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GENETIC PURITY ASSESSMENT IN LINSEED (*LINUM USITATISSIMUM* L.) VARIETIES USING MICROSATELLITE MARKERS

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ABSTRACT
The present investigation was undertaken in the Department of Genetics and Plant Breeding, IGKV, Raipur, India during *Rabi* 2012-13 and 2013-14 season. The present investigation was carried out with the objectives to identify informative SSR marker allele (s) for fingerprinting popular linseed cultivars, and to identify specific SSR markers in seed genetic purity assessments. A set of 38 SSR markers located across the 30 chromosomes of linseed were used, out of which 28 SSR markers were observed to be polymorphic for cultivars considered in the study. These loci were screened in breeder seed of four popular cultivars of linseed. Out of These Thirty-eight microsatellite markers six microsatellite (LU-1,LU-7,LU-8,LU-9,LU-24,LU-25) markers were found polymorphic which are used for fingerprinting in population. Based on the polymorphic status, 6 SSR markers were found to be informative for discriminating the genotypes and showed a total of 24 alleles. To validate the utility of the SSR markers in genetic purity assessment, seed lots of R 552, Deepika, Indira Alsi 32 and RLC 92 were assessed for their genetic purity using both SSR marker analysis and 18 morphological characters in a grow-out test (GOT). The results indicated practical utility of the SSR markers in assessing the genetic purity of the linseed cultivars and their fingerprinting.
Abbreviations: CTAB-Cetyl Trimethyl Ammonium Bromide, DUS-Distinctness Uniformity Stability, PAGE-Poly Acryl-amide Gel Electrophoresis, PCR-Polymerase Chain Reaction, PIC-Polymorphic Information Content, SPS-Single Plant Selection, SSR-Simple Sequence Repeats, UPOV-Union for Protection of New Plant Varieties.

INTRODUCTION

Linseed (*Linum usitatissimum* L.) belongs to the genus Linum of the family Linaceae commonly known as "Alsi" and has 2n = 30 chromosomes. Linseed is one of the important *rabi* oilseed crops of India, cultivated in light soil under one or two irrigation in Madhya Pradesh, Chhattisgarh, Uttar Pradesh, Maharashtra, Rajasthan, West Bengal, Karnataka, Orissa and Bihar. In Chhattisgarh, linseed is grown mostly rainfed as "utera" as well as in crop fields (Pali and Mehta, 2014). It is one of the oldest crops cultivated by man. Based on diversity of plant types, linseed has two centers of origin *i.e.,* South West Asia, particularly in India (Vavilov, 1935; Richharia, 1962) and the Mediterranean region of Europe (Darlington, 1963). In industrial oil production, it ranks first in the country. It is a long day, self pollinated crop and is cultivated in 34 countries of the world. Genetic diversity in sesame, based on morphological, biochemical, metabolic, and molecular markers, has been reported by many researchers worldwide (Tripathi *et al*., 2013; Yol and Uzun 2012).

Fingerprinting with molecular markers allows precise and rapid cultivar identification, which has been proved to be an efficient tool for crop germplasm characterization, collection and management. SSR markers have been widely used for genetic analysis and cultivar identification because of their abundance, co-dominance, inheritance, high polymorphism, reproducibility and ease of assay by PCR (Kuleung *et al*., 2004 and Xie *et al*., 2011). Fingerprinting of the commercial linseed popular varieties based on molecular markers is a crucial measure for unambiguous and quick identification of similar or closely-related cultivars.

The genetic purity of varieties is conventionally determined by the grow-out test (GOT), which is based on assessment of morphological and floral characters called "descriptors" in plants grown to maturity. However, it is a time and resource consuming exercise, influenced by environmental factors and the results are often subjective. The above limitations can be managed effectively by employing molecular markers, particularly DNA based markers which can be assessed through the technique of PCR.

