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Evaluation and characterization of advance varietal trial of linseed (*Linum usitatissimum* L.) using morphological and molecular markers

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Abstract
Linum usitatissimum L. is the binomial name for linseed, which belongs to the *Linum* genus of the Linaceae family and advance varietal trial was constituted by the entries promoted from initial varietal trial on the criteria specified. Morphological characters namely days to the first flower (33 to 40days), days to 50% flowering (first 50% flower from 46 to 52 days), height of plant (55 to 77.75 cm), number of primary branches per plant (branches varied from 2.00 to 7.00), number of capsules per plant (ranged from 26.5 to 67.50), number of seeds per capsule(ranged from 7.5 to 10.00 seeds per capsule), 1000 seeds weight (g) (from 5.52 to 8.68g), color of flower (white and blue) were recorded in AVT. Eight ISSR and Five SSR Primers were utilized for genetic diversity analysis. The dendrogram produced from linseed genotypes show three main cluster.

Keywords: Evaluation, characterization, varietal, morphological, *Linum usitatissimum* L.

Introduction
The advance varietal trial is constituted by the entries promoted from Initial Varietal Trial (IVT) on the criteria specified. A limited number of entries in Advance Varietal Trial (AVT) not exceeding 16 is tested along with a minimum of three checks comprising of the national check, zonal check, and local check. All these entries are evaluated in a randomized block design with 3-4 replications at the different locations. The monitoring is done by the same team as given for IVT. Besides the agronomic and morphological observations, the additional data may be generated by the co-operators on disease and insect-pest resistance, and quality. Again, if a variety gives significant superior performance by a margin of 10% over the, best performing Scheck-in combination with other attributes is promoted to the next stage. (Chand. S. *et al*., 2020) [6].

Linseed (*Linum usitatissimum* L.) is a versatile plant. Alpha-linolenic acid, an omega-3 fatty acid, is responsible for the majority of the biological activities of linseed oil. Linseed also contains soluble and insoluble fiber. Insoluble fiber aids in bowel motions and improves laxation, whereas soluble fiber lowers blood cholesterol levels and helps to balance blood sugar. Genomic studies allow researchers to gain valuable insight into how to crop genomes are organized, as well as a variety of practical applications such as variety identification via DNA fingerprinting, developing genetic maps that facilitate indirect selection of economic traits, cloning of important genes, and evolutionary and phylogenetic studies. Uses of genetic markers based on PCR are nowadays widely used in plantsystems for genotyping, identification, and authentication of samples. According to the current literature on DNA markers in linseed, the SSR markers reported are mostly newly developed genomic SSRs or EST-SSRs, and they are mostly found in exotic flax genotypes (Kumari *et al*., 2017 [8] and Soto-cerda *et al*., 2014). As a result, there is a great need and opportunity to use such novel DNA markers for screening, identifying, and validating specific positive markers linked to yield major components, and oil content of linseed genotypes developed in Indian backgrounds.

Materials and Methods
The linseed genotypes for present study were obtained from Oilseed Research Station, Latur (M.S). Towards fulfillment of morphological and molecular diversity study among these cultivars.

