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RESEARCH PAPER

Association analysis for morphological and nutraceutical traits in Linseed (*Linum usitatissimum* L.) using microsatellite markers

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Abstract: In linseed, marker-trait associations were studied for 15 morphological and nutraceutical traits using a 50 microsatellite markers with a set of 170 elite genotypes. Using regression based association analysis, each of the 15 traits were regressed on 111 available polymorphic alleles. A total of 21 SSR markers gave significant associations with at least one of the traits. The number of markers associated with individual traits ranged from 1 to 4. Plant height showed significant regression with only one SSR marker, while 1000 seed weight and seed yield per plant showed significant regression on 4 SSR markers. SSR markers Lu144a-1, Lu151-2 and Lu138-4 were associated with number of capsules per plant at 1% significance level. Similarly three SSR markers *viz.* Lua49B (allele number one), Lua60 (allele number one) and Lua125a (allele number three) were significantly associated alpha linolenic acid content at 0.1% significance level ($P \leq 0.001$). Four SSR markers namely Lu151-2, Lua68-2, Lu138-3 and Lu143-1 were associated with 1000 seed weight. SSR markers Lu236-2, Lu144a-1, Lu151-2, and Lu143-2 were significantly ($P \leq 0.001$) associated with seed yield per plant. Lub14-4 and Lu143-1 were associated with oil content in seeds. Association explained 7 to 44.2 per cent of the variation of individual traits. Markers identified by this study will be useful for marker-assisted breeding after necessary validation.

Keywords: Association analysis, Linseed, Microsatellites, Nutraceutical crops

Introduction

Linseed (*Linum usitatissimum* L.) is a dual purpose crop having utility as oilseed and fibre. It is a self pollinated annual crop species belonging to the family *Linaceae* and order Geraniale which includes 14 genera and over 200 species. Cultivated species *L. usitatissimum* is reported to have wide genetic diversity which is distributed over six continents. The cultivation of the crop as such goes back to more than 5000 years. The ancient Greeks and Romans cultivated linseed for its seed and fibre. Recently researchers reported spun and dyed wild linseed fibres in a prehistoric cave in the Republic of Georgia which shows that the plant was already in use by humans as early as of 30,000 BC. All parts of the linseed plant have extensive and varied commercial uses. Although use of linseed is long known from ancient times, the interest in the crop is reinvented during the last decade due to its nutraceutical importance.

Linseed oil contains upto 60 per cent of alpha linolenic acid (ALA), which is an essential omega-3 fatty acid for human diet. ALA acts as a precursor for biologically active longer chain polyunsaturated fatty acids (PUFA) such as Eicosapentaeonic acid (EPA) and Docosahexaeonic acid (DHA). Linatine, a vitamin B_6 compound found in linseed is known to have antagonist properties (Klosterman *et al.,* 1967). Linseed is also a good source of a major lignan, Secoisolariciresinol diglucoside (SDG) that reduces the development of hypercholesterolemic atherosclerosis and slows down the growth of cancer cells in humans. Linseed, with its high levels of alpha-linolenic acid (ALA), good amount of fiber, mucilage (long chain carbohydrates), and cancer-fighting lignans, is recommended as a healthy food supplement.

India is the second largest producer of linseed, with an area of 525.5 thousand hectares, a production of 153,000 metric tonnes per annum and productivity of 403 kg/ha. Although India has 18.8 per cent of worlds recorded linseed area, it produces less than 10 per cent of total world production. This may be attributed to lack of superior cultivars and its market potential. Hence there is a need for developing high yielding, nutraceutically enriched varieties of linseed.

The progress in developing a superior variety depends largely on the genetic basis of selection such as diverse parents and the breeding approach followed. However, linseed is one of the neglected oilseed crops of developing countries, grown on marginal land with poor management. A poor yield of this crop is attributed to non-availability of improved cultivars to suit the diverse agro climatic conditions. Hence, development of high yielding cultivars becomes the top priority to overcome the poor yield levels.

Most of the important morphological and nutraceutical traits such as yield and fatty acid composition in linseed are quantitative in nature. They are under polygenic control and are influenced by the environment in which they are grown. Hence identifying the Quantitative trait loci (QTL) governing these traits are valuable in marker assisted selection and further in cloning.

Molecular markers associated with agronomically important QTLs are generally identified by utilizing planned populations such as recombinant inbred lines (RILs) or near isogenic lines (NILs). But, such populations are not available for minor crops like linseed and it takes substantial time to develop the same.