Among the molecular markers, microsatellites also called Simple Sequence Repeat (SSR) markers are most suitable for many applications because of the ease in handling, reproducibility, co-dominant inheritance and genome-wide coverage (McCouch *et al*., 1997). The use of SSR markers for assessing genetic purity of parental lines and hybrids has been reported in agricultural crops like rice (Sundaram *et al*., 2008) and maize (Mingsheng *et al*., 2006).

The above limitations can be managed effectively by employing molecular markers, particularly DNA based markers which can be accessed through the technique of

PCR. However, this is the second attempt of fingerprinting of the main commercial linseed varieties under cultivation at present in Chhattisgarh state of India. The present study therefore aimed to identify informative microsatellite markers of flax which can serve as distinct molecular fingerprints/IDs for popular Indian flax cultivars and to validate their utility in monitoring genetic purity in different seed classes.

MATERIALS AND METHODS

Plant materials

The present study was carried out at Plant Molecular Biology Laboratory, Department of Genetics and Plant Breeding, Indira Gandhi Agricultural University (IGAU), Raipur, India during *Rabi* 2011-2012. The plant material for this study comprised of SPS nucleus seeds of four popular cultivars of flax (Table 3) collected from National Seed Project (NSP), IGKV, Raipur and certified seeds collected from Chhattisgarh State Seed Certification Agency (CGSSCA), Raipur, India.

Genomic DNA extraction

Genomic DNA was extracted from young leaves of the flax plants according to the CTAB method as described by (Kang *et al*., 1998) with some modifications. The concentrations and quality of the genomic DNA samples were estimated on spectrophotometer ND-2000 (Nanodrop, USA). Finally, all the genomic DNA samples were diluted to a final concentration of 40 ng/ìL with TE buffer (10 mM Tris-HC1, pH 8.0; 1 mM EDTA) and stored at -20ºC for further use.

SSR-PCR amplification

A total of thirty-eight flax SSR primers covering all the chromosomes of flax were used in this study. Polymerase chain reaction for amplifications of DNA preparations were carried out in a 20 μL volume for SSR (Table 4 and Table 5). All PCR reactions were carried out in a Veriti Thermal Cycler (Applied Biosystems, USA). PCR products were separated using 5% PAGE, stained with ethidium bromide and photographed under UV light using Image Gel Doc Lab™ software Version 2.0.1 (Bio-Rad, USA).

Data analysis

The band profiles were scored only for distinct, reproducible bands as present (1) or absent (0) for each SSR primer pair. The polymorphism information content (PIC) value of SSR markers was calculated using the following formula (Anderson *et al*., 1993).

$$PIC = 1 - \sum_{i=1}^{k} P_i^2$$

Where, k is the total number of alleles (bands) detected for one SSR locus and P_i is the frequency of the i^{th} allele (band) in all the samples analyzed.

Cluster analysis

Cluster analysis Jaccard's similarity was carried out based on the similarity index data derived from UPGMA based Jaccard's coefficient.

Genetic purity analysis through SSR markers

A total of 80 individual plants were randomly selected from grow out test field from three different genetic purity lots. DNA was extracted from each leaf sample of 80 individual plants using the protocol as mentioned above described by Kang *et al*. (1998). The isolated DNA was amplified using the SSR marker and PCR product were separated using 5% PAGE, stained with ethidium bromide and photographed under UV light using UV light using image Gel Doc Lab ™ software version 2.0.1(Bio–Rad, U.S.A).

RESULTS AND DISCUSSION

A set of 38 SSR markers located across the 30 chromosomes were employed for fingerprinting the chosen varieties. Based on the polymorphic status, 28 SSR markers were found to be informative for discriminating the different classes of seeds of four linseed varieties and showed a total of 61 alleles. (Fig: 1, Fig: 2).The number of alleles amplified by each primer pair ranged from 2-4 with an average value of 2.17. The polymorphic information content (PIC) of these 28 SSR markers ranged from 0.37 to 0.75 with an average of 0.41. Based on the result of the present study, SSR markers selected could be considered as highly informative as evident from their PIC value. The SSR markers LU-1, LU-7 and LU-24 generated