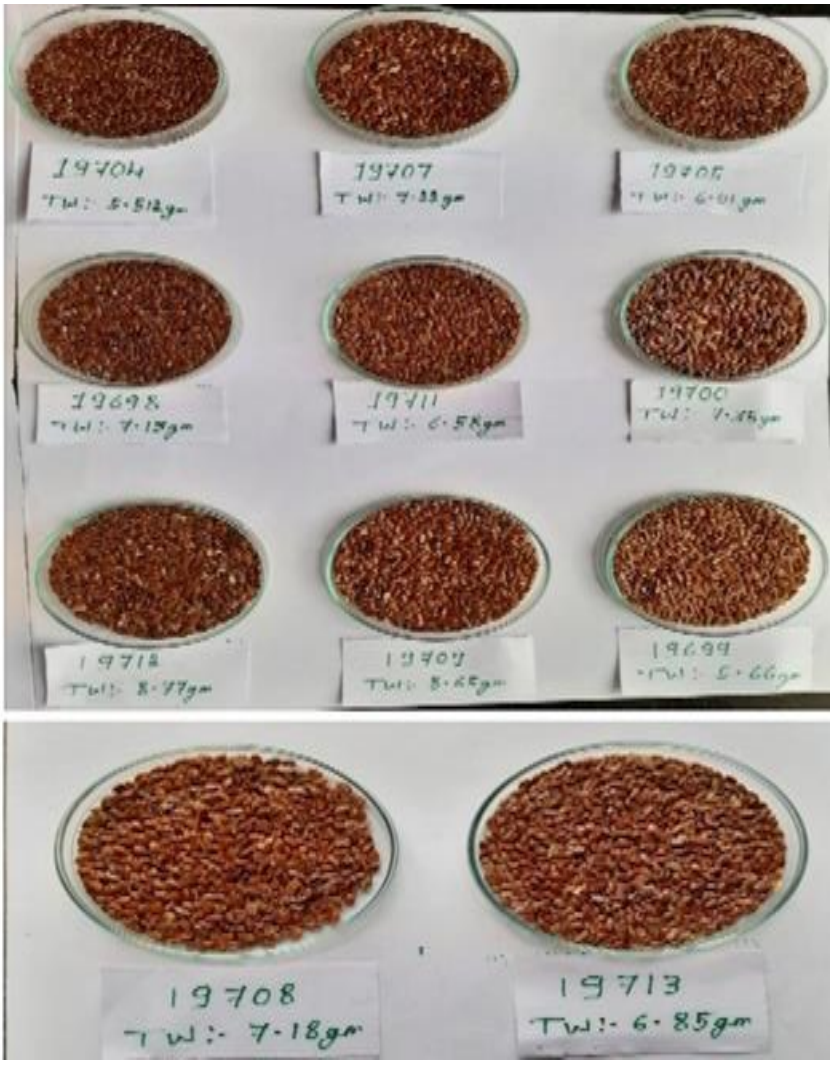

Fig 1: Linseed varieties collection from Oilseed Research Station, Latur

Table 1: Pedigree and codes of Linseed entries of AVT evaluated during 2019-20.

Sr. No	Code	Entries	Pedigree	Source
1	19698	RLC 171	POLF 22 X JRF 5	Raipur
2	19699	T 397(NC)	T 491 X T 1193-1	Kanpur
3	19700	SLS 121	JLS 73 X JLT 215	Sagar
4	19704	-	-	-
5	19705	OL 10-2	OL 18-4 X OLC 10	Keonjhar
6	19707	JLS 95 (ZC)	JLS 27 X EC 1387	Sagar
7	19708	-	-	-
8	19709	BAU 14-09	Shekhar X Garima	Kanke
9	19711	RLC 172	GS 234 X R 552	Raipur
10	19712	SLS 122	JLS 73 X JLS 66	Sagar
11	19713	NL 356	R 552 X Sheela	Nagpur

Morphological Characterization

Days to the first flower, Days to 50% flowering, Color of flower, Plant height at maturity (cm), Number of primary branches per plant, Number of capsules per plant, Number of seeds per capsule, 1000 seed weight (g).The results obtained for morphological characters were analyzed statistically like arithmetic mean, coefficient of Range, standard error etc.

Molecular Characterization

Isolation of DNA

High quality genomic DNA was isolated from fresh and young leaves of 11 genotypes The genomic DNA was extracted from leaf tissue of field grown plants following CTAB (Cetyl Trimethyl Ammonium Bromide) extraction method (Kang *et al*. 1998) [5] with some modifications. Integrity and intactness of DNA samples was checked on 0.8% agarose gel and quantity were measured by recording absorbance ratio A260/280using Nano photometer™. Individual samples were then diluted in sterile Milli-Q water to final concentration of 50 ng/µl and stored at 4 °C for further use. Amplification of 20 ng DNA was performed in 25µl reaction volume containing 109 PCR buffer (10 mM Tris HCl, pH 8.3; 50mM KCl, 1.5mM MgCl2), 0.1 mM dNTPs, 0.4, 0.3 lM primer and 0.48 U Taq DNA polymerase (Bangalore Genei, India) using PTC 225 thermal cycler (MJ Research, USA) programmed for 45 cycles. After initial

