keeping this in view, the present investigation was conducted to evaluate the molecular variation and relationships among released linseed varieties, which are grown under different agro-climatic zones of the country, using ISSR profiles.

MATERIALS AND METHODS

Plant material

The present study was carried out at Oilseed Section, Department of Plant Breeding and Genetics, PAU Ludhiana, India. The plant material comprised of 19 commercial linseed varieties released for general cultivation at state and national level. The varieties were selected from PAU linseed germplasm collections which were procured from different Indian linseed breeding programmes. All the 19 commercial linseed varieties were evaluated for important agro-morphological, oil quality traits in the year 2014-15 and molecular characterization of the same varieties using ISSR markers was done in the subsequent year.

Phenotypic and biochemical evaluation

The experiment was carried out in *rabi* 2014-15 crop season in a randomized complete block design with three replications. All the standard package of practices were followed to raise a healthy crop (*Package of Practices for Crops in Punjab, Rabi*, 2014). Observations for agro-morphological traits included the days to flowering, days to maturity, plant height, seed weight and oil quality traits including oil content and fatty acid profile, recorded from 10 plants of each genotype.

The oil content in intact seeds was estimated by nuclear magnetic resonance (MQC23, Oxford Instruments, UK) which is a non-destructive, quick, easy to perform and simple to calibrate (Alexander *et al.*, 1967). A standard sample containing 4 g seeds of linseed with known oil content was set to calibrate the instrument. Oil content in standard samples were estimated by petroleum ether extraction using standard method (AOAC, 2007) and these samples were used for standardizing Newport analyzer (MK IIIA). The operating conditions of the instrument were: gate width 1.5 g, Rf level 100 μA and integration time 32 sec.

The fatty acid composition was determined by gas chromatography of fatty acid methyl esters. These were prepared as per the procedure developed by Appelqvist *et al.* (1968) and analyzed on M/s Nucon Engineers AIMIL gas chromatograph (solid state) model 5700 series equipped with flame ionization detector fitted with 6% butane-diol succinate (BDS) on Chromosorb WAW/DWCS column 180 cm in length X 0.65 cm outer diameter. The temperatures of oven, detector and injector were maintained at 200, 240 and 250°C, respectively. Individual fatty acids were expressed as percentages of the total fatty acids.

Genomic DNA extraction and ISSR-PCR amplification

Total genomic DNA was isolated from fresh leaf samples of each variety using CTAB DNA extraction protocol (Murray and Thompson, 1980). DNA quality and concentration were examined by Biophotometer Plus (Bio Rad). A total of 100 ISSR primers were synthesized from the Canada Biotechnology Laboratory, University of British Columbia (Canada). PCR amplifications were

performed in 25 µL reaction mixtures containing 20 ng template DNA, 1 µM primer, 1 unit *Taq* DNA polymerase, 0.25 mM of each dNTP and 1× reaction buffer supplied with the enzyme. PCR products were separated and visualized using an automated high-throughput electrophoresis system (Caliper Lab Chip GX, version 3.0.618.0).

Analysis of amplification products

The electrophoresed PCR products for each primer was scored using system generated genotype profile. The band profiles were scored for only distinct and reproducible bands as present (1) or absent (0) for each ISSR primer. Allelic polymorphic information content (PIC) was calculated in each case using the formula: PIC = 1 - ∑(pi)2, where pi is the frequency of the i^{th} allele in the set of analyzed genotypes calculated for each ISSR locus (Botstein *et al.*, 1980). A data matrix was assembled and analyzed using Paleontological Statistics (PAST) software package for education and data analysis to estimate genetic distances and Neighbour joining algorithm was used to construct dendrogram (Hammer *et al.*, 2001).

