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Balaji C Jadhav
College of Agricultural Biotechnology Latur, V. N. M. K. V. Parbhani, Maharashtra, India

Mahendra S Dudhare
College of Agricultural Biotechnology Latur, V. N. M. K. V. Parbhani, Maharashtra, India

Kalyani More
College of Agricultural Biotechnology Latur, V. N. M. K. V. Parbhani, Maharashtra, India

Hanuman A Malge
College of Agricultural Biotechnology Latur, V. N. M. K. V. Parbhani, Maharashtra, India

Devendra Payasi
Regional Agricultural Research Station, Sagar, J.N.K.V.V, Jabalpur, Madhya Pradesh, India

MK Ghodke
Oilseeds Research Station, Latur, V. N. M. K. V. Parbhani, Maharashtra, India

Corresponding Author:
Mahendra S Dudhare
College of Agricultural Biotechnology Latur, V. N. M. K. V. Parbhani, Maharashtra, India

Genetic diversity and molecular characterization of initial varietal trials of linseed lines using molecular markers (ISSR and SSR)

Balaji C Jadhav, Mahendra S Dudhare, Kalyani More, Hanuman A Malge, Devendra Payasi and MK Ghodke

Abstract
Two types of molecular markers, Inter Simple Sequence Repeats (ISSR) and simple sequence repeat (SSR), were used to determine the genetic diversity and molecular characterisation of 21 linseed lines. The present SSR primers investigation revealed that genotypes BAU 14-03 and Sharda (ch) (72%) were found to be the most diverse linseed lines and primer LU-10 (0.79) and LU-9 (0.78) showed highest PIC value and show the 100% polymorphism. The cluster analysis based on SSR markers across various linkage groups data revealed a relatively broad genetic background of the indigenous linseed lines. Two distinct and major clusters (I and II) were found to represent unique grouping of 21 linseed genotypes. The number of genotypes in each cluster varied from 5 to 16 and ISSR investigation revealed that genotypes SLS 127 and SLS 128 (25%) were found to be the most diverse and primer UBC-825(0.29) and UBC-815(0.24) showed highest PIC value and show the 100% polymorphism. The cluster analysis based on ISSR markers across various linkage groups data revealed four distinct and major clusters (I, II, III and IV) were found to represent unique grouping of 21 linseed genotypes. The number of genotypes in each cluster varied from 2 to 10.These results indicated good sources of genetic analysis using ISSR and SSR primers which will help breeders to evaluate genetic diversity and genetic relationship among different flax genotypes.

Keywords: Genetic, molecular, characterization, molecular, ISSR and SSR, *Linum usitatissimum* L.

Introduction
Flax (*Linum usitatissimum* L.) also called as linseed, is an annual herb which is the third largest natural fiber crop and one of the five major oil crops in the world (Deng *et al*., 2011) [3]. Linseed (*Linum usitatissimum* L.) is an important oilseed crop with a chromosomal number of 2n = 30. Linseed is a self-pollinated annual crop that belongs to the family Linaceae and is presumed to be originated in Southwest Asia particularly in India (Vavilov, 1935; Richharia, 1962) [13, 9].
An IVT includes the maximum number of locations across the country to evaluate varietal adaptation and performance (Chand *et al*., 2020) [2]. Inter-simple sequence repeat (ISSR) technique, which was first published by (Zietkiewicz *et al*., 1994) [16] is simple, quick and does not require previous knowledge of sequence of the genome. ISSR are Polymerase Chain Reaction (PCR)-based molecular markers that have shown higher reproducibility, levels of variability and simplicity as compared with other dominant marker systems (Wolfe and Liston, 1998 and Reddy *et al*., 2002) [14, 8] used ISSR-PCR primers to generate multi-locus markers. ISSR markers are highly polymorphic and are useful in studies of genetic diversity, phylogeny, gene tagging, genome mapping and evolutionary biology.
Genetic diversity analysis is an important task in plant breeding as diversity in plant genetic resources (PGR) provides an opportunity for plant breeders to develop new and improved cultivars with desirable characteristics. ISSR technique was chosen for making fingerprint for newly cultivated and foreign flax genotypes to evaluate the genetic diversity among them, with the aim of providing facts and tools to increase the assortment for future flax genotypes propagation and assisting in developing and planning breeding strategies for crop improvement programs.
More recently, a better approach utilizing simple sequence repeats (SSRs), or microsatellite DNA, has been developed. SSRs are short, randomly repeating nucleotide motifs (1–6 bp long) that are widely distributed in genomes of eukaryotic organism's genomes including flax (Tautz, 1989; Temnykh *et al*., 2001) [11, 12].