Germplasm-regression-combined marker trait association can be used as an alternative to marker trait association in planned populations. GRC marker analysis is based on the evaluation of the phenotypes in diverse germplasm collection and associating it with DNA fragments using regression technique (Wright and Movers, 1994; Yonash *et al.,* 2000; Chatterjee and Pradeep, 2003; Pradeep *et al.*, 2007; Srivastava *et al.,* 2007). The germplasm-regression-combined (GRC) association studies allow mapping of QTLs with higher level of confidence.

GRC analysis method is increasingly adopted to identify the associations of molecular markers with desirable traits in many plant breeding programs such as rice (Virk *et al.*, 1996), wheat (Roy *et al.*, 2006), tea (Mishra and Sen-Mandi, 2004), Alfalfa (Obert *et al.,* 2000; Maureira-Bulter *et al.,* 2007), mulberry (Vijayan *et al.,* 2006; Kar *et al*., 2008), coconut (Shalini et al., 2007), birch (Wang *et al.,* 2008; Xia *et al.*, 2008), oat (Achleitner *et al.,* 2008) and sea buckthorn (Ruan *et al.,* 2010). These studies identified associations between DNA fragments and quantitative traits (QTLs) without the necessity of the planned mapping populations and the linkage map.

Genetic markers such as microsatellites, the heritable entities that are associated with economically important traits are used by plant breeders as selection tools. Simple sequence repeat markers (SSR) are being extensively used in genome studies, marker-assisted selection, and cultivar identification and are well-known for their versatility in providing a quick assay and for their highly informative data.

Germplasm-regression-combined marker trait association can be used as an alternative to marker trait association in planned populations. It is conducted through the combination between the present germplasm and the regression technique (Srivastava *et al.,* 2007) and increasingly adopted in many plants (Maureira-Butler *et al.,* 2007). The germplasm-regression-combined (GRC) association studies not only allow mapping of genes/QTLs with higher level of confidence but also allow detection of genes/QTLs.

Recently, several SSR markers are developed in linseed. Hence developing association between these markers and useful agronomic traits such as ALA content is possible. This will be useful in breeding programs to develop plants with desirable yield and quality attributes. Hence, present study was conducted to analyse the genetic diversity present in the germplasm collection and to identify putative marker alleles associated with economically important traits through GRC analysis.

Material and methods

Plant materials and field experiments: The experimental material comprised of 167 linseed (*Linum usitatissimum* L.) germplasm accessions and three checks (S-36, NL115 and Indira Alsi) obtained from Project Coordinating Unit, AICRP on linseed, Chandra Shekar Azad University of Agricultural and Technology (CSAUAT), Kanpur. The accessions were diverse in nature both from the point of geographical area and other yield attributing characters. The experiment was laid out in an augmented design. It consisted of seven blocks and three checks were repeated in each block. The experiment was conducted in medium black soils under rain fed conditions with protective irrigation during *rabi* 2012-2013 at the MARS, Dharwad, Karnataka. Each germplasm entry was sown in a single row of two meters length. A spacing of 30cm between the rows and 4cm between the plants in a row was maintained. All recommended agronomic practices were followed during the crop growth period. 100 elite and diverse genotypes were selected from this germplasm collection (Appendix-I) for molecular analysis.

Morphological evaluation: Data from 5 plants of each genotype were recorded on the following economically important traits: days to flowering, days to maturity, plant height (cm), number of primary branches per plant, number of secondary branches per plant, number of capsules per plant, number of seeds per capsule, 1000 seed weight (g), and seed yield per plant (g). Oil content in seeds was determined with the help of Pulsed NMR (Nuclear Magnetic Resonance) spectrometer at Regional Agricultural Research Station (RRS), Raichur. Fatty acid composition of seeds was measured using standardized Near Infrared Spectroscopy (NIRS) at Seed Quality and Research Laboratory, UAS, Dharwad. This was estimated from well matured seeds of linseed. Randomly chosen samples of seeds were obtained from each line and used for fatty acid analysis. Mathematical procedures on the spectral information were carried out with WinISI II Project Manager software, version 1.50 (Infrasoft International, LLC).