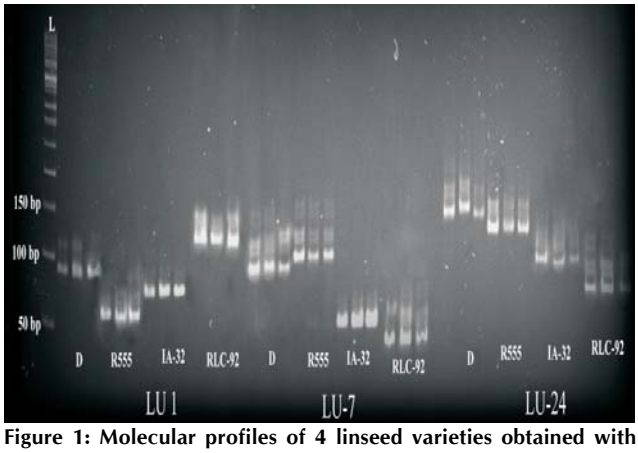

Figure 1: Molecular profiles of 4 linseed varieties obtained with microsatellite marker LU-1, LU-7 and LU-24. The number of lanes 1 to 36 correspond to the linseed varieties: where D = Deepika, RLC-92, IA-32 = Indira Alsi-32 and R552, L = Ladder

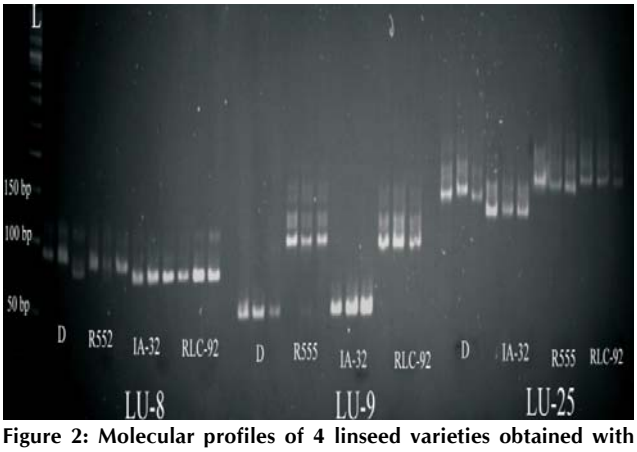

Figure 2: Molecular profiles of 4 linseed varieties obtained with microsatellite marker LU-8, LU-9 and LU-25. The number of lanes 1 to 36 correspond to the linseed varieties: where D = Deepika, RLC-92, IA-32 = Indira Alsi-32 and R552, L = Ladder