The abundance, highly polymorphic nature, heritability, distributionn, reproducibility and generally co-dominant nature of SSR markers make them highly suitable. Development of SSR markers in Flax MAS and genetic diversity studies in flax, large numbers of genomic SSR markers have already been developed (Sandip *et al*., 2012) [10].

Material and Methods

The experimental materials of twenty one (21) lines of initial varietal trials of linseed genotypes obtained from Oilseed Research Station (ORS), Latur (MS). Genomic DNA was extracted from the leaves of 21 linseed genotypes following the Cetyltrimethyl Ammonium Bromide (CTAB) method with some modifications as described by Doyle and Doyle (1987).

ISSR and SSR analysis:

A total of four ISSR and Five SSR primers were used in analysis. PCR amplifications were performed in 25µL of reaction volume containing the following reagents: 25-30ng of genomic template DNA, 0.1µmol/L of primer, 3U Taq polymerase, 1x PCR buffer, 2.0 mmol/L $MgCl_2$, 100 µmol/L of each dNTP (Zietkiewicz *et al*., 1994) [16]. Some adjustments in the annealing temperature and changes in the number of amplification cycles were made to the original program to improve the results. The amplification protocol was 94 ºC for 5 min to prede nature, followed by 45 cycles of 94 ºC for 1 min, 36 ºC (for ISSR analysis) or 58-60 ºC (for SSR analysis) for 1 min and 72 ºC for 1 min, with a final extension at 72ºC for 10 min. Amplification products were visualized using 1.8% (for ISSR analysis) or 2.5% (for SSR analysis) agarose gel electrophoresis stained with ethidium bromide. The each fragment length (bp) in the amplified product was determined with reference 100bp and 1000bp DNA marker ladder.

Table 1: The 21 linseed lines used in the study

Sr. No.	Code	Entries	Pedigree	Source
1	19601	DLV 10	Mutant of Indira Alsi	Dharwad
2	19602	OL 12-4	OL 98-14-3 x Padmini	Keonjhar
3	19603	Sharda (ch)	(Shubhra x J1) x (J1 x Kiran)	Mauranipur
4	19604	LCK 1929	Sheela x GS 234	Kanpur
5	19608	PKVNL 367	RL 21120 x H 34	Nagpur
6	19610	BAU 14-03	Pusa 2 x R 552	Kanke
7	19611	RL 15594	RL 29202 x RL 914	Kota
8	19613	T 397 (NC)	T 491 x T 1193-1	Kanpur
9	19614	BRLS 103	BAU 06-5 x RL 26018	Sabour
10	19616	RLC 176	Polf 22 x RLC 92	Raipur
11	19617	SLS 128	R 2 x Laxmi 2	Sagar
12	19619	IA 32 (Ch)	Kiran x RLC 29	Raipur
13	19620	RLC 175	RLC 92 x LCK 88062	Raipur
14	19621	LMS 2017-R-8	NL 97 x Surabhi	Mauranipur
15	19622	RL 15593	RL 27010 x Triveni	Kota
16	19623	DLV 6	Mutant of NL 115	Dharwad
17	19624	SLS 127	JLS 73 x Kiran	Sagar
18	19625	LMS 2017-R-5	J-23 x JRF 5	Mauranipur
19	19626	TL 114	TLM 5 x EC 41598	BARC, Mumbai
20	19627	LCK 1933	T 397 x Neela	Kanpur
21	19628	JLS 95 (ZC-III)	JLS 27 x GS 281	Kanke

Fig 1: Linseed genotypes used for Experimental trial

Data analysis: ISSR and SSR data were scored (1) for presence and (0) for absence, each band was regarded as a locus. Two matrices, one for each marker, were generated. Based on the similarity matrix, a dendrogram showing the genetic relationships between genotypes, was constructed using the un weight edpair group method with arithmetic average (UPGMA) 24 through the software NTSYs-pc version 2.02i

Results and Discussion

Characterization of Initial Varietal Trials of linseed lines using molecular markers (ISSR and SSR): A set of 5 SSR primers were used to carry out PCR amplification of 21 genomic DNAsIn the present study using SSR primers a total number of 147 amplicons were generated by 5 SSR primers. 105 amplicons were found to be polymorphic with an average polymorphism of 90% on an average each primer produced 29 amplicons. The size of amplification product ranged from 300bp-150bp.

