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Genetic diversity in linseed (*Linum usitatissimum*) cultivars based on RAPD-analysis*

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Linseed (*Linum usitatissimum* L.) is an annual, non-edible, self-pollinating, winter (*rabi*) season oilseed crop. This crop is grown under diverse agronomic conditions for its fibre (fibre flax), seed oil (linseed) or both fibre and seed oil (dual-purpose linseed). Fifty years of breeding efforts under All India Co-ordinated Research Project on Oilseeds (Linseed) have produced more than 51 improved varieties and made significant impact on Indian linseed production and productivity. However, genetic relatedness among these cultivars and genetic erosion that might have occurred during the breeding process is not known. It is well known that information on the extent and distribution of genetic variation among the linseed germplasm are essential for target specific breeding programmes. Traditionally, morphological characters have been used to establish the description of genotypes based on distinctness, uniformity and stability (Table 1). But these characters are often influenced by environment.

Recently, molecular markers such as Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Random Amplified Polymorphic DNA (RAPD) and Simple Sequence Repeat (SSR) have been developed with distinct superiority over morphological markers and allozymes in numbers and polymorphisms. Polymerase Chain Reaction-based molecular markers, like RAPD are relatively quick, need low amount of DNA and detect good number of polymorphisms without prior sequence information of target genome.

The present study on linseed was conducted using PCR-based molecular marker (RAPD) to find out the genetic diversity and also assess the genetic relationship among the major cultivated linseed varieties developed by Chandra Shekhar Azad University of Agriculture and Technology, Kanpur.

The experimental materials comprised 10 improved varieties of linseed developed between 1964 and 2007 by C S Azad University of Agriculture and Technology, Kanpur. The leaves from same age linseed cultivars (Table 1) grown during 2007–08 under DUS Project at the student instructional farm of the university were harvested and washed thrice with sterilized water. The moister was soaked with sterilized blotter paper and 100 mg sample of each variety was used for DNA isolation. The leaves of each cultivar were ground separately in pre-cooled mortar with liquid nitrogen and powder was transferred into 1.5 ml tubes containing 0.5 ml of pre-warmed (65° C) CTAB extraction buffer [(10 ml of 1 M Tris-HCl (pH 7.6), 14 ml of 5 M NaCl and 4 ml of 0.5 M EDTA (*p*H 8.0) were mixed together and 1 g DDT and 1 g CTAB were dissolved in this solution. The final volume was made upto 100 ml by adding ultra pure water]. The tubes were incubated at 65° C for 15 min., followed by centrifugation at 13 000 rpm at 20° C for 2 min. The supernatant was transferred in to a fresh tube and rest of the protocol was followed according to the instructions of manufacturer of SiMax Genomic DNA Extraction Kit. The isolated DNA was gel electrophoresed in 1% agarose gel and stained with ethidium bromide and visualized by gel documentation system to know the quality and quantity.

The random primer kit (SBS-B) used for amplification was procured from SBS Genetech Co., Ltd, China (Table 2). The PCR reaction was setup by using Easy-Do PCR PreMix Kit supplied in lyophilized form. The reaction mix contained Taq DNA polymerase-2 units, 10×PCR reaction buffer-5 ìl, dNTPs (10 mM each)-200 ìM each, loading dye-5 ìl, stabilizer-5 ìl. In this pre-mix, about 50 ng DNA template and 50 pmol of RAPD primer were added, and the final volume of 50 ìl was made up by adding nuclease free water. The DNA amplification was performed in an Eppendorf

*Short note

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Table 1 List of the linseed cultivars and their characteristics used for RAPD analysis

Varieties/ characters	Days to 50% flowering	Flower Size of corolla	Flower shape	Flower colour	Flower aestivation	Flower venation colour	Stamen colour of distal part of filament	Anther colour	Plant growth habit	Plant height	Capsule size	Capsule dehiscence	Seed colour	Seed size (length of seed)	Seed weight (1000 seed/g)	Oil content (%)
'T–397'	Medium	Medium	Funnel	Violet	Semi-twisted	Violet	Blue	Grey	Bushy	Medium	Small	Non-dehiscent	D. brown	Medium	Low	40.56
'Neelum'	Late	Large	Disk	Blue	Twisted	Blue	White	Blue	Erect	Medium	Bold	Semi-dehiscent	Brown	Large	High	42.02
'Shekhar'	Medium	Medium	Disk	Blue	Twisted	Blue	White	Grey	Semi-erect	Medium	Medium	Non-dehiscent	Brown	Medium	High	44.83
'Garima'	Medium	Medium	Disk	Blue	Twisted	Violet	White	Grey	Erect	Medium	Medium	Non-dehiscent	L.brown	Medium	Medium	45.44
'Padmini'	Medium	Large	Disk	Violet	Semi-twisted	Violet	White	Grey	Semi-erect	Dwarf	Medium	Semi-dehiscent	L.brown	Medium	High	45.67
'Parvati'	Late	Large	Disk	Blue	Twisted	Violet	White	Grey	Erect	Tall	Medium	Semi-dehiscent	L.brown	Medium	High	43.32
'Sharda'	Early	Small	Funnel	White	Twisted	White	White	Cream	Semi-erect	Dwarf	Medium	Non-dehiscent	Brown	Medium	High	42.98
'Sweta'	Medium	Small	Funnel	Blue	Semi-twisted	White	White	Cream	Erect	Medium	Medium	Non-dehiscent	L.brown	Medium	Medium	45.73
'Shikha'	Late	Large	Disk	Blue	Semi-twisted	Blue	Blue	Blue	Erect	Tall	Medium	Non-dehiscent	Brown	Medium	Medium	40.22
'Gaurav'	Late	Large	Disk	Red violet	Twisted	Violet	White	Cream	Erect	Tall	Medium	Non-dehiscent	Fawn	Medium	Medium	45.55