DNA extraction and PCR amplification conditions: High quality total genomic DNA was isolated from the young linseed leaves by following Cetyl trimethyl ammonium bromide procedure (Ghosh *et al.*, 2009) with minor modifications. The quality and quantity of the extracted DNA was measured by using Nano Drop (UV technologies, USA). DNA concentration and purity was also checked by running the samples on 0.8% (w/v) Agarose gel with known concentration of uncut lambda DNA. The DNA samples were then diluted to 10.0ng/µl working concentration. The list of 50 SSR primers used in the present study is given in Table 3. Amplification was carried out using Master Cycler Gradient 5331 (Eppendorf, Germany) in a total volume of 20µl containing 2.0 µl of 10X Taq buffer, 2µl of 2 mM dNTP, 0.5µl of forward and reverse primer (10pM), 0.3U of Taq DNA polymerase, 50-100ng of template DNA, and 13µl of ddH_2O. The amplification profile consisted of an initial denaturation of 94°C for 5 minutes, followed by 35 cycles each one including one minute at 94° C, 1 minute for annealing at 45° -60° C (depending on the primer used) and 2 minutes for elongation at 72° C. A 5 minutes step at 72° C was programmed as a final extension. Amplification reaction products were separated and visualized on 3.5% (w/v) Agarose gel with Ethidium Bromide.

Data analysis: The mean values of genotypes were used for the analysis of variance. The significance of the differences among all the genotypes was tested by F-test using the error variance. (Rao 1952).

For SSR analysis, only intense and clearly resolved amplicons on the gels were scored in binary format by giving 0 for absence of the amplicon and 1 for presence of the amplicon. Polymorphic information content (PIC) for each marker was calculated using the Power Marker V 3.0. In order to detect the marker-trait association, multiple regression analyses were followed. The association analysis was conducted using multiple regression option of SPSS 15. The morphological traits served as dependent variables and the SSR marker data as independent variable. Markers showing significant regression values are considered as associated with the quantitative trait under consideration.

Results and discussion

The analysis of variance (Table: 1a and 1b) indicated significant differences for all the 15 characters. This study showed moderate values of PCV and GCV for plant height at maturity, days to 50 percent flowering seed yield per plant, palmitic acid and linoleic acid. The results were in accordance with Akbar *et al.* (2003) for plant height. In contrast to the present investigation, Mirza *et al.* (1996) and Singh (2001) reported high variability for plant height, whereas, Singh (2001) reported high variability for days to 50% flowering. Further, Tadesse *et al.* (2009) reported low values for plant height. Thus the presence of

Table 1a. ANOVA for yield and yield contributing traits of linseed genotypes

Source of Variation	Df	Days to 50% flowering	Days to maturity	Plant height	No. of Primary branches	No. of secondary branches	No. of capsules per plant	No. of seeds per capsule	1000 seed weight	Seed yield per plant
Block (Eliminating Checks+ Varieties)	14	0.629	0.850 *	0.479	0.110	0.316	0.157	0.091	0.129	0.067
Entries (Ignoring Blocks)	167	39.224 **	85.401 **	86.876 **	5.342 **	221.571 **	329.734 **	3.892 **	1.343 **	0.617 **
Checks	2	260.000 **	172.089 **	10.544 **	64.820 **	754.009 **	364.696 **	36.884 **	3.499 **	4.572 **
Varieties	164	36.494 **	83.140 **	67.339 **	3.135 **	70.852 **	126.737 **	3.454 **	0.361	0.339 **
Checks vs Varieties	1	45.414 **	282.828 **	3443.749 **	248.239 *	23874.69*	33551.320 **	9.807 **	158.032 **	38.227 **
Error	28	0.928	0.399	0.249	0.174	0.438	0.235	0.143	0.213	0.102
CV		3.16	6.78	8.38	5.16	4.16	4.67	4.95	6.55	6.13

Table 1b. ANOVA for nutritional traits of linseed genotypes

Source of Variation	df	PAL	STE	OLE	LIN	ALA	Oil content
Block(Eliminating Checks+Var)	14	1.026 **	1.868 **	53.326 **	16.770 **	11.47	0.463
Entries (Ignoring Blocks)	167	1.213 **	1.488 **	12.272 **	2.486	13.262 **	1.554 *
Checks	2	3.002 **	29.383 **	120.347 **	4.336	10.196 **	25.010 **
Varieties	164	1.196 **	1.138 **	10.763 *	2.478	12.060 **	1.258 *
Checks vs Varieties	1	0.352	3.114 **	43.571 *	0.121	216.505 **	3.156 *
Error	28	0.160	0.205	4.324	2.034	1.120	0.738
CV		5.29	8.61	10.15	11.73	4.93	5.15