The polymorphic information content (PIC) value per primer varied from 0.71 to 0.79 with an average of 0.74. highest informative marker with the PIC value of 0.79 was SSR primer LU-10 with 100% polymorphism while the least informative primer was found to be SSR primer LU-2 with PIC value of 0.71 which were closer to those reported by Bickel, *et al*., (2011) observed polymorphism information content (PIC) was estimated, with a range of 0.1049–0.8642, with an average of 0.47.

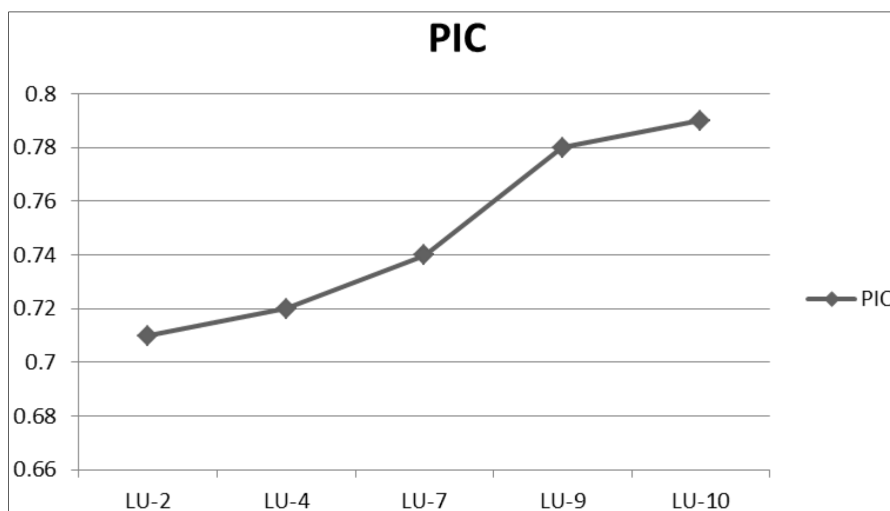

Fig 2: Graphical representation of PIC value of SSR markers:

Table 2: SSR primers used for linseed molecular characterization

Primer name	Sequence
LU-2	F-TCCGGACCCTTTCAATATCA
	R-AACTACCGCCGGTGATGA
LU-4	F-TTATTTCCGGACCCTTTCAA
	R-AAACTACCGCCGGTGATGAT
LU-7	F-CATCCAACAAAGGGTGGTG
	R- GGAACAAAGGGTAGCCATGA
LU-9	F- TTGCGTGATTATCTGCTTCG
	R- ATGGCAGGTTCTGCTGTTTC
LU-10	F- GCCTAAAGCTGATGCGTTTC
	R- TGTCAGGCTCCTTCTTTTGC