RESULTS AND DISCUSSION

Morphological and biochemical variability

The descriptive statistics values exhibited large variation for all the agro-morphological and biochemical/quality traits (Table 1). Among the 19 cultivars, two cultivars *viz.*, 'LCP 147' and 'Pratap Alsi' were dual purpose having commercial importance for seed oil and fiber, while remaining 17 have significance as oilseed type. The days to 50% flowering varied from 78 to 115 days with an average of 99±2.03 days. The days to maturity ranged from 144 to 169 days with genotypes 'Deepika' and 'SLS 71' being early matured (144 days) varieties, whereas 'Himani' matured in 169 days. The range of plant height was from 47.11 cm in 'Sharda' to 81.20 cm in 'LCP 147'. Similarly, the maximum seed weight of 8.76 g was recorded in 'Shubhra' and the lowest of 4.45 g in 'LC 185' with an average of 6.61±0.31 g. The varieties were evaluated for five major fatty acids with predominance of 18 carbon species namely palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2) and linolenic (18:3) acids exhibited a wider range of fatty acid variation. Among the fatty acid, linolenic acid ranged from 33.43 (LCP 147) to 58.48% (Surbhi) with an average of 48.59%. Linoleic and oleic acid recorded a range of 6.15 to 23.64 and 20.30 to 33.47%, respectively; whereas palmitic acid had lowest range of 4.66 to 7.03%. Characterization of crop germplasm is a first and crucial step in plant breeding for quantification of genetic variability and diversity present in crop species. Using these information, the plant breeders can develop cultivar by selection of most desirable and diverse parents to widen the breeding gene pool (Fu, 2006). The morphological traits have been utilized for characterization and has important role to discriminate the flax varieties (Smykalova *et al.*, 2013). There was significant difference between the accessions for all measured traits, indicating the presence of a wide range of variation across the accessions for these traits, including days to flowering, days to maturity, plant height and seed weight and quality. Among fatty acid, the highest range was identified for linolenic acid 25.05%, followed by linoleic acid (17.49%) and oleic acid (13.17%) in the cultivars. Rajwade *et al.* (2010) showed a wide range for fatty acid in Indian released varieties recommended at national and state level. A wide range of diversity for fatty acid composition has been reported in flax varieties of India (Diederichsen, 2001). Examining the studied morphological and biochemical traits, all the 19 cultivars showed grater variability from each other in one or more individual characters. This confirmed that the cultivars exhibit more genetic variations confirming the source of improvement in these traits.

Cultivars relationship and genetic diversity

Initially 71 ISSR primers were used to investigate the level of polymorphism in 19 linseed varieties and 24 primers were selected for their ability to produce clear and reproducible patterns of

polymorphism with multiple bands. The reproducibility of ISSR amplifications was assessed with different DNA samples isolated independently from the same cultivar and amplified at different times. A set of 24 ISSR primers showed multiple bands in each cultivar. This primer set amplified a total of 74 bands (alleles) out of which 66 were polymorphic with high frequency of 89%. The percentage of polymorphic loci for all the primer ranged from 50 (UBC 842, UBC 868) to 100%. Fifteen primers showed the polymorphism for all the amplified loci. The amplified alleles ranged from 1 to 12 across primes with an average of 3.08 (Table 2).

Table 1: Mean values of agro-morphological and biochemical/quality traits of 19 cultivars of linseed