Table 1: Characteristics of the 38 microsatellite markers of *L. usitatissimum*

S.No	Primers	Sequence 5′—> 3′	Annealing temperature (°C)
1	LU 1	F:TCATTCATCTCCTTCCACTAAAA R:TTGAAAGCCCTAGTAGACACCA	58
2	LU 2	F:TCCGGACCCTTTCAATATCA R:AACTACCGCCGGTGATGA	58
3	LU 3	F: GCTCGTGATCTCCTTCATCC R: AAAACCACGTCCAGATGCTC	58
4	LU 4	F: TTATTTCCGGACCCTTTCAA R: AAACTACCGCCGGTGATGAT	58
5	LU 5	F:GTCACTGGGTGTGTGTTTGC R:AGCAGAAGAAGATGGCGAAA	58
6	LU 6	F:CCCCATTTCTACCATCTCCTT R:CAACAGCGGAACTGATGAAA	58
7	LU 7	F:CATCCAACAAAGGGTGGTG R:GGAACAAAGGGTAGCCATGA	58
8	LU 8	F:TCCCGTAATATTCTATGTTCTTCC R:TGAGTTGGACCTTACAAGACTCA	58
9	LU 9	F:TTGCGTGATTATCTGCTTCG R:ATGGCAGGTTCTGCTGTTTC	58
10	LU 10	F:GCCTAAAGCTGATGCGTTTC R:TGTCAGGCTCCTTCTTTTGC	58
11	LU 11	F:ATGGCAGGTTCTGCTGTTTC R:TTGCGTGATTATCTGCTTCG	58
12	LU-12	F: GGGATTGAGAAGAGGGCATA R:GTTGGGGTGAAGAGGAACAA	58
13	LU 13	F: AAGATGACGTCGGTGGTGAT R: CGGAACCTTCCATTTTCCTC	58
14	LU 14	F: GCTTGCGAGAAGAAGGAGAA R: TCACCAAAGGCATTCACAAA	58
15	LU 15	F:TGGACGACGATGAAGATGAA R:CCGCCGGGTACACTACTACT	58
16	LU 16	F: TTATTCTTGCCTGCCAATCG R: TCCAGCTCTTGCTCGTTCTT	58
17	LU-17	F: GCTGGACCTTACAAGCCTCA R:TTGGTGGGAGAACAACAAGA	58
18	LU 18	F:AGAGGCGGAGGGCATTAC R:TTGGAGAGTTGGAATCGAGA	58
19	LU-19	F: TCTCAGCTCCCTTTTATTTACCC R:GCAGTCTCGAGTGCTGAGTG	58
20	LU 20	F:TTCAACCAGGCAAATTTCAA R:CAAGAAGAGGCCCAGAATTG	58
21	LU 21	F:AAGGGTGGTGGTGGGAAC R:GTTGGGGTGAAGAGGAACAA	58
22	LU 22	F:GATGGGGTTGAAGCCAGTAG R:CCCACCCCATCTATCATTTG	58
23	LU-23	F: GAGAATCGAAGCCAAAACCA R:CGCAGAGGAGAGAAGAGGAA	58
24	LU 24	F:ATGGCAGGTTCTGCTGTTTC R:TTGCGTGATTATCTGCTTCG	58
25	LU 25	F:TCTACAGAGTTCAATTCCCGTAA R:GTTGGACCTTACAAGACTCACTG	58
26	LU-26	F: CCTGCAGGAAAAAGTGAAGC R:CTGCAAACAGCACAACTTCC	58
27	LU 27	F:GTTTGAGAAGAGGGCATCCA R:GTTGGGGTGAAGAGGAACAA	58
28	LU-28	F: GCTGTTAGCACTCAGCAGCA R:CACGTTGACCAACAAAACCA	58
29	LU 29	F:GGGCAGTGATTGATTGGTTT R:GGCGGCAATTGCTACATT	58
30	LU-30	F: TCTTGACCATCAGCATCACC R:GAGGCACAGGGAAACTAACG	58
31	LU 31	F:TCTTTGTTTGGTGCCAAAGTT R:TTCATGATCTCACCTAACCTGA	58
32	LU 32	F:ACGCGTAAACTTTCCGTTTC R: ATAATGTCGGCTGCTTCTGC	58
33	LU 33	F:TTCTCCATCATCTCACATCCA	58

Table 1: Cont.....

S.No	Primers	Sequence 5′—> 3′	Annealing temperature (°C)
		R: CCAAATCAGAATGTGCGTGT	
34	LU 34	F:GGAAGAATTGGAAGAGGAAGG R:CCTTCTCCCATGATCAAACAA	58
35	LU 35	F: CCAACGGATCATCCTCTAGC R: GGACAGAAAGGGGAAAGGAA	58
36	LU-36	F: GAAGTTCGTGGGAGGAGTTG R:CCACATGAACCGCTGTAGAA	58
37	LU-37	F: AGTACCCAACGCCCAGATT R:AACAGCAGTCCTGGCAGTCT	58
38	LU-38	F: GATCTTGTTGCCTGGGAAAG R:TTCGTTTGCAATACGTCAGC	58

Table 2: Flax (*Linum usitatissimum* L.) cultivars used for analysis of polymorphism microsatellite markers