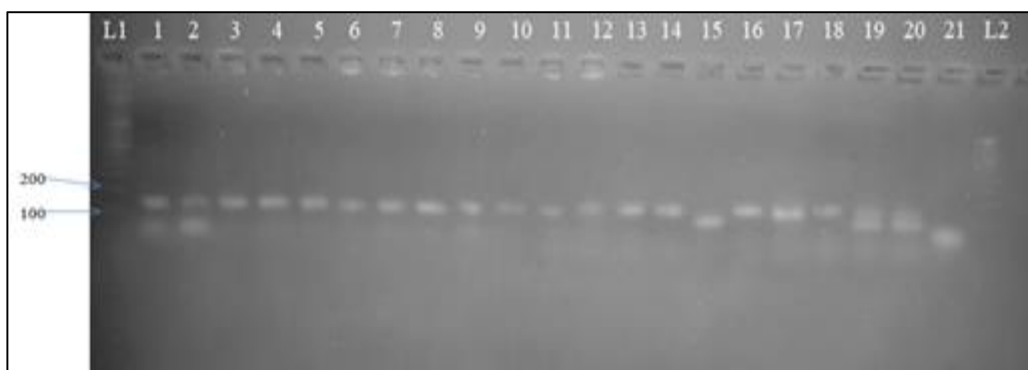

Fig 3: SSR profiling of 21 linseed lines obtained with primer LU-2

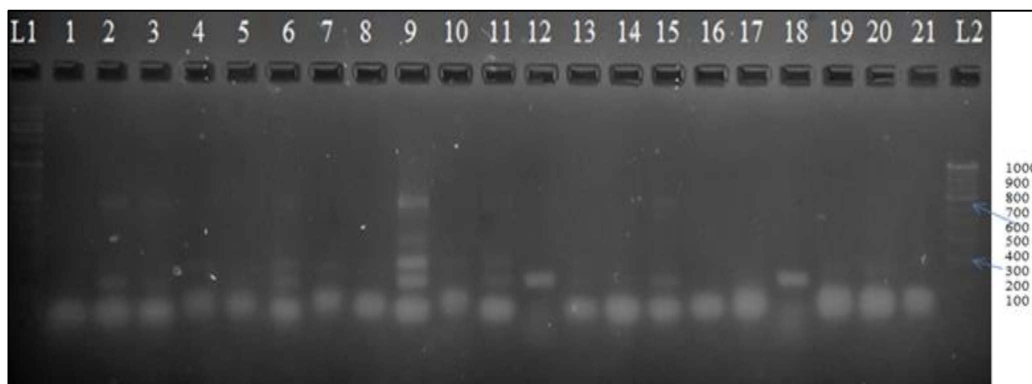

Fig 4: SSR profiling of 21 linseed lines obtained with primer LU-10

ISSR analysis: In the present investigation using four ISSR primers a total number of 105 amplicons were generated by 4 ISSR primers. 84 amplicons were found to be polymorphic with an average polymorphism 87.5%. On an average each primer produced 26.25 amplicons. The size of amplification product ranged from 600bp-7500bp. Primer UBC 819 produced maximum number of amplicons (34), whereas primers UBC 825 and UBC 855 produced 31 and 25 amplicons each respectively and UBC 815 produced minimum number of amplicons (15). Least percent of polymorphism was shown by primer UBC 819 (50%). is equivalent to findings of Godwin *et al*., (1997) [5] and Zientkiewicz *et al*., (1994) that reports multiplication of 10 to 60 fragments per genotype during the ISSR. The size of fragments ranged from 210.5 to 1750bp.

Graphical Representation of PIC value of ISSR markers

The polymorphic information content (PIC) value per primer varied from 0.22 to 0.29 with an average of 0.25. highest informative primer with the PIC value of 0.29 was ISSR primer UBC-825 with 100% polymorphism while the least informative primer was found to be ISSR primer UBC-855 with PIC value of 0.22. The mean PIC value (0.25) detected in the present study was higher than reported in Indian flax genotypes by Rajwade*et al*., 2010, who detected average PIC of 0.18 using only ISSR markers.Ziarovska*et al.* (2012) reported PIC values from four ISSR primers ranged from 0.12 to 0.37 respectively from 18 accessions collected from 11 countries. These values were similar to the range of results from the present study.

Table 3: ISSR primers for linseed genetic diversity

Primer name	Sequence
UBC-815	CTCTCTCTCTCTCTCTG
UBC-825	ACACACACACACACACT
UBC-855	ACACACACACACACACYT
UBC-819	GTGTGTGTGTGTGTGTA