Cultivars	Pedigree	DTF	DTM	PH (cm)	SW (g)	Oil content (%)	Fatty acid profile				
							Palmitic (16:0)	Steric (18:0)	Oleic (18:1)	Linoleic (18:2)	Linolenic (18:3)
Surbhi	LC 216 X LC 185	100	154	57.5	5.48	39.40	5.36	3.65	22.72	9.61	58.48
Kiran	(R1 X AFG-8) X R1	101	154	65.5	6.35	40.60	6.71	5.16	26.59	12.19	49.10
Garima	T 126 X Neelum	99	154	57.0	7.69	40.00	5.58	4.42	22.99	9.63	56.95
Sheela	Gaurav X Janki	100	150	57.1	7.19	37.80	5.66	4.25	32.29	13.55	44.21
LC 185	NP(RR)-37 X Kangra Local	115	152	58.3	4.45	39.80	6.41	5.39	27.89	13.99	45.50
J 23	EC-9832 X Hira	103	154	65.0	6.40	38.20	5.03	5.24	30.10	11.68	48.40
SLS 71	EC 11411 X LCK 88062	78	144	63.0	8.38	36.80	5.37	5.73	30.29	15.76	42.70
T 397	T 491 X T-1103-1	96	149	56.7	5.35	37.20	6.14	4.64	25.11	11.57	51.70
Shubhra	Mukta X K2	98	149	60.0	8.76	42.00	5.45	4.41	20.30	23.64	45.12
Suyog	(Kiran X KL 168) X Kiran-1	98	151	63.0	8.38	36.10	6.48	5.70	26.21	11.49	50.34
Sharda	(Shubhra X J1) X (J1 X Kiran)	97	159	47.1	8.49	36.00	5.70	4.04	30.90	11.35	48.01
Indira Alsi 32	Kiran X RLC 29	100	161	50.7	6.95	34.80	5.34	4.83	29.41	11.73	48.10
LCP 147	Belley X ACC NO 2251-2	109	160	81.2	6.90	39.00	6.43	7.26	32.30	20.20	33.43
Binwa	Flake1 X SPS-47/7-10-3	102	166	54.1	5.84	32.80	4.66	4.26	24.37	10.80	55.71
Pratap Alsi	ACC 750 X RL 29-8	89	145	77.7	7.74	37.30	4.72	4.60	29.76	13.25	48.12
Deepika	Kiran X Ayogi	87	144	50.1	4.84	36.80	6.74	5.18	33.47	6.15	48.46
Baner	EC 21741 X LC 216	108	166	66.4	6.29	38.20	5.62	7.11	25.23	14.38	47.17
Kartika	Kiran X LCK 88062	87	148	54.0	5.50	34.00	5.08	4.96	21.31	16.04	52.40
Himani	DPL20 X KLS-I	109	169	55.0	4.67	36.00	7.03	5.45	27.94	10.28	49.28
Mean ± SE		98.7 ±2.0	154.2 ±1.7	60.0 ±2.0	6.61 ±0.31	37.52 ±0.53	5.76 ±0.16	5.07 ±0.22	27.33 ±0.89	13.02 ±0.90	48.59 ±1.27

DTF = Days to 50% flowering, DTM = Days to maturity, PH = Plant height, SW = 1000-seed weight

The ISSR primer (UBC 808) produced the maximum 12 alleles, followed by UBC 868 with six alleles. The lowest number of alleles was observed in UBC 809 with one allele only. The size of detectable allele ranged from 120 bp (UBC 842) to 12 kb (UBC 835). The PIC value ranged from 0.33 (UBC 889) to 1.0 (UBC 835,846, 809 &866). None of the genotype exhibited identical ISSR profiles signifying the robustness of primers used in the study.

The ISSR amplification pattern was used to establish genetic relationship among the genotypes by cluster analysis using Neighbour joining similarity coefficient with a cophenetic correlation of 0.74. A dendrogram was generated using PAST programme based on binary data matrix of the total allele which clustered the 19 cultivars into two main groups (Fig. 1). Bootstrap analysis using 1000 replicates showed that forks had 100% bootstrap support and 21 of 28 forks had greater than 50% bootstrap support. The first group included 10 genotypes of which 8 were seed type namely ‘Binwa’, ‘Suyog’, ‘Indra Alsi-32’, ‘Deepika’, ‘Baner’, ‘Shardha’, ‘Kartika’ and ‘Himani’ and two were dual purpose type *viz.* ‘Pratap Alsi’ and ‘LCP-147’. Out of 19 tested cultivars only two cultivars were dual purposes that were much closer to each other in the first cluster. Out of remaining seven, five cultivars derived from ‘Kiran’ which was used as a single parent or three ways cross. The genotype ‘Binwa’ ‘Suyog’, ‘Indra Alsi-32’, ‘Deepika’, ‘Baner’, ‘Shardha’, ‘Kartika’ and ‘Himani’ and two were dual purposes that were much closer to each other in the first cluster. Out of remaining seven, five cultivars