denaturation at 94 ^{0}C for 5 min, each cycle comprised 30 s denaturation at 94 ^{0}C, 45 s annealing at 53 ^{0}C or 60 ^{0}C depending on the annealing temperature of primers used and 2 min extension at 72^{0}C with 5 min final extension at 72^{0}Cat the end of 45 cycles. The amplification products were resolved on 1.5% agarose gel and electrophoresed in 0.59 TAE buffer. The gels were stained with ethidium bromide and documented on Image Master VDS gel documentation system.

Result and Discussion

Agro-morphological Traits Analysis

The phenotypic data were recorded on 11 advance varietal trials genotypes. Theagro-morphological characterizations was attempted and have been described as follows,

1. Days to the first flower

For the character days to the first flower and the average range observed was from 33(SLS 121) to 40 (19704) with a mean of 36.20. Among all linseed lines, the earliest of days to first flower was observed in SLS 121, SLS 122 (33 days) followed by BAU 14-09, RLC 172(34.50 days), JLS 95(ZC) and T 397(NC) (both at 35.5 days), OL 10-2 and 19708 (both at 37.50 days) and NL 356 (39.5 days) and followed by 19704 (40 days).

2. Days to 50% flowering

Day to 50% flowering observed amongst plants ranged from 46 to 54 days and the average range with genotype being 46.50 SLS 122 to 52.50(OL 10-2) with a mean of 49.72. Among all linseed lines early days to 50% flowering was observed in-state check SLS 122 (46.50 days) followed by SLS 121 (47 days), JLS 95(ZC) (48.75 days) and 19704, RLC 172 and T 397(NC) are at (49 days) Among all lines, late days to 50% flowering was observed in OL 10-2 (52.50 days) followed by NL 356 and RLC 171 both (51 days) and 19708 at (50 days).

3. Height of plant (cm)

Plant height various from 54 to 78cm and average ranged between 55cm to 77.75cm with a mean of 63.95. Among all lines, the tallest plant was observed T 397 (NC) (77.75cm) followed by 19708 and RLC 171 both (70cm), RLC 172 (65cm), 19704 (64.50cm), JLS 95(ZC) (63cm) and smallest plant height was observed in lines SLS 122 (55 cm) followed by SLS 121 (57cm), NL 356 (57.77cm), BAU 14-09 (60.25cm) and OL 10-2 (62.25cm).

4. Number of primary branches per plant

A moderate range of variation was observed for the character number of primary branches per plant. The number of primary branches varied from 2 to 7 in the collection with an overall mean of 4. Several primary branches observed minimum in OL 10-2 (2) followed by T 397(NC) (2), SLS 121 (3), RLC 171 (3) and RLC 172 (4). While a maximum number of primary branches were recorded in JLS 95(ZC) (7) followed by BAU 14-09 (5), NL 356 (5), SLS 122 and 19708 both at (5) and 19704 (4).

5. Number of capsules per plant

A number of capsules per plant ranged from 25 to 68 and wide average ranged of variation 26.5 to 67.50 were observed for this character with an overall mean of 47.63. capsules per plant in linseed lines. SLS 122 (67.50) had a maximum number of capsules followed by NL 356 (64.5), JLS 95(ZC) (56.50), RLC 172 (51.75). While a minimum number of capsules per plant were observed in T 397(NC) (26.5), OL 10-2 (30.50), 19708 (41.5), BAU 14-09 and 19704 both (47.00) and SLS 121 (48.75).

6. Number of seeds per capsule

The number of seeds per capsule ranged from 7 to 11 were recorded on an average of 8.45 seeds per capsule with a range varying from 7.5 to 10.00 seeds per capsule. The maximum number of seeds in the capsule was found in the genotype19704 (10.00) followed by SLS 121 (9.75), T 397(NC) (9.5), OL 10-2 (9.00). While the minimum number of seeds in the capsule were observed in NL 356 (7.5) followed by BAU 14-09 and 19708 both (7.25), RLC 171 (8.25), JLS 95(ZC) and SLS 122 both (8.50).