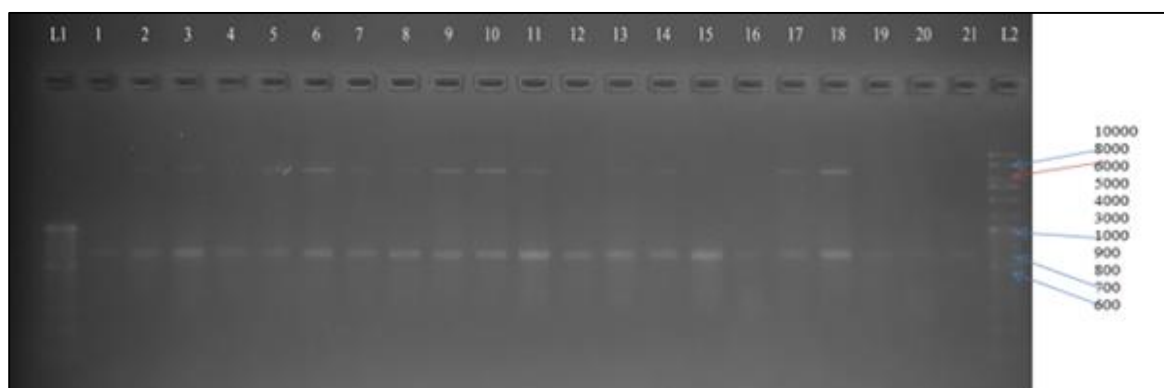

Fig 5: ISSR profiling of 21 linseed lines obtained with primer UBC-819

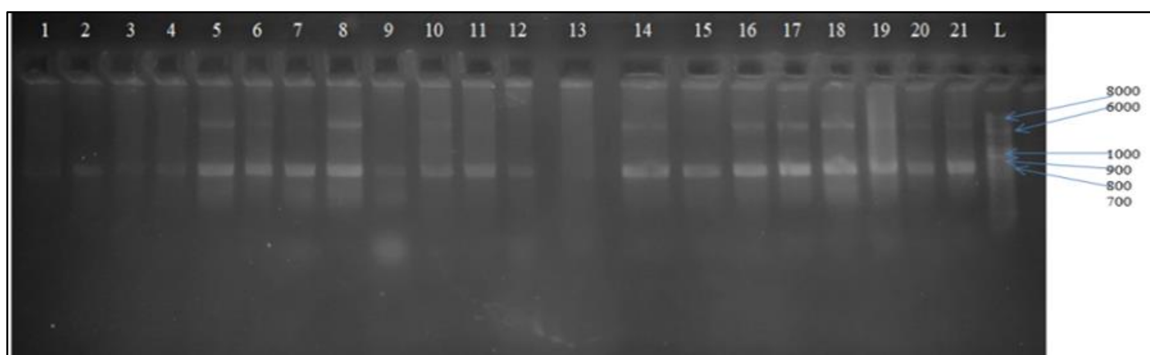

Fig 6: ISSR profiling of 21 linseed lines obtained with primer UBC-825

Genetic diversity analysis: A dendrogram was generated based on the similarity matrix by the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) which clearly discriminated the flax genotypes. The cluster analysis was done using Jaccard's similarity coefficients to study the genetic relationships among these flax genotypes**.** The composition of clusters obtained using ISSR markers alone (Fig. 7) and SSR markers alone (Fig. 8).

Similarity matrix of 21 linseed genotypes by ISSR analysis: The similarity matrix among 21 linseed accessions ranged from 0.25 (SLS 127-SLS 128) to 1.0 (BAU14-03-Sharda (ch), RL 15594-LCK1933, LMS 2017-R-8-LCK 1933, LMS 2017-R-8-RL 15594) from ISSR analysis. Thus, the most dissimilar genotypes *viz*., SLS 127 and SLS 128 with each other showing minimum similarity index (0.25) were noticed.

Clustering analysis based on ISSR markers: The cluster analysis based on ISSR markers across various linkage groups data revealed a relatively broad genetic background of the indigenous linseed lines. four distinct and major clusters (I, II, III and IV) were found to represent unique grouping of 21 linseed genotypes. The number of genotypes in each cluster varied from 2 to 10.

In major cluster-I, two minor cluster are formed IA and IB were found to be similar with each other at around 62% cut-off on the scale which contain 10 linseed lines, In minor cluster IA had two genotypes Sharda (ch) and BAU 14-03 were appeared to be similar with each other at around 79%. IB minor cluster had subdivided in two subclusterIBa and IBb similar with each other at around 71% cut-off on the scale which contain 8 linseed lines, Subcluster IBa is further divided into IBa1 and IBb2 with similar with each other at around 75% cut-off on the scale. Subcluster IBa1 had two genotypes TL 114 and PKVNL 367 with 86% similarity. While, in subcluster IBa2 had three genotypes LCK 1933, RL 15594 and LMS 2017-R-8 shows 100% similarity and RLC 176 found to be out grouped in minor subcluster IBa2. Subcluster IBb had two genotypes SLS 128 and LMS 2017-R-5 similar with each other at around 79% cut-off on the scale.