Table 2: ISSR primers used for DNA profiling of 19 linseed cultivars

Primer	Primer sequence	Total number of amplified bands	High frequency polymorphic band	% age polymorphism	Average PIC value
UBC 806	(AG)9T	5	5	100	0.770
UBC 808	(AG)9G	12	9	75	0.900
UBC 809	(GA)9T	1	1	100	1.000
UBC 811	(GA)9A	5	5	100	0.430
UBC 825	(AC)8T	2	2	100	0.370
UBC 834	(AG)7YT	3	3	100	0.650
UBC 835	AG)8YC	1	1	100	1.000
UBC 836	(AG)8YG	2	2	100	0.800
UBC 842	(GA)8YG	2	1	50	0.100
UBC 846	(CA)8RT	2	2	100	0.100
UBC 847	(CA)8RC	4	4	100	0.640
UBC 848	(CA)8RG	4	4	100	0.410
UBC 849	(GT)8YA	3	3	100	0.660
UBC 850	(GT)8YC	2	2	100	0.430
UBC 857	$(AC)_8$YG	3	3	100	0.630
UBC 864	(ATG)6	3	2	67	0.660
UBC 866	$(CTC)_6$	1	1	100	1.000
UBC 868	(GAA)6	6	3	50	0.820
UBC 878	$(GGAT)_4$	1	1	100	1.000
UBC 887	DVD$(TC)_8$	3	3	100	0.580
UBC 888	BDB$(CA)_8$	2	2	100	0.500
UBC 889	DBD$(AC)_8$	2	2	100	0.330
UBC 890	VHV$(GT)_8$	3	3	100	0.640
UBC 891	HVH$(TG)_8$	2	2	100	0.470

derived from ‘Kiran’ which was used as a single parent or three ways cross. The genotype ‘Binwa’ was most distinct from others and did not come together with any other genotype in first cluster. The second group included nine cultivars namely ‘Shubhra’, ‘T 397’, ‘J 23’, ‘SLS 27’, ‘Surbhi’, ‘Garima’, ‘LC 185’, ‘Sheela’ and ‘Kiran’. The second group could be divided into two sub-groups as the cultivar ‘Shubhra’, ‘T 397’, ‘J 23’, ‘SLS 27’ formed first sub-group of this cluster. ‘Shubhra’ and ‘T 397’ varieties were closer to each other and shared approximately 60% similarity. The second sub-cluster included cultivars namely ‘Surbhi’, ‘Garima’, ‘LC 185’, ‘Sheela’ and ‘Kiran’ and has 70% similarity. The ‘LC 185’ was most diverse, followed by ‘Surbhi’ and ‘Kiran’ whereas ‘Sheela’ and ‘Garima’ were the sister lines in this sub-cluster.

The cluster analysis based on marker data showed no clear grouping of cultivars with regard to the breeding centre and pedigree record (Fig. 1). The principle component analysis (PCA) was also performed and the results based on first PCA were comparable to those obtained in Neighbour joining similarity cluster analysis (Fig. 2). The genotypes ‘Surbhi’, ‘Garima’, ‘LC 185’, ‘Sheela’ and ‘Kiran’ were placed away from the other genotypes in second cluster and formed a sub-cluster.

The studied conducted on discrimination among plant cultivars between mid-2006 and mid-2009 revealed that 292 papers published on locus-specific microsatellite analysis (SSR) showed it as the choice of marker (36%), followed by RAPD (27%), ISSR (13%), AFLP (11%), other nuclear DNA-based methods (10%, including for example CAPS, DAMD, IRAP, REMAP, SNPs, SCAR and SRAP) and organellar DNA-based methods (3%, mostly cpDNA) (Nybom and Weising, 2010; Hilde Nybom1, 2014). Among the molecular markers ISSR is a popular approach because of its reproducibility, used for unknown and non-sequenced genome and widely used for population and phylogenetic studies as well as crop varietal authentication. Twenty cultivars of oilseed discriminated using ISSR primers, however the corresponding association was not found for cultivars based on their parentage or pedigree record and breeding centre by examining grouping patterns. Wiesnerova and Wiesner (2004) reported 72.6% polymorphism using 9 anchored ISSR primers for 53 Czech flax

