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ASSESSMENT OF GENETIC DIVERSITY IN GERMPLASM COLLECTION OF LINSEED (*Linum usitatissimum* L.)

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ABSTRACT
Molecular characterization of 72 linseed (*Linum usitatissimum* L.) germplasm accessions was carried out using 30 ISSR markers. Out of 350 amplified alleles 344 alleles showed polymorphism among evaluated germplasm accessions. Private bands and % heterozygosity were high in breeding material generated at PAU, Ludhiana and exotic germplasm collected from different breeding centres worldwide. There was 78.15 ± 7.85 avarage % ISSR polymorphism resided among these 72 accessions, highest 89.68 % among breeding and exotic accessions, 76.79 % and 56.45 % Indian and flax varieties respectively. Also these two groups had high mean heterozygosity in comparison to Indian and flax varieties. Clustering based on Neighbor-Joining (NJ) cluster analysis wherein the genotypes were clustered into eight major clusters lacking clear grouping among clusters. Pair wise population Nei genetic distance and identity was highest between Indian and flax varieties (0.094) and lowest between breeding and exotic lines (0.017). Accessions constitutes breeding material and exotic collection were more diverse than Indian and flax varieties. These findings are useful for selection for identification of suitable diverse parental genotype for hybridization in linseed cultivars development programme.

INTRODUCTION

Linseed (*Linum usitatissimum* L., 2n = 30) is a member of the Linaceae DC. (Dumort) family comprising 22 genera and about 300 species distributed worldwide with a wide center of origin in the Middle East, although secondary diversity centers were identified in the Mediterranean basin, Ethiopia, Central Asia, and India (Zohary and Hopf, 2000). It is an important agro industrial oilseed species cultivated for fibre and for its oil rich seeds. Globally linseed is an important crop and its production is 21.23 lac tones from 21.12 lac ha with an average yield of 1006 kg/ha, while our national production is 1.54 lac tones from an area of 3.42 lac ha with productivity of 449 kg/ha. India ranks second in area after Canada in the world, but is at fourth position in terms of production after Canada, China and USA. It contains 33-45% oil and 24% crude protein. Linseed oil possesses a very healthy fatty acid profile, particularly, Omega-3 (Alpha Linolenic Acid), richest source only in linseed (58%). ALA provides beneficial effects in numerous clinical conditions such as, cardiovascular diseases, inflammatory disorders, immune functions and cancer etc. Almost every part of the linseed plant is utilized commercially either directly or after processing. The linseed oil is industrial oil primarily goes to industries for manufacture of paints, varnish, linoleum, oil cloth, patent leather, printer ink, pad ink, enamels, stickers, tarpaulins, soaps etc. The fiber extracted from straw is used to produce strong yarns such as sewing threads, linen fabrics and linen threads. The coarser grades are used for making twines, canvas bags, *Linum usitatissimum* L. quality papers (Savita, 2006).

Developments of superior varieties with genetic control for biotic and abiotic stresses are prime objective of any crop breeding programme. Germplasm characterization of targeted traits and assessment of genetic variability is important preliminary work in linseed cultivar improvement. Despite its nutritional and commercial significance linseed varieties developed through conventional breeding methods *viz:* introduction, selection, and hybridization. Earlier morphological and isozyme markers were popular for assessment of genetic diversity in flax. Oh *et al*. (2000) first reported the use of DNA-based markers (RAPD and RFLP techniques) to generate a preliminary genomic map and study of genetic diversity. The low genetic variability has been reported by Fu *et al*. (2002) using RAPD markers in 61 flax varieties including Canadian cultivars and land races. In other study Fu *et al*. (2003) used RAPD markers to analyze the genetic variation and genetic erosion in 54 North American flax cultivars. The ISSR technique for flax fingerprinting was optimized by Wiesner and Wiesnerova (2003) using re-amplification method. Similarly, Rajwade *et al*. (2010) was to analyze the genetic relationships, using PCR-based ISSR markers, among 70 Indian flax (*Linum usitatissimum* L.) genotypes.