The major cluster-II was further found to be divided into two minor clusters IIA and IIB with four genotypes. The minor cluster IIA had further found to be divided into two clusters IIAa and IIAb with three genotypes similar with each other at around 70% cut-off on the scale. Cluster IIAa had two genotypes DLV 6 and LCK 1929 similar with each other at around 79% cut-off on the scale and BRLS 103 was found to be outgrouped within the cluster IIAb. RL 15593 was found to be outgrouped within the cluster IIB.

The major cluster-III was further found to be divided into two minor clusters IIIA and IIIB with four genotypes. The minor cluster IIIA had two genotype SLS 127 and RLC 175 similar with each other at around 83% cut-off on the scale and minor cluster IIIB had two genotype DLV 10 and T 397 (NC) similar with each other at around 87% cut-off on the scale

The major cluster-IV had two genotype IA 32 (ch) and OL 12-4 similar with each other at around 60% cut-off on the scale.

Similar result were reported by Rajwade*et al*., (2010) reported in a study conducted on Indian flax genotypes, 'Sheetal' was the most diverse variety among the evaluated germplasm.

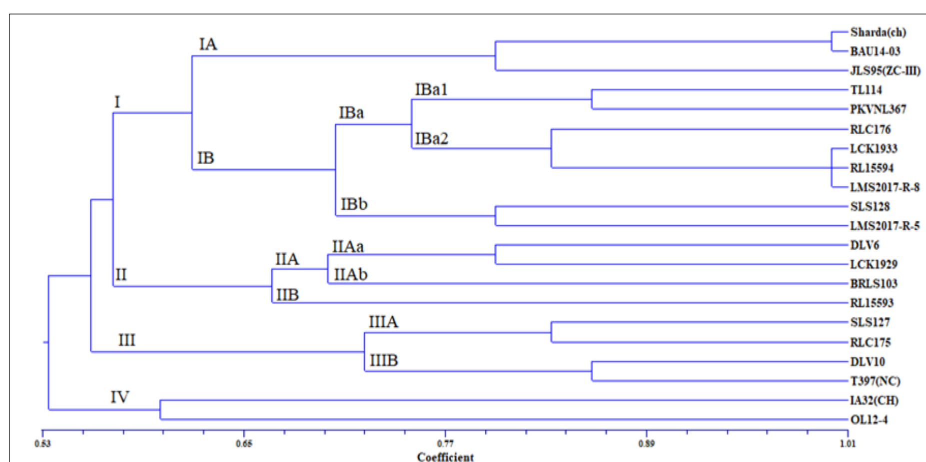

Fig 7: Dendrogram generated by UPGMA analysis based on ISSR markers

Similarity matrix of 21 linseed genotypes by SSR analysis
The similarity matrix among 21 linseed accessions ranged from 0.23 (BAU 14-03-Sharda (ch)) to 1.0 (PKVNL 367-SLS 127, SLS 127-DLV 10L, T397(NC)-RLC 176, PKVNL 367-DVL 10, RL 15594-JLS 95 (ZC-III)) from SSR analysis. Thus, the most similar genotypes *viz*., PKVNL 367-SLS 127, SLS 127-DLV 10L, T397(NC)-RLC 176, PKVNL 367-DVL 10, RL 15594-JLS 95 (ZC-III) with each other and the most dissimilar genotypes *viz*., BAU 14-03-Sharda (ch) were noticed. Similar result were obtained by Kumari S, *et al*., (2020). The binary data from the SSR markers were used for computing similarity coefficients. The similarity coefficient ranged from 0.30 to 0.90.