*= Significant at 5 percent
**= Significant at 1 percent

Table 2. Genetic variability parameters for quantitative characters in linseed

Characters	Range			Variance		%				
	Min	Max	Mean	Phenotypic	Genotypic	GCV	PCV	h^2	GA	GAM %
Days to 50% flowering	34.00	58.00	44.81	30.84	29.91	12.19	12.38	97	11.10	24.73
Days to Maturity	80	114	92.41	69.99	69.59	9.02	9.05	94	17.14	23.75
Plant height (cm)	41.2	81.5	61.59	56.67	56.42	12.17	12.20	96	15.44	25.03
Number of Primary branches per plant	4.20	12.1	7.56	2.66	2.49	20.99	21.72	93	3.14	41.81
Number of Secondary branches per plant	8.20	58.2	25.49	59.66	59.22	30.71	30.83	93	15.79	63.04
Number of capsules per plant	3.9	59.00	23.06	106.63	106.39	46.02	46.08	91	11.34	70.26
No. of seeds per capsule	3.20	13.50	7.74	2.93	2.78	21.5	22.05	95	3.35	43.20
1000 seed weight (g)	5.02	9.20	6.68	0.34	0.12	5.3	8.74	37	0.44	6.64
Seed yield per plant (g)	3.42	6.70	5.00	0.30	0.20	8.96	11.02	66	0.75	15.01
Oil content (%)	30.70	45.2	40.08	1.18	0.44	1.66	2.72	37	0.83	2.09
PAL	5.97	7.9	7.48	1.03	0.87	12.36	13.45	84	1.77	23.40
STE	3.60	6.53	5.30	0.99	0.78	16.57	18.62	79	1.62	30.41
OLE	14.62	27.49	20.01	9.74	5.42	11.23	15.07	56	3.57	17.26
LIN	11.07	14.05	12.55	2.41	0.37	5.02	12.76	36	0.50	4.08
ALA	46.57	60.25	53.97	10.32	9.20	5.59	5.92	89	5.90	10.87

abundant genetic variability as indicated by the above results for the said characters provides greater scope for improvement of this character by direct selection. On the other hand, days to maturity, 1000 seed weight, oil content, and alpha linolenic acid content exhibited low variability in the present investigation. Pujar (2012) also reported low variability for oil content. However, Akbar *et al.* (2003) reported moderate values of PCV and GCV for days to maturity, Chandrashekhar *et al.* (1998) reported high PCV and GCV for 1000 seed weight. Genetic variability estimates including genotype mean, range, genotypic, phenotypic and environmental variance, PCV, GCV, heritability, genetic advance and genetic advance as per cent mean for different quantitative characters are presented in Table 2.

A total of 117 alleles were detected for the 50 microsatellite markers, out of which 111 were polymorphic (Table 3). Polymorphism percentage was 97.14. The number of alleles detected per primer pair ranged from 2 to 5 with an average 2.34. The maximum number of five amplified products were observed in the profile of the primer Lu138, followed by Lub14 and Lu273 having 4 alleles. The primers Lub11 (66.66%), Lub14, Lua47 (66.66%), Lu138 (80%), Lu146 (66.66%) and Lu273 (75%) were proved to be less polymorphic and rest were 100% polymorphic.

PIC was highest for the SSR primers Lu273 (0.81), followed by Lu236 (0.77) and NCL_26 (0.75). PIC was lowest (0.14) for the primer Lu144a, followed by Lu154 and Lua133 having 0.15 each. The mean PIC value was 0.45. The results are presented in Table 3. The higher the PIC value, the more informative is the SSR marker. Hence, primers Lu273, Lu236 and NCL_26 were found to be highly informative.