Master Cycler Gradient (Eppendorf, Hamburg) by using the following programme: 1 cycle of 94°C for 5 min., 45 cycles of (94°C for 1 min., 36°C for 1 min., 72°C for 2 min.) followed by a final extension of 5 min. at 72°C.

The amplified DNA was analyzed on 1% Agarose gel in 1XTAE (40 mM Tris-acetate and 1 mM EDTA, pH 8.0) by electrophoresis at 60 V for 2 hr. The bands were visualized by adding ethidium bromide stain (0.5 ìg/ml) in the gel. The gels were photographed using Gel Doc System. Bands were scored as 1 (present) or 0 (absent). Reproducibility of the bands was confirmed by repeating the experiment twice (extraction and amplification).

RAPD bands were scored as present (1) or absent (0) for all the linseed genotypes. Each band was assumed to represent a unique genetic locus. Its presence was interpreted as either a heterozygote or dominant homozygote and the absence as a recessive homozygote. The pattern and extent of RAPD variation were analyzed with respect to primer, polymorphic locus and cultivar. Data entry was done into binary data matrix and statistical analysis was carried out using NTSYS-pc, 2.01 versions (Rolf 1993). Pair-wise comparisons of samples were used to estimate Jaccard's similarity co-efficient (GS): a/(n-d) where a=no. of +ve coincidences, n=total sample size and d=no. of –ve coincidences. Genetic distances between pair of cultivars were estimated as 1-GS. Jaccard's similarity co-efficient was used to generate dendrogram using unweighted pair group method with arithmetic mean UPGMA (Sneath and Sokal 1973).

All the 20 decamer primers used in the present study were found to display reliable amplification profiles, which have been summarized in Table 2. Some of the primers did not produce any scorable bands in some linseed genotypes eg. primer SBSB04 amplified only two bands in only one variety 'Garima'. In total, 111 bands were generated, of which 70 bands were polymorphic. Two primers (SBSB06 and SBSB14) could not generate any polymorphic band. The average number of bands/primer was 5.55 and the number of amplified products/primer varied from 2 (SBSB04) to 9 (SBSB01 and SBSB11). Maximum level of polymorphism (100%) evident with SBSB04, B08, B09, B11, B12, B13 and B19 indicate the capability of RAPD primers to amplify the highly repeated regions of the DNA. Higher number of co-migrating bands monitors the reproducibility of amplification pattern, while polymorphic ones provided the key to genotype identification (Mathew *et al.* 2000). Primer B-06 amplified two similar bands of H"300 bp and H"500 bp in all the linseed genotypes used. Eleven primers have produced genotype specific bands that would be highly useful for the identification of genotypes and designing future breeding strategy. Primer SBSB17 amplified 2 unique bands of H"700 bp one each in 'Sweta' and 'Shikha'. The results suggest that RAPDs are powerful markers and can be used for varietal identification and purification in linseed. The diversity revealed by RAPD is in agreement with the earlier reports