Clustering analysis based on SSR markers
The cluster analysis based on SSR markers across various linkage groups data revealed a relatively broad genetic background of the indigenous linseed lines. Two distinct and major clusters (I and II) were found to represent unique grouping of 21 linseed genotypes. The number of genotypes in each cluster varied from 5 to 16
In major cluster-I, two minor cluster are formed IA and IB were found to be similar with each other at around 54% cut-off on the scale which contain 5 linseed lines, IA minor cluster had subdivided in two subcluster IAa and IAb similar with each other at around 62% cut-off on the scale which contain 4 linseed lines, subcluster IAa had two genotypes Sharda (ch) and SLS 128 similar with each other at around 73% cut-off on the scale and IA 32 (ch) and TL 114 grouped as subcluster IAb similar with each other at around 78% cut-off on the scale. The genotype LMS 2017-R-8 was found to be out grouped from rest of all the members from cluster IB
The major cluster-II at around 56% similarity cut off emerged as the largest one, comprising 16 accessions, was further found to be divided into two minor clusters IIA and IIB with 16 genotypes. LMS 2017-R-5, DLV 6 and BAU 14-03 were found to be out grouped within the clusters II. The minor cluster IIA had eleven accessions, which have shown 59% similarity. In second minor cluster (IIB) had two genotypes RLC 175 and RL 15593 were appeared to be similar with each other at around 71%.minor cluster IIA is further divided into two subcluster IIAa and IIAb with similar with each other at around 59% cut-off on the scale. Subcluster IIAa is further divided into IIAa1 and IIAa2 with similar with each other at around 67% cut-off on the scale. subcluster IIAa1 had five genotypes, SLS 127, DLV 10, PKVNL 367, T 397 (NC), RLC 176 While, in subcluster IIAa2 had only two accessions JLS 95 (ZC-III) and RL 15594 were observed in one subcluster which were 100% similar. Subcluster IIAb were Further divided into two subcluster IIAb1 and IIAb2 similar with each other at around 72% cut-off on the scale. two accessions namely OL 12-4 and LCK 1929 were grouped in subcluster IIAb1 and genotype BRLS 103 with 87% similarity and LCK 1933 were grouped in subcluster IIAB2 with 76% similarity.

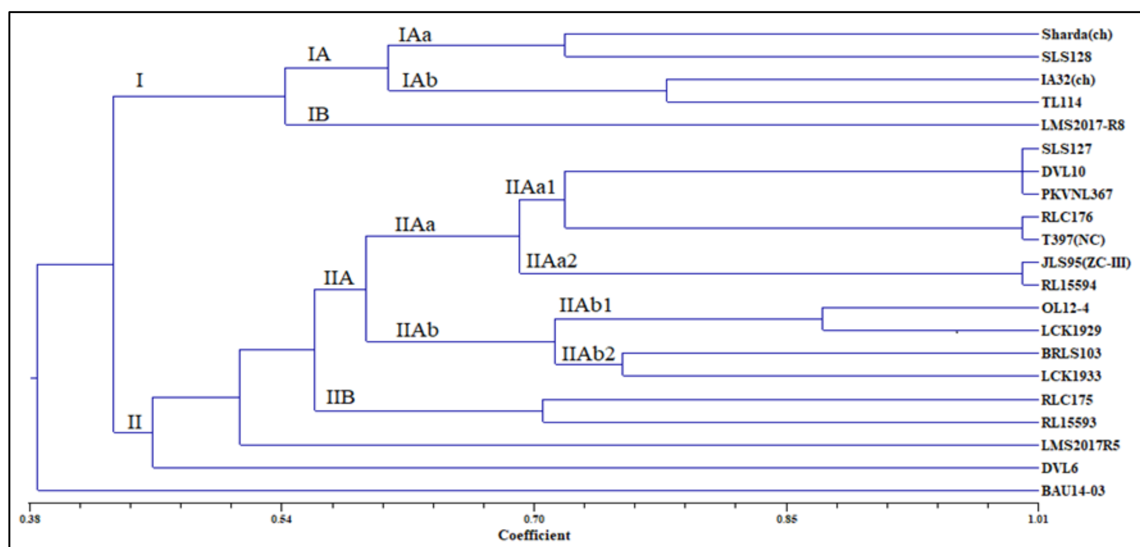

Fig 8: Dendrogram generated by UPGMA analysis based on SSR markers.

In conclusion of the genetic diversity assessment of these set of linseed germplasm accessions can be very useful in breeding for rapid and early identification of most diverse individuals allowing for the improvement of linseed breeding programs. However, a further investigation is needed in this crop using high throughput modern genomic technology for genetic understanding and improvement.

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