ISSR marker genotyping is popular approach for genotyping of nonsequenced genomes and widely used for phylogenetic studies as well as in crop varietal fingerprinting (Zietkiewicz *et al.*, 1994). Linseed production is low due to many limiting factors such as low productive genotypes with susceptibility to biotic and abiotic stress (drought, heat, salt). So, there is need for development of new genetic material for generation of new recombinants with desirable traits. Therefore, the present investigation was carried out to analyze genetic

diversity using ISSR markers for effective evaluation and utilization in linseed breeding programs.

MATERIALS AND METHODS

A germplasm collection of 72 linseed accessions which included 37 exotic accessions from different country, 18 advanced breeding lines of university breeding programme, 12 indigenous and 5 flax type released cultivars were planted at oilseeds section research field of Punjab Agriculture University, Ludhiana, Punjab. Each entry sown in the small plastic pot in green house with two replicates during to raise the healthy plants followed by recommended agronomic practices from package and practices. Fresh leaf samples were collected in liquid nitrogen from five individual plants of each variety of 15 days old plants. Genomic DNA was extracted with liquid nitrogen dried tissue by using protocol of CTAB DNA extraction method (Murray and Thompson, 1980). The final concentration of DNA was estimated by Spectrophotometer Plus, using known concentration of Lambda DNA (Genei, 300ng/î l) as a standard. The working concentration of DNA was adjusted to 10 ng/ul for ISSR analysis. A set of 100 ISSR were synthesized on the basis of the sequence information published by Biotechnology Laboratory website by University of British Columbia (UBC), Vancouver, Canada. All the 100 primers were first screened on 5 accessions for standardization of the protocol and to identify the polymorphic markers before testing on 72 accessions. Thirty ISSR primers detected to show the polymorphism in the representative genotypes. PCR amplifications were performed in 25 î L of reaction volume containing the following reagents: 25-30ng of genomic template DNA, 1 î M of primer, 1 Unit of Taq DNA polymerase, 0.25 mM of each dNTP and 1 × reaction buffer supplied with the enzyme (Zietkiewicz *et al.*, 1994). Some adjustments in the annealing temperature and changes in the number of amplification cycles were made to the original program to improve the results. Amplification products were visualized using agarose gel electrophoresis stained with ethidium bromide.

The ISSR amplification profiles of the genomic DNA were scored visually. The each fragment length (bp) in the amplified product was determined with reference 100bp DNA marker ladder (Fermentus). In ISSR amplification for presence (1), absence (0) and uncertain (9) in each sample to generate a binary data matrix. A few gels were scored twice by two individuals to verify and minimize possible scoring errors. Polymorphism information content (PIC) of each primer pair were calculated by using the following formulas: PIC = (1-″ pij2)/n, where pij is the frequency of jth allele in the ith primer and n is the total number of accession (Anderson *et al.*, 1993). The genetic diversity parameters and genetic distance in four linseed groups were carried out by using with GenAlEx 6.4 software (Peakall and Smouse, 2006). The genetic distance and cluster analysis of accessions was carried out using Neighbour Joining (NJ) method and a dendrogram was drawn by Paleontological Statistics (PAST version 2.11) software package for education and data analysis (Hammer *et al.*, 2001). Bootstrap analysis with 5000 replicates was performed to obtain the confidence of branches of the tree. Principal Component Analysis (PCA) was also carried out to show the distribution of individual accession in scatter diagram and a scatter 2-dimention PCA plot was drown using PAST version 2.11.

RESULTS AND DISCUSSION

ISSR polymorphism

Thirty primers were selected after screening on 5 representative genotypes for clear, polymorphic and reproducible