Cultivar	Origin	Release year	Parentage	Seed category	Plant type	Special features
Deepika	IGKV Raipur	2006	Kiran × Ayogi	Breeder	Seed	Medium in height and early maturity, blue flower, brown seeded, resistant to powdery mildew, oil content- 41.39%.
Indira Alsi-32	IGKV Raipur	2005	Kiran × RLC 29	Breeder	Seed	Dwarf statured, blue flower, dark brown seeded, resistant to powdery mildew, oil content- 39.18%.
R552 (Jawahar 552)	IGKV Raipur	1984	No.55/R 67	Breeder	Seed	Brown medium to large seeded, tolerant to wilt, rust and powdery mildew.
RLC-92	IGKV Raipur	2008	Jeevan × LCK 9209	Breeder	Seed	Tall in height, tinge blue flower, brown seeded, tolerant to bud fly and resistant to wilt, powdery mildew, oil content- 39%.

Table 3: PCR components using SSR markers

Components	Microsatellite (SSR) (in ¼l)
Nanopure water	13.5
10 X PCR Buffer	2
dNTPS 10mM	1
Primer (F) 5 pmol	0.5
Primer (R) 5 pmol	0.5
Taq 1U/ ¼l	0.5
Template DNA (40 ·g/¼l)	2
Total volume	20

maximum number of four alleles while, LU-8, LU-9 and LU-25 exhibit three polymorphic alleles and rest of markers generate only two alleles. Out of 28 polymorphic SSR markers, a set of three markers (LU-1, LU-7 and LU-24) exhibited unique alleles for four linseed varieties.(Fig3 a ,b,c and d) These can serve as molecular IDs for these varieties.

This is the pioneer attempt of fingerprinting of four main commercial linseed varieties under cultivation at present in Chhattisgarh state of India using SSR markers, and the fingerprinting data obtained can be used for variety identification in practice. Distinctiveness, uniformity and stability (DUS) testing based on DNA markers can effectively augment the process of characterization of the four main commercial linseed varieties under cultivation at present in Chhattisgarh state of India (Pali *et al.,* 2013) and (UPOV,1995).

Cluster analysis was carried out based on the similarity index data derived from the SSR markers which grouped the four linseed varieties into two major clusters (Fig 4). The Jaccard's similarity ranged from 0.05 to 0.30 with an average similarity index of 0.11. Indicating that three varieties used in the present study were having common ancestors in their pedigree. R-552 shared maximum similarity with Indira Alsi-32 with similarity index of 0.21. Cluster – I comprised of only one variety (Deepika) sharing a similarity of 11%. Cluster II consisted of three varieties *viz.,* Indira Alsi-32, RLC-92 and R-552 which were further divided into two minor sub-clusters. Sub-cluster I with (R-552 and Indira Alsi-32) shared 56 per cent of genetic similarity, while sub-cluster II consist of one variety (RLC-92) having 16 per cent. The average genetic similarity within four varieties was about 11 per cent and the genetic diversity among these varieties was observed to be high, RLC-92 was observed to be divergent from Deepika, Indira Alsi-32 and R-552. However, phenotypically, RLC-92 is different for these three varieties due to its tall stature.

For genetic purity in field, four linseed varieties (Deepika, Indira Alsi -32, RLC-92 and R-552) for which unique 'molecular fingerprints' or 'molecular identities (IDs)' were selected. Eighty plants of different 3 seed class (certified, breeder and mixture)

Table 4: Temperature profile used for PCR amplification for SSR markers.

Steps	Temperature (°C)	Duration (min)	Cycles	Activity
1	95	4	1	Initial denaturation
2	95	1		Denaturation
3	58	1	32	Annealing
4	72	1		Extension
5	72	7		Final extension
6	4	24 hrs		Storage