Table 2 List of the decamer primers and the polymorphism observed in the study

Primer	Primers sequences(5'–3')	Total no of bands	No. of polymorphic bands	Per cent	Genotype distinguished
SBSB–01	GTT TCG CTC C	9	6	67.67	'Garima'
SBSB–02	TGA TCC CTG G	6	4	67.67	'Neelum'
SBSB–03	CAT CCC CCT G	6	5	83.33	
SBSB–04	GGA CTG GAG T	2	2	100.00	'Garima'
SBSB–05	TGC GCC CTT C	9	4	44.44	'Garima'
SBSB–06	TGC TCT GCC C	3	0	00.00	
SBSB–07	GGT GAC GCA G	7	4	57.14	'T397', 'Shikha'
SBSB–08	GTC CAC ACG G	7	7	100.00	
SBSB–09	TGG GGG ACT C	6	6	100.00	'Shekhar'
SBSB–10	CTG CTG GGA C	8	3	37.50	'Gaurav'
SBSB–11	GTA GAC CCG T	9	9	100.00	'Garima'
SBSB–12	CCT TGA CGC A	5	5	100.00	
SBSB–13	TTC CCC CGC T	4	4	100.00	'Parvati'
SBSB–14	TCC GCT CTG G	3	0	00.00	
SBSB–15	GGA GGG TGT T	5	1	20.00	
SBSB–16	TTT GCC CGG A	4	2	50.00	'Shekhar'
SBSB–17	AGG GAA CGA G	6	2	33.33	'Sweta', 'Shikha'
SBSB–18	CCA CAG CAG T	4	1	25.00	
SBSB–19	ACC CCC GAA G	3	3	100.00	
SBSB–20	GGA CCC TTA C	5	2	40.00	
Total		111	70		
Average		5.55	3.5	63.06	

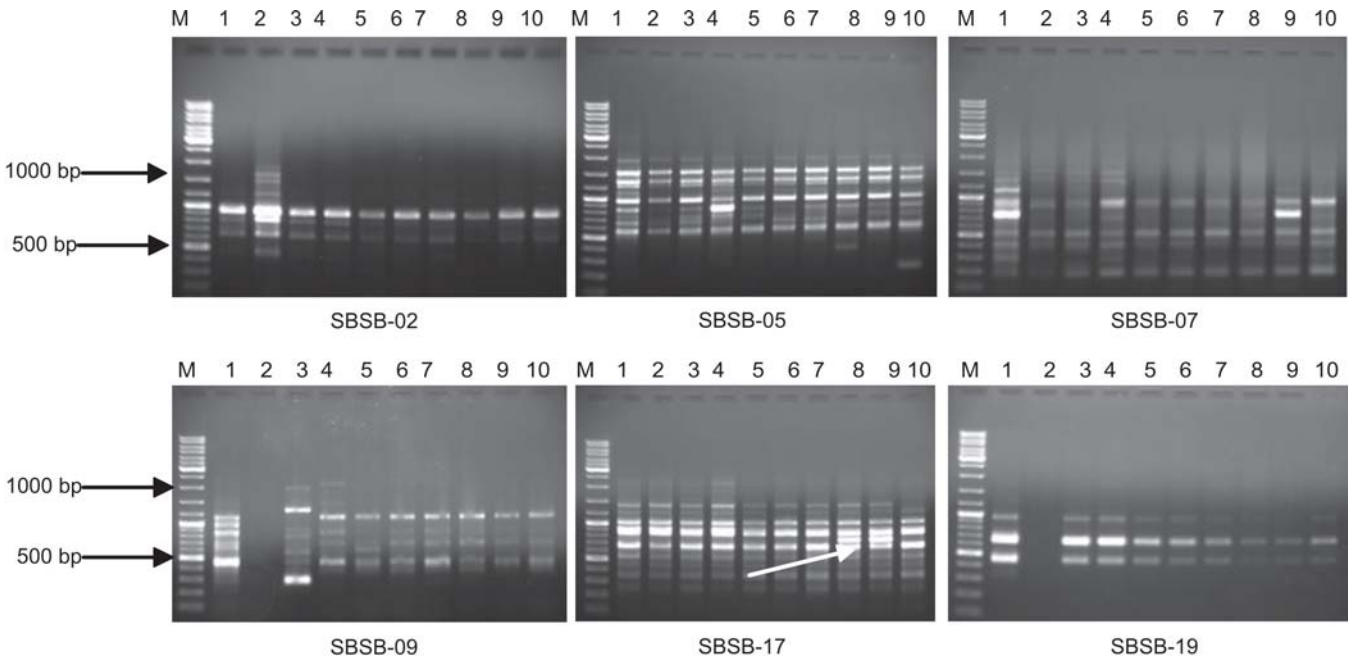

Fig 1 RAPD variation patterns of the 10 linseed cultivars observed with primers SBSB-02,05,07,09,17 and 19. (Lane M=1 kb DNA ladder-Fermentas;
Lane 1= 'T397';Lane 2= 'Neelum'; Lane 3= 'Shekhar'; Lane 4= 'Garima'; Lane 5= 'Padmini'; Lane 6= 'Parvati'; Lane 7= 'Sharda'; Lane 8= 'Sweta'; Lane 9= 'Shikha'; Lane 10= 'Gaurav')

(Yong *et al.* 2002, 2003 and Yong 2005).