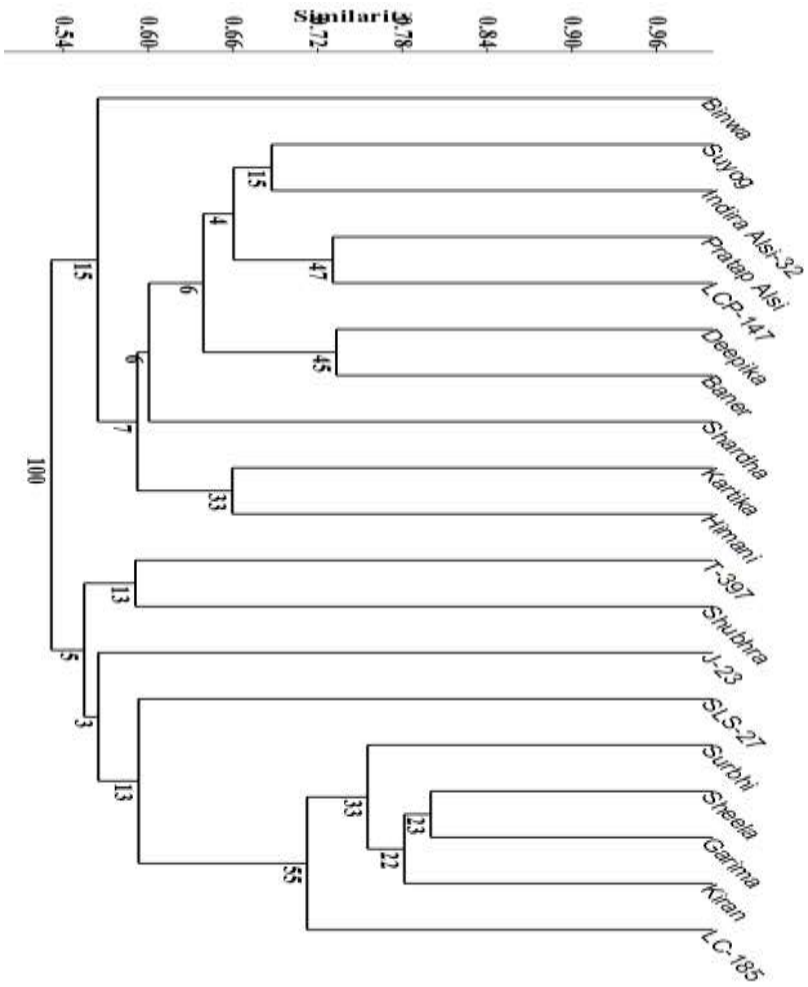

Fig. 1: Neighbour Joining based dendrogram showing genetic relationship of 19 linseed cultivars in clusters based on 24 ISSR markers

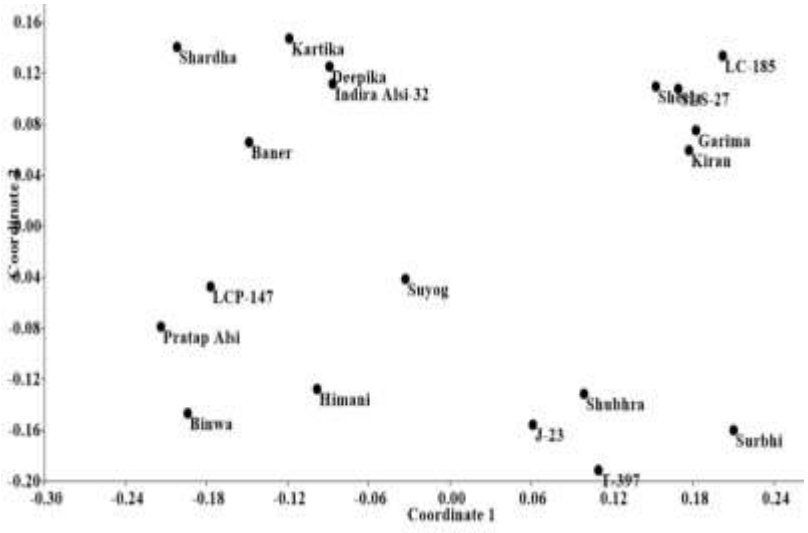

Fig. 2: Principle component analysis (PCA) of 19 linseed cultivars based on 24 ISSR markers