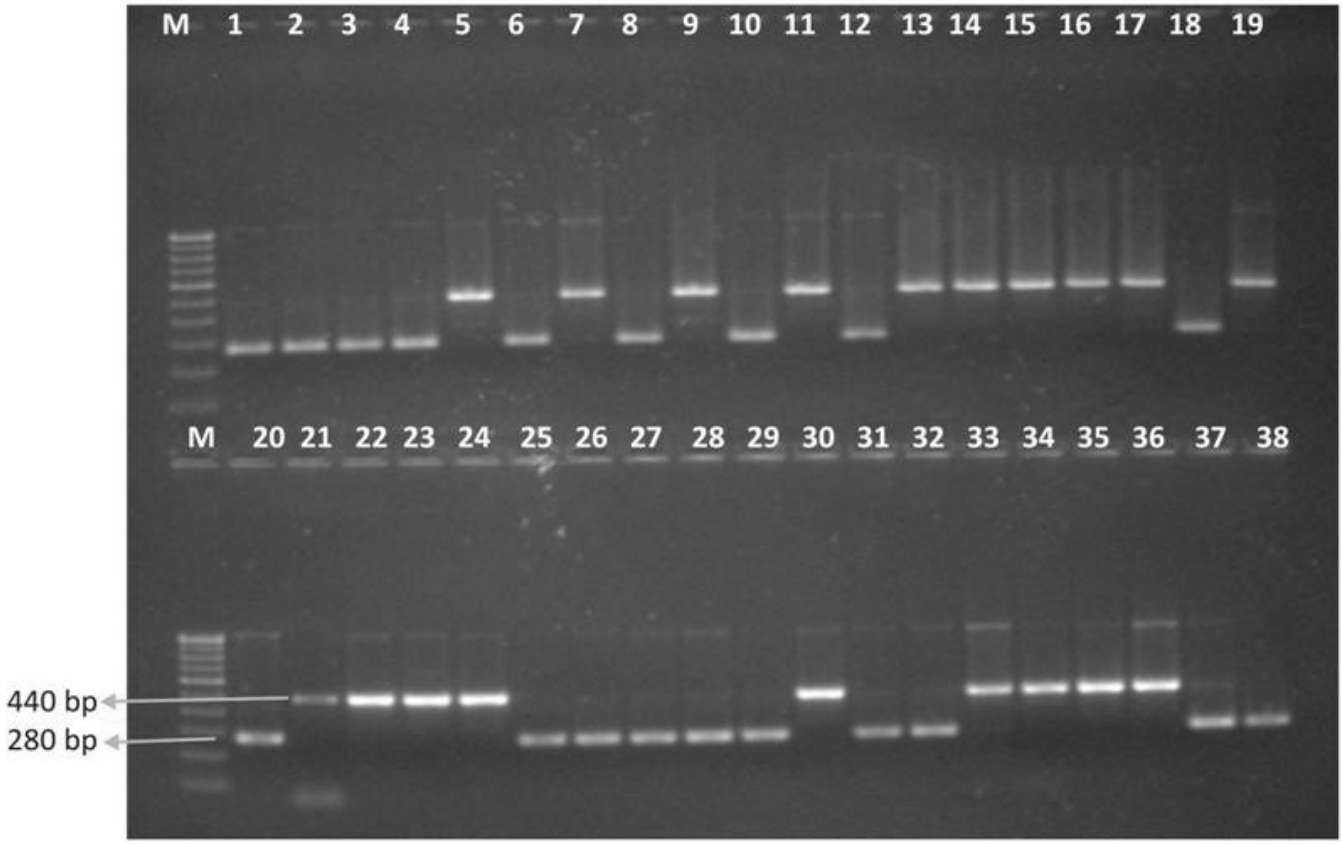
Significant regression was observed for 8 traits on a total of 21 SSR alleles of the 111 polymorphic SSR alleles. Details of multiple regression analysis are presented in Table 4. The number of markers associated with individual traits ranged from 1 to 4. Among 8 traits, plant height showed significant regression with only one SSR marker (Lua129 allele number one), while 1000 seed weight and seed yield per plant showed significant regression on 4 SSR markers. The association explained 7 to 44.2 per cent of the variation of individual traits. The highest variation was accounted by Lua129-allele number one. SSR marker Lu151 (allele number two) was linked with three traits, number of capsules per plant, 1000 seed weight and seed yield per plant.

SSR markers Lub11-2 and Lua133-2 were associated with days to maturity at 1% significant level. Number of secondary branches found to have two associated markers (NCL_4-2, Lu236-2). SSR markers Lu144a-1, Lu151-2 and Lu138-4 were associated with number of capsules per plant at 1% significance level. Similarly three SSR markers *viz.,* Lua49B (allele number one), Lua60 (allele number one) and Lua125a (allele number three) were significantly associated alpha linolenic acid content