The first time determination of genetic relationships between employed released varieties is important for further linseed crop improvement. Nevertheless, RAPD markers revealed marginal genetic diversity among cultivated varieties with genetic similarity ranging from 67% (between

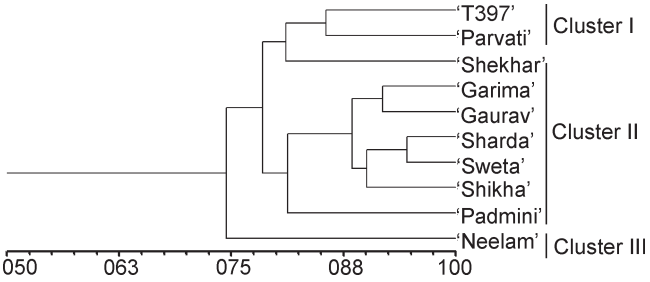

Fig 2 UPGMA dendogram depicting relationships among 10 linseed cultivars

'Neelum' and 'Parvati', 'Neelum' and 'Padmini') to 94% between 'Sharda' and 'Sweta'. Interestingly, the results indicated the existence of adequate genetic diversity between prime cultivars ('Padmini', 'Parvati') and 'Neelum', an old and bold seeded variety, developed during 1964 under Indian Central Oilseeds Scheme. These divergent varieties can be used as parents in recombination breeding programme. Maximum similarity was observed between the varieties 'Sharda' and 'Sweta' which have funnel-shaped white flower, white flower venation, white stamen, cream anther and recommended for rainfed ecology. This, in turn, indicated that flower characteristics have played a role in clustering pattern and might be strongly linked with each others. These estimates would provide useful information on how significant the genetic base is and how fast it has been narrowed during the breeding programme. Obviously, effort is needed to diversify the genetic base of breeding materials as like land races, expected to harbour more genetic variation than linseed cultivars.

The UPGMA dendogram obtained from this cluster analysis using Jaccard's similarity co-efficient (Fig 2) showed that similarity co-efficient between all pairs of varieties ranged between 0.70 and 1.00. At 0.83 similarity level, all the cultivars combined to form 3 major clusters. Cluster I combined three genotypes 'T397', 'Parvati' and 'Shekhar', whereas cluster II comprised of six genotypes, viz 'Garima', 'Gaurav', 'Sharda', 'Sweta', 'Shikha' and 'Padmini'. It was interesting to observe that 'Neelum' was distinct from all the other nine varieties and sole constituent of cluster III. Morphologically similar genotypes, like 'Parvati' and 'Shikha' (both are dual-purpose cultivar with tall stature, disk-shaped blue flower of big size and late maturity duration) were genetically dissimilar and grouped into different clusters.

SUMMARY

Molecular characterization of ten phenotypically distinct linseed varieties released and recommended for different agro-ecological situations was performed for the first time with RAPD markers. All the 20 primers used in this study were highly reproducible and generated 111 bands with an average of 63.06% polymorphism. The number of amplification product per primer ranged from 2 (SBSB04) to 9 (SBSB01 and SBSB11) with an average number of 5.55 bands/primer. Two primers (SBSB06 and SBSB14) could not generate any polymorphic band, whereas seven primers produced 100% polymorphism. Eleven primers produced genotype specific bands that would be helpful in their identification. Genetic relationship was established based on Jaccard's similarity co-efficient which ranged from 0.67 to 0.94. UPGMA cluster analysis generated dendogram with three clusters at 0.83 similarity co-efficient. Cluster I and II comprised three and six genotypes, respectively, whereas cluster III was having only 'Neelum'. The most distant pairs of linseed varieties 'Neelum' and 'Parvati', followed by 'Neelum' and 'Padmini' and 'Neelum' and 'Shekhar' can be utilized in future breeding programme to get promising recombinants. The present study has proved that linseed varieties 'Parvati' and 'Shikha' reported to be similar in morphology may be genetically different.

REFERENCES

Mathew L, Babu R C, Souframanien J, Chezhian P, Shanmugasundaram P, Nagarajan P and Sadasivam S. 2000. DNA polymorphism among rice (*Oryza sativa* L.) accession differing in drought tolerance. *Journal of Plant Biology* **27**: 145–52.

Rolf F J. 1993. *NTSYS-pc Numerical taxonomy and multivariate analysis system*, version 2.01. Exter Software, Setauket, NY.

Yong B F, Diederichsen A, Richards K W and Peterson G. 2002. Genetic diversity within a range of cultivars and landraces of flax (*Linum usitatissimum* L.) as revealed by RAPDs. *Genetic Resources and Crop Evolution* **49**: 167–74.

Yong B F, Rowland G G, Duguid S D and Richards K W. 2003. RAPD analysis of 54 North American Flax cultivars. *Crop Science* **43**: 1510–15.

Yong B F. 2005. Geographic patterns of RAPD variation in cultivated flax. *Crop Science* **45**: 1048–91.