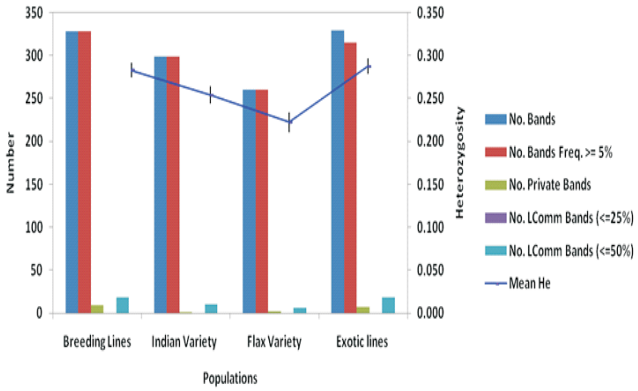

Figure 1: Genetic diversity parameters in different groups

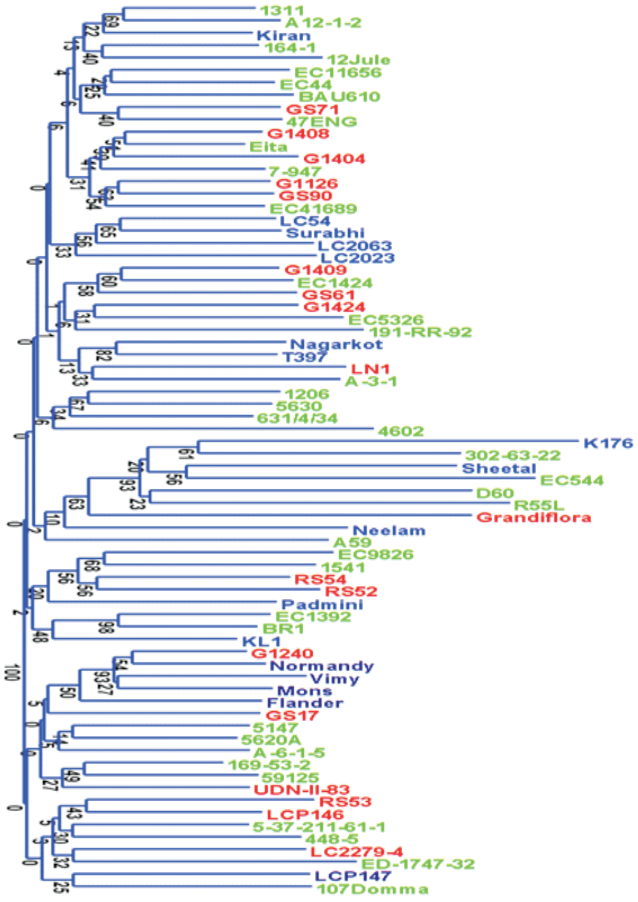

Figure 2: Dendrogram based on Nei genetic distance of 72 flax genotypes

Table 1: Variation of ISSR primers in number of amplified products, polymorphic band and polymorphism information content observed in 72 flax genotypes

Sl. No.	Primers	Sequence (52 '→32)	Total Amplified band	Polymorphic band	PIC value
1	UBC 847	(CA)8RC	14	13	0.91
2	UBC 845	(CT)8RG	14	14	0.91
3	UBC 898	GATCAAGCTTNN NNNNATGTGG	5	4	0.74
4	UBC 857	$(AC)_8$YG	10	10	0.88
5	UBC 866	$(CTC)_6$	8	8	0.86
6	UBC 827	(AC)8G	15	14	0.91
7	UBC 888	BDB$(CA)_8$	13	12	0.86
8	UBC 889	DBD$(AC)_8$	15	15	0.91
9	UBC 890	VHV$(GT)_8$	17	17	0.91
10	UBC 887	DVD$(TC)_8$	14	14	0.92
11	UBC 891	HVH$(TG)_8$	13	13	0.89
12	UBC 892	TAGATCTGATATCT GAATTCCC	11	11	0.89
13	UBC 878	$(GGAT)_4$	7	7	0.81
14	UBC 842	(GA)8YG	5	5	0.79
15	UBC 844	(CT)8RC	10	10	0.89
16	UBC 850	(GT)8YC	10	10	0.86
17	UBC 853	(TC)8RT	7	7	0.69
18	UBC 859	$(TG)_8$RC	8	7	0.84
19	UBC 836	(AG)8YG	15	15	0.88
20	UBC 840	(GA)8YT	12	12	0.86
21	UBC 841	(GA)8YC	5	5	0.75
22	UBC 834	(AG)7YT	22	22	0.93
23	UBC806	(AG)9T	13	12	0.89
24	UBC808	(AG)9G	11	11	0.87
25	UBC809	(GA)9T	15	15	0.86
26	UBC810	(GA)9C	11	11	0.86
27	UBC 811	(GA)9A	18	18	0.91
28	UBC 825	(AC)8T	15	15	0.91
29	UBC 826	(AC)8C	6	6	0.82
30	UBC 821	(TC)8A	11	11	0.85
		Total	350	344	0.862