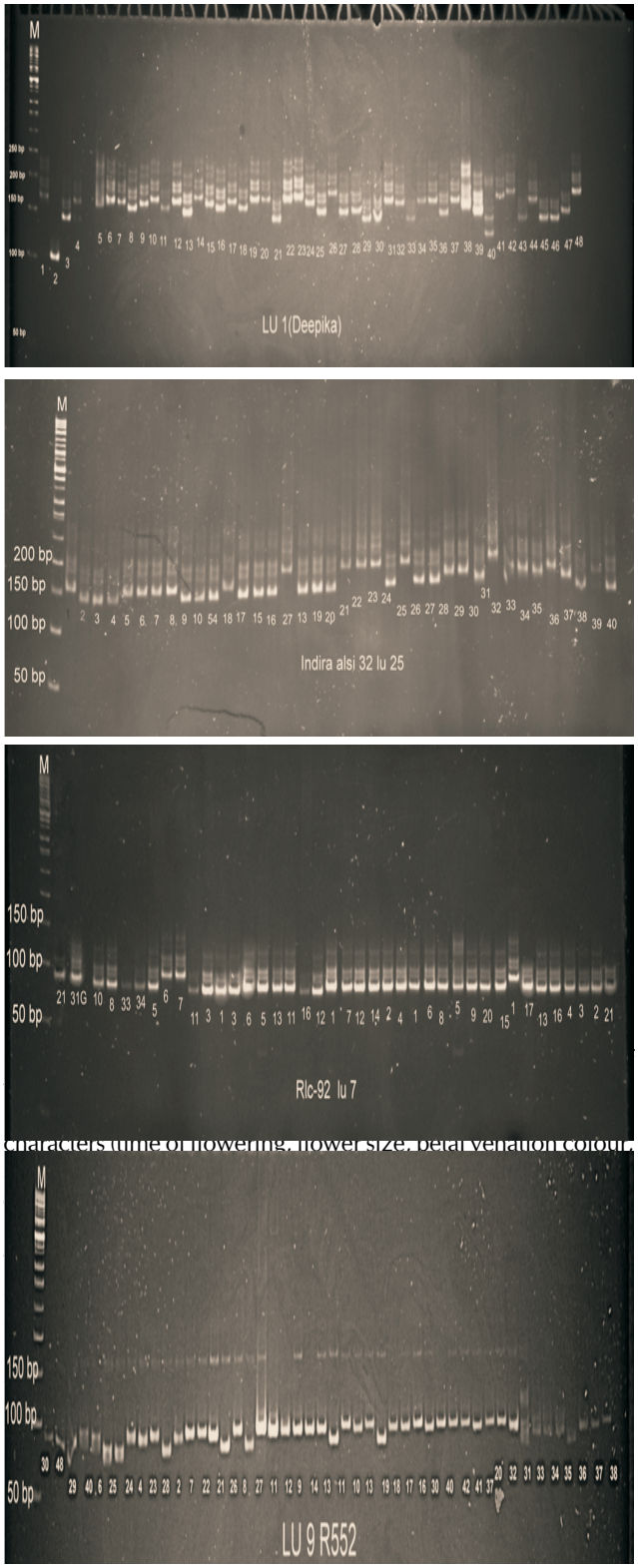

Figure 3. (a ,b,c and d) Detection of impurities in the Indian flax cultivar- M- 50 bp DNA ladder

lot, some additional impure plants were detected based on SSR analysis. This was not detected by field observation. This demonstrated the better discriminatory power and efficiency

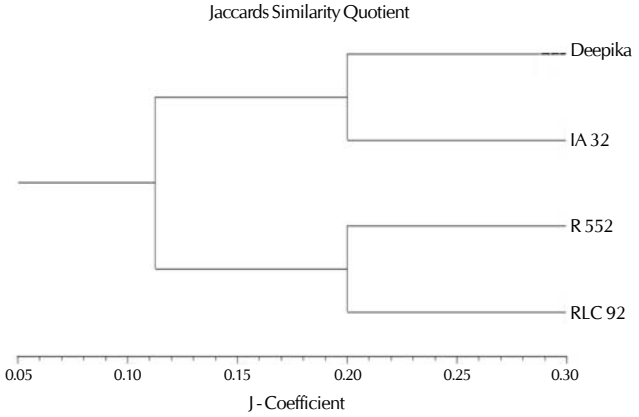

Figure 4: Dendrogram constructed using UPGMA based on Jaccard's coefficient of 4 linseed varieties (SSR Markers)

of SSR markers in genetic purity assessments and these markers could even accurately detect residual heterozygosity in the seed. Based on the results obtained from this study, it is clear that there is a need to critically assess the genetic purity of popular varieties of premium quality at each and every stage of seed multiplication and processing with the help of molecular markers so that the seeds cultivated by farmers are true-to-type and fetches premium price.