cultivars, while in present study, we detected 93.4% average polymorphism in 19 flax genotypes using 24 ISSR primers. Smykal *et al.* (2011) conducted a study using inter-retro-transposon amplified polymorphism (IRAP) markers with genetic diversity among 708 accessions of cultivated flax comprising 143 landraces, 387 varieties and 178 breeding lines. The maximal genetic diversity was detected among wild *Linum* species, while diversity within cultivated germplasm decreased from landraces (58%, 0.63) to breeding lines (48%, 0.85) and cultivars (50%, 0.81). In a study 20 flax genotypes using RAPD (random amplified poly-morphic DNA) markers showed no evidential grouping based on straight pedigrees relationships, but wider genetic background of genotype can be seen (Ahsan Iqbal, 2014).

DNA fingerprinting and cultivar authentication based on ISSR profiling

Based on the genotypic amplification patterns/profile of the selected ISSR primers, a digital fingerprint for each genotype constituted. Cultivar-specific alleles have been identified that distinguished according to its unique fingerprint profile. The 15 cultivars were characterized by the presence and/or absence of specific bands (Table 3). Primer UBC 849 gave a diagnostic amplification in 'Garima' and 'Shubhra' cultivars by

Table 3: Cultivar-specific bands revealed by ISSR fingerprinting for 15 linseed cultivars

Cultivars	Characterized by presence of band	Characterized by absence of band
Shubhra	UBC 849 (451bp)	UBC 835 (1100 bp), (800 bp)
Surbhi		UBC 848 (304 bp)
Garima	UBC 849 (451 bp)	-
LC 185	-	UBC 846 (373 bp)
SLS 27	-	UBC 825 (410 bp)
T 397	-	UBC 847 (555 bp), UBC 887 (850 bp), UBC 890 (400 bp)
J 23	UBC 888 (875 bp), UBC 878 (900 bp), UBC 842 (275 bp), UBC 836 (600 bp)	UNC 806 (450 bp), UBC 848 (304 bp), UBC 849 (392 bp)
Suyog		UBC 847 (555 bp)
Baner	UBC 888 (875 bp)	UBC 808 (360 bp), (440 bp)
Sharda	UBC 846 (327 bp)	-
India Alsi	UBC 847 (317 bp), UBC 848 (331 bp), UBC 849 (682 bp)	-
Pratap Alsi	UBC 888 (700 bp), UBC 836 (600 bp)	-
Binwa	UBC 888 (700 bp), UBC 847 (317 bp)	UBC846 (373 bp), UBC 836 (700 bp)
Kartika	-	UBC 806 (450 bp), UBC 835 (1100 bp)
Himani	-	UBC 835 (1100 bp)

amplifying of 451bp fragment and 682 bp fragment in 'Indira Alsi 32'. The 392 bp amplification product by UBC 849 was absent only in 'J 23' cultivar. Cultivar-specific ISSR bands were obtained for 15 of the 19 linseed cultivars tested that was able to distinguish 15 out of 19 cultivars (Table 3). ISSR marker profile has been used to generate an amplification profile for fingerprinting, to detect the varietal diagnostic markers for identification and differentiate cultivars and species that might be difficult to characterize and identify due to similar morphological characteristics or indistinct traits. PCR-based ISSR profiling markers has been used extensively for the identification of genotypes in crop plants and has gained importance due to its simplicity, efficiency and non-requirement of sequence information. In this study cultivar-specific alleles have been identified that distinguished cultivars from others based on its unique fingerprint profile and specially using the unique/private alleles. The genetic purity and marker authentication has been performed in agricultural crops (Sundaram *et al.*, 2008), maize (Wu *et al.*, 2006) and safflower (Naresh *et al.*, 2009). In conclusion, the variation in agro-morphological, quality traits and genotypic profile generated by 24 polymorphic ISSR markers can be used for identification of diverse parent based on relatedness for future breeding. Identification of diagnostic markers can play important role in seed genetic purity assessment and securing of plant variety right.

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