Table 2: Total Band Patterns for ISSR marker data by different group

Population	sample size	No. Bands	No. Bands Freq. > = 5%	No. Private Bands	%P	No. L Comm Bands (< = 50%)	Mean He
Breeding Lines	18	328	328	9	89.68	18	0.283
Indian Variety	12	299	299	1	76.79	10	0.254
Flax Variety	5	260	260	2	56.45	6	0.222
Exotic lines	37	329	315	7	89.68	18	0.287
Mean					78.15		
SE					7.85		

Table 3: Pairwise population Nei genetic distance and Nei genetic identity values

Pop1	Pop2	Nei GD	Nei ID
Breeding Lines	Indian Variety	0.039	0.962
Breeding Lines	Flax Variety	0.079	0.924
Indian Variety	Flax Variety	0.094	0.910
Breeding Lines	Exotic lines	0.017	0.983
Indian Variety	Exotic lines	0.033	0.967
Flax Variety	Exotic lines	0.074	0.928

amplification out of 100 UBC ISSR primers (Table 1). The reproducibility of the ISSR amplifications was assessed using different DNA samples, isolated independently from the same genotype and amplified at different times. Under the optimized PCR conditions, the banding profiles were found consistent among PCR experiments. These selected primers amplified a total of 350 DNA fragments of which 344 were polymorphic across the 72 flax genotypes (Table 1).

Each ISSR primers showed multiallelic patterns in each genotype and no band was detected in any negative control

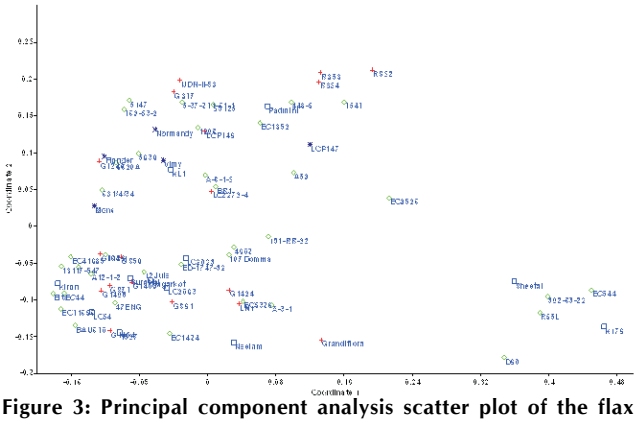

Figure 3: Principal component analysis scatter plot of the flax genotypes (The coloured symbol indicates different groups)

amplification. The average number of loci per primer and polymorphic loci per primer were 11.6 and 11.5, respectively. Among the tested linseed genotypes, approximately all amplified ISSR primer was polymorphic. The maximum numbers of polymorphic alleles (22 alleles) were amplified by UBC 834 whereas the lowest (5 alleles) were amplified by UBC 841, UBC 842 and UBC 898. The number of polymorphic alleles detected for one primer ranged from 22 (UBC 834) to 4 (by UBC 898), thus the maximum amplified alleles were polymorphic in all the evaluated lines.

PIC value is a measure of variability at a specific locus gives the information about the probability that polymorphism will exist between two randomly selected genotypes at that locus. Polymorphisms between individuals mainly results from sequence differences in the primer binding sites and are visible as the presence or absence of a particular amplification product. The polymorphic information content calculated from the frequency of polymorphic alleles ranged from 0.69 to 0.93 with an average 0.86 across all genotypes (Table 1). The primer UBC 853 and UBC 898 exhibited the lowest PIC value of 0.69 and 0.74, respectively. The highest PIC value (0.93) was obtained by the UBC 834 primer which amplified 22 polymorphic loci.