The present study clearly demonstrated the ability of SSR markers to generate locus specific allelic information for the elite Indian linseed varieties. SSR markers in seed genetic purity assessments have been validated and compare with morphological observation in the field study. Primers identified in the study could be utilized for routine genetic purity testing of varieties. The SSR markers information developed through this study will be of immense help for seed certification programme to select appropriate marker combinations and assess genetic purity of the crop.

REFERENCES

Anderson, J. A., Churchill, G. A., Sutrique, J. E., Tanksley, S. D. and Sorrells, M. E. 1993. Optimizing parental selection for genetic linkage maps. *Genome*. **36:** 181-186.

Darlington, C. D.1963. Psychology, Genetics and the process of History. *British J. Psychol*. **54:** 293-298.

Kang, H. W., Yong, G. C., Ung, H. Y. and Moo, Y. E. 1998. A rapid DNA extraction method for RFLP and PCR analysis from a single dry seed. *Plant Mol. Biol. Rep*. **16:** 1-9.

Kuleung, C., Baenziger, P. S. and Dweikat, I. 2004. Transferability of SSR markers among wheat, rye and triticale. *Theor. Appl. Genet*. **108:** 1147-1150.

McCouch, S. R., Chen, X., Panaud, O., Temnykh, S., Xu, Y. and Cho, Y. G. 1997. Microsatellite marker development, mapping and applications in rice genetics and breeding. *Plant Mol. Biol*. **35:** 89-99.

Mingsheng, Wu, Xihai, Jia and Lei, Tian 2006. Rapid and reliable purity identification of F1 hybrids of maize (*Zea mays* L.) using SSR markers. *Mol. Plant Breed*. **14(3):** 381-384.

Pali, V., Verma, S., Xalxo, S., Saxena, R. R., Mehta, N. and Verulkar, S. B. 2014. Identification of icrosatellite markers for fingerprinting popular Indian flax (*Linum usitatissimum* L.) cultivars and their utilization in seed genetic purity assessments. *Australian J. Crop Science*.

8(1):119-126.

Pali, Vikas and Mehta, Nandan. 2014. Combining ability and heterosis for seed yield and its attributes in Linseed (*Linum usitatissimum* L.). *The Bioscan*. **9(2):** 701-706.

Richharia, R. H. 1962. Linseed. *India Central Oilseeds Committee*, India.

Tripathi, A., Bisen, R., Ahirwal, R. P., Paroha, S., Sahu, R. and Ranganatha, R. G.. 2013. Study on genetic divergence in sesame (sesamum indicum l.) Germplasm based on morphological and quality traits. *The Bioscan*. **8(4):** 1387-1391.

UPOV. 1995. Guidelines for the conduct of tests for distinctness, uniformity and stability on linseed (*Linum usitatissimum* L.). UPOV TG/57/3.

Vavilov, N. I. 1935. Theoretical basis of Plant Breeding. *VIR, St. Petersberg, USSR*.

Xie, R. J., Zhou, J., Wang, G. Y., Zhang, S. M., Chen, L. and Gao, Z. S. 2011. Cultivar identification and genetic diversity of chinese bayberry (*Myrica rubra*) accessions based on fluorescent SSR markers. *Plant Mol. Biol. Rep*. **29:** 554-562.

Yol, E. and Uzun, B. 2012. Geographical patterns of sesame accessions grown under mediterranean environmental conditions, and establishment of a core collection. *Crop Sci*. **52:** 2206-221.