7. 1000 seeds weight (g)

Test weight various from 5.43g to 8.83g an overall mean for 1000 seeds weight was 6.95g with a range varied from 5.52 to 8.68. The lines recorded 19704 lowest test weight 5.52g followed by T 397(NC) (5.59g), OL 10-2 (6.11g), RLC 172(6.58g) and NL 356 (6.91g). The lines show the highest weight SLS 122 (8.68g) followed by BAU 14-09 (8.64g), SLS 121 (7.23g), JLS 95(ZC) (7.14g) and 19708 (7.09g).

8. Colour of flower

The linseed lines genotype did not show any significant variation concerning flower colour. While out of 11 lines JLS 95(ZC), RLC 172,19708 and NL 356 show white in colour and 7 lines were in blue.

Similarity matrix of 11 linseed genotypes based on eight morphological characters

According to Jaccard's estimate of similarity matrix was assessed and mentioned in (Table 4.2) for a set of 11 advanced varietal trials linseed genotypes. The similarity ranged from 0.12 in between (OL 10-2 and JLS 95 (ZC)) to 0.88 in between (SLS 122 and SLS 121) among these genotypes.

Clustering of linseed lines on the basis of eight morphological characters analysis

The similarity matrix was used to depict the dendrogram through UPGMA cluster analysis by using software NTSYS-pc (Version 2.02i). The dendrogram showing a phylogenic relationship based on UPGMA cluster analysis reveals closed genetic similarity in selected linseed lines genotyped using eight morphological characters. The 11 AVT lines were grouped into 2 clusters and three outgroups. The least similarity observed between genotype OL 10-2 and JLS 95 (ZC), NL 356 and T 397 (NC) was found to be only 12% similar and higher similarity observed between genotype SLS 122 and SLS 121was found to be 88% similar.Cluster I consisted of 14 genotypes, cluster II of 05 and cluster III of 15 genotypes. Different clustering patterns were also reported in linseed by some workers.(Negash *et al.* 2005, Diederichsen *et al.* 2006) [9] Figure UPGMA dendrogram for the linseed lines based on eight morphological characters analysis using Jaccard's similarity coefficient (NTSYSv2.02i, Rohlf, 2000)

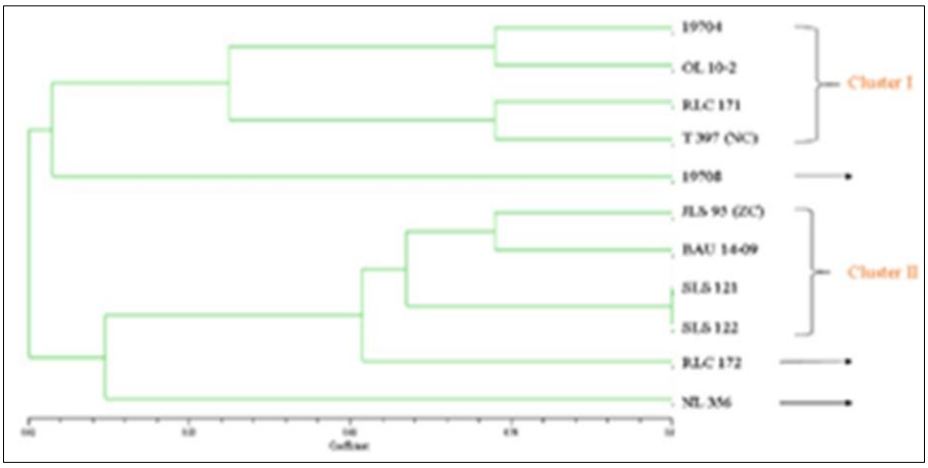

Fig 2: Dendrogram revealed linseed lines for morphological characters.

Genetic diversity at molecular level

Inter-Simple Sequence Repeat (ISSR) analysis

Ten ISSR primers were used for screening 11 lines for the assessment of genetic diversity. Out of 10 ISSR primers, eight primer pairs were showed a polymorphic banding pattern. Primers namely, UBC-807, UBC-810, UBC-818, UBC-825, UBC-840, UBC-841, UBC-850 and UBC-855 were revealed to be highly poly morphic and reproducible; whileUBC-815 and UBC-819 were monomorphic.

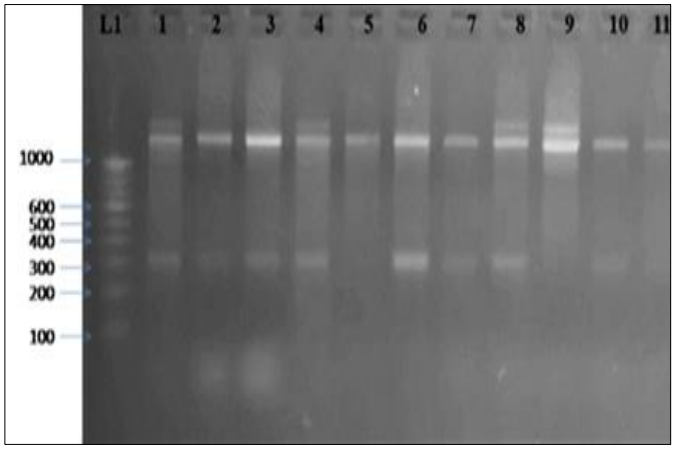

Plate 1: ISSR profiling of 11 linseed lines obtained with primer UBC- 807

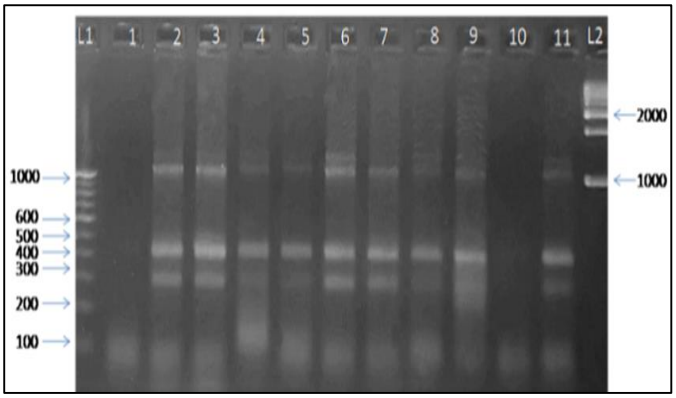

Plate 2: ISSR profiling of 11 linseed lines obtained with primer UBC- 825

The total of three different loci were amplified by primer UBC-807. This primer enabled to amplify 24 amplicons in total, found to be poly morphic and recorded 66% polymorphism. The amplicons size was ranged from 0.3 to 1.75kb, which was near about similar (0.5-1.9 kb) to the applicant mentioned by kumara *et al*., (2014). However, the 1.7kb allele occurred only in four accessions as BAU 14-09, 19704, NL 356, SLS 121; whereas, 1.5 kb allele was present in all lines and 0.3 kb allele was occurred all in but expect in lines SLS 122,19708, respectively.

In respect of UBC-810, three allelic loci were produced with a total number of 17amplicons, found to be polymorphic 66%.In primer, UBC-818 produced the lowest polymorphism i.e 33% and three allelic loci with 30 amplicons. The total of four different loci was amplified by primer UBC-825. This primer enabled to amplify 34 amplicons in total, found to be polymorphic and recorded75% polymorphism. In respect of UBC-840, four allelic loci and two polymorphic loci were produced by primer with a total number of 30 amplicons, were found to be polymorphic and recorded 50% polymorphism. The primer UBC-841 produces 100% polymorphism and three allelic loci with three polymorphic loci with 22 amplicons and UBC-850, three loci were produced with a total number of 18 amplicons, were found to be polymorphic and recorded 100% polymorphism. The total of four different loci was amplified by primer UBC-855.

Wiesnerova *et al*., (2004) had also reported 72.6% polymorphism by using 9ISSR primers across 53 Czech flax cultivars; while63.9% average polymorphism was detected by Rajwade *et al*., (2010) [10] in 70 flax genotypes by using12 ISSR primers as compared to reported values by fewer primers in our present study that revealed 70.62% polymorphism.

A total of 194 amplification products (fragments)were scored, and found to be polymorphic from a corresponding total of 26 loci amplified and scored across eleven lines via ISSR analysis. ISSR primers namely, UBC-841 and UBC-850 revealed the height polymorphism of 100% whereas, UBC-818 exhibited the lowest polymorphism i.e.,33% among the seeight polymorphic primers.

The overall average of 70.62% polymorphism was recorded based on banding patterns or amplified polymorphic loci of each genotype (row-wise bands polymorphism). Thus, 18 out of a total of 26 loci (alleles) were found to be polymorphic across the set of eleven lines edlines. In the present study, the obtained polymorphic information content (PIC) value per primer ranged between 0.19 to 0.44 with Anoverall average value of 0.29 per primer. ISSR primers *viz*., UBC-841

revealed the highest PIC value of 0.44 followed by UBC-818(0.40), UBC-850(0.28), UBC-810(0.27), UBC-825(0.26) whereas UBC-840exhibited the lowest PIC value i.e., 0.19.The mean PIC value (0.746) detected in the present study was higher than the PIC value (0.385) obtained by Kumari *et al*., 2014 [8] in 28 genotypes of flax characterized by 11 polymorphic ISSR primer and was also higher than reported in Indian flax genotypes by Rajwade *et al.*, 2010 [10], who detected average PIC of 0.18usingonlyISSRmarkers.

Simple Sequence Repeats Analysis

Total of 10 primers were used for screening 11 genotypes for the assessment of genetic diversity. Out of 10 SSR primers, five primers pair were amplified with polymorphic banding patterns among the linseed lines. These five primers produce 11amplicons.

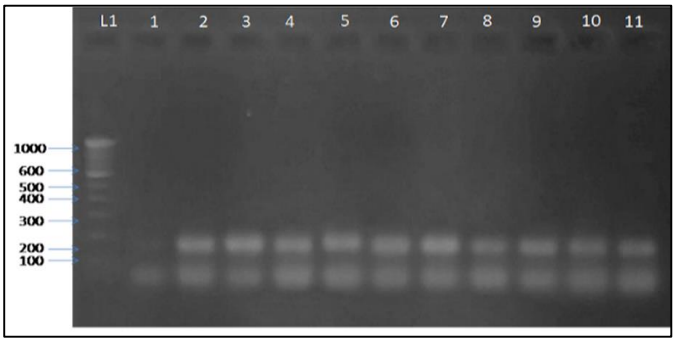

Plate 3: SSR profiling of 11 linseed lines obtained with primer LU-1

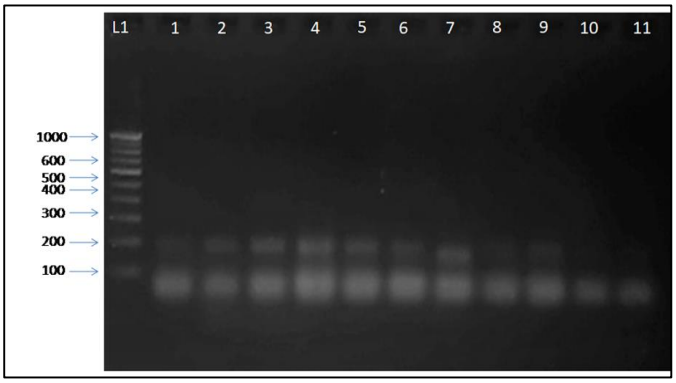

Plate 4: SSR profiling of 11 linseed lines obtained with primer LU-3

A set of 10 pre-selected SSR primers mostly of series 'LU' designs in linseed (oil content-related SSRs as per Soto-Cerda *et al*., 2014. Out of 10 SSR primers, 5 were found to be polymorphic. A total of 11 alleles were amplified by 5 primers, all of which were found to be polymorphic. 100% polymorphism was demonstrated by 2 SSR primer pairs, while the remaining 3 SSR showed 50% polymorphism, respectively in the given AVT set during the present investigation.

The SSR primer, LU-3 revealed highest PIC value i.e., 0.82 followed by LU-1, LU-9 both at (0.66) and LU-5 (0.52); whereas the primer LU-8revealed the lowest (0.07) PIC values. A totalof5SSRprimerswerescreened, out of which 2 primers were found to be 100% polymorphic and the rest of 3primers have shown 50%polymorphism respectively, same results were observed by Rana *et al.*, 2017, Dash *et al.*, 2016 [5]. In respect of LU-1, two allelic loci and one polymorphic locus were produced by these primers with a total number of 20 amplicons, with 50% polymorphism and primer LU-3 produces 50% polymorphism and two allelic loci with one polymorphic locus was produced by these primers with 21 amplicons. The allelic size ranges from 100 to 200 bp. A total of three different loci was amplified by primer LU-5. This primer enabled to amplify 21 amplicons in total, found to be polymorphic and recorded 100% polymorphism. The ampli con size was ranged from 100 to 320bp. 240bp unique band in the line BAU 14-09 and JLS 95(ZC), which would be considered as informative and can be distinguished from others during DNA finger printing with high diagnostic value. However, 170bp allele was occurred in lines except as RLC 171 and OL 10-2; whereas, 100bpallele was present in all lines but expect in one lineOL 10-2 respectively.

A total of 100% polymorphism were present in primer LU-8 and two allelic loci were produced by these primers with a total number of 14 amplicons, which were found to be polymorphic (Plate 4.12). 220bpalleles occurred in only three lines in JLS 95(ZC), RLC 171 and SLS 121. Where this primer produced 100 bp amplicons, which were monomorphic (commonly shared) among all the lines, respectively.

Clustering of accession based on combined markers analysis

Based on the combined (ISSR and SSR) markers UPGMA analysis, the genetic similarity values ranged from 0.41 to 0.87with an average of 0.60 among these eleven promising genotypes and using Jaccard's coefficient of similarity the dendrogram was generated.

The combined cluster analysis of all two marker systems (ISSR and SSR) data showed a relatively wide genetic background of the present in digenous linseed germplasm accessions. Three distinct major clusters (A, B and C) were found to represent a unique grouping of 11 member-genotypes. Cluster A, emerged as the one, comprising 3 accessions, and one separated grouped i.e., BAU 14-09. While JLS 95(ZC), RLC 171 and RLC 172 they found to be similar to each other at 82%.

The major cluster B comparing 4 accessions, was found with lines 19704, NL 356 SLS 121 and 19708 they are similar to each other at 78%. While lining 19704 and NL 356 which are similar to each other. Line 19708 occurred in out grouped. In Cluster (C) two genotypes (OL 10-2, T 397(NC)) were appeared to be similar with each other at around 65%.

The least similarity observed between genotype SLS 121 and BAU 14-09 was found to be only 41% similar and higher similarity observed between genotype NL 356 and 19704 was found to be 87% similar.

Khan *et al.* (2013) clustered 55 genotypes into thirteen clusters, in which clusters I and II genotypes were found to be most suitable for hybridization. Adugna *et al.* (2006) grouped 60 genotypes into 18 clusters across which the genotypes of four clusters were observed to be highly genetically diverse. Dikshit *et al*. (2015) also reported a similar type of results in lines edgeno types.

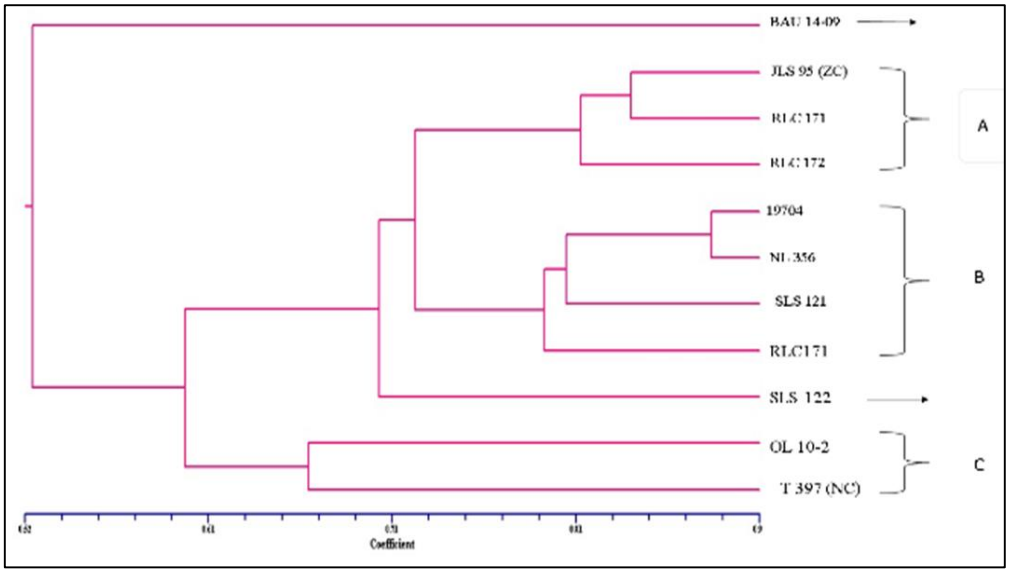

Fig 3: UPGMA dendrogram for the linseed accessions based on Combined (ISSR and SSR) marker analysis using Jaccard's similarity coefficient (NTSYSv2.02i, Rohlf, 2000)

Conclusion

In the present study the investigation was done on linseeds at morphological and molecular level. It is observed that the earliest first flower was observed in SLS 121, SLS 122 (33 days), late flower 19704 (40 days). Day to 50% flowering range from 46.50 SLS 122 to 52.50(OL 10-2). Among all linseed lines earliest 50% was observed in SLS 122 (46.50 days) followed by SLS 121 (47 days), JLS 95 (ZC) (48.75 days), and 19704 (49 days). The tallest plant are observed T397 (NC) (77.75cm). The smallest plant height was observed in lines SLS 122 (55cm). The number of primary branches per plant varied from 2.00 to 7.50 in the collection with an overall mean of 4.06. Maximum number recorded in JLS 95 (ZC) (7.50) capsules per pant in linseed lines. SLS 122 (67.50) had a maximum number of capsules. The maximum number of seeds in the capsule was found in the genotype 19704 (10.00).While the minimum number of seeds in the capsule was observed in NL 356 (7.5).The lines recorded 19704 lowest test weight 5.52g.The lines show the highest weight SLS 122 (8.68g).While out of 11 lines JLS 95(ZC), RLC 172,19708, and NL 356 show white in color, and 7 lines were in blue.

The overall average of 70.62% polymorphism was recorded based on banding patterns. In the present study, the obtained polymorphic information content (PIC) value per primer ranged between 0.19 to 0.44 with an overall average value of 0.29 per primer. ISSR primers *viz*., UBC-841 revealed the highest PIC value of 0.44 followed by UBC-818 (0.40), UBC-850(0.28), UBC-810(0.27), UBC-825(0.26), whereas UBC-840 exhibited the lowest PIC value i.e., 0.19.A total of 11 alleles were amplified by 5 primers and all alleles were found to be polymorphic. 100% polymorphism was demonstrated by 2 SSR primer pairs, while the remaining 3 SSR showed 50% polymorphism, respectively in the given AVT set during the present investigation. The SSR primer, LU-3 revealed the highest PIC value i.e., 0.82 followed by LU-1 and LU-9 both at (0.66) and LU-5 (0.52); whereas the primer LU-8 revealed the lowest (0.07) PIC values. Based on the combined (ISSR and SSR) markers UPGMA analysis, the genetic similarity values ranged from 0.41 to 0.87 with an average of 0.60 among these eleven promising genotypes and using Jaccard's coefficient of similarity the dendrogram was generated.

The present morphological analysis revealed that most dissimilar genotype *viz*., OL 10-2 and JLS 95(ZC), NL 356 and T397 (NC) 12% were noticed.

By studying the correlation between morphological and molecular data further we can use diverse parents 59% dissimilar (SLS 121 and BAU 14-09) among these 11 lines for future breeding program.

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