Genetic parameters

Prior to genetic diversity analysis relevant genetic parameters were calculated in different groups (Table 2). The advanced breeding lines amplified total 328 alleles with 89.68 % polymorphism and 0.283 mean heterozygosity. The highest nine unique/specific bands were also amplified in this set of accessions. Indian varieties amplified 299 alleles, flax variety 260 alleles and exotic lines 329 alleles with 1, 2 and 7 private/ unique alleles, respectively. The percentage polymorphism was highest 89.68% in the advanced breeding lines and exotic lines, owing to the fact that the assessed breeding lines have been developed from the exotic germplasm. The lowest polymorphism (56.45%) was estimated in the flax variety group with the average 78.15% ± 7.85%. The heterozygosity was highest in the exotic accessions (0.287) followed by advanced breeding lines (0.283), Indian variety (0.254) and Flax lines (0.222) (Fig 1). Total number of amplified bands, % polymorphism, unique bands, least common bands and % heterozygosity are reflecting narrow genetic base in Indian and flax varieties collection than exotic germplasm and breeding lines collection.

ISSR-based genetic relationships among the linseed genotypes

The clustering analysis were carried out using data set matrix from DNA profiling of 72 linseed accessions from 30 primer amplified 345 polymorphic loci. Pair wise population Nei genetic distance and identity (Table 3) was calculated which is highest between Indian and flax variety (0.094) and lowest between advanced breeding lines and exotic lines (0.017). The Neighbor-Joining (NJ) algorithm partitioned the 72 accessions into eight major groups and 17 sub clusters (Fig 2). Cluster I and III comprised 8 genotypes in each, Cluster II consisted of 12 genotypes and Cluster V and VII comprised 4 genotypes in each. The nine genotypes namely K176, 302-63-22, 'Sheetal', EC 544, D 60, R 55 L and Grand flora (wild species), were the most distinct genotypes which formed the cluster IV. Rajwade *et al.* (2010) reported in a study conducted on Indian flax genotypes, 'Sheetal' was the most diverse variety among the evaluated germplasm. The cluster VI is comprised 10 genotypes. The cluster VIII was the largest cluster with 17 genotypes from different group. Cluster I included three group genotypes 3 Breeding lines, four Exotic lines and one Flax type cultivar group. Cluster III comprised 8 genotypes four from exotic lines, 2 from advanced breeding lines and 2 Indian varieties group. Distribution of the accessions across clusters or subclusters or sub-subclusters was not based on proximity to their position in breeding cycle. The genotypes form different group of germplasm are scattered into different cluster confirming that the breeding lines derived from germplasm collection. Similarly, Ziarovska *et al.,* 2012 showed lack of corresponds between linseed or fiber flax and also no geographical distribution of the germplasm.

The Principle components analysis (PCA) was performed and 2-dimension graph was drawn using the PAST statistical package. The PCA graph (Fig 3) showed the greatest differentiation between groups as well as between the genotypes from all groups. PCA in combination with cluster analysis is a useful tool to extract maximum information from molecular marker data (Messmer *et al.,* 1992). The results obtained from PCA graph were comparable to those obtained with cluster analysis (dendrogram). The genotypes namely K176, 302-63-22, 'Sheetal', EC 544, D 60, R 55 L and Grandiflora placed in cluster IV were the most differentiated genotypes and were also placed away from the rest of the genotypes.

Different molecular markers are being used for diversity study in linseed including inter-retrotransposon amplified polymorphism (Smykal *et al.,* 2011), random amplified polymorphic DNA (Fu, 2005), inter-simple sequence repeat (Wiesnerova and Wiesner, 2004), and simple sequence repeat (Cloutier *et al.,* 2009; Singh *et al.,* 2015), and in different plant species *viz*: ISSR in chickpea (Pandey *et al.,* 2014) and SSAP markers in Pigeonpea (Patil *et al.,* 2015). The other studies conducted in flax germplasm using RAPD, ISSR, AFLP and SSR markers supported our results as a narrow genetic diversity in the respective studied material (Wiesnerova and Wiesner 2004; Fu, 2005; Cloutier *et al.,* 2009). Taken together, these studies show that cultivated flax has low genetic diversity compare to wild relatives or some other crops. The narrow genetic variation in the presented and supportive studied

possibly resulting from a domestication bottleneck; as a consequence of only modern cultivars are used (Tanksley McCouch, 1997). Genetic diversity assessment could also be used for understanding flax domestication and its primary gene pool (Uysal *et al.*, 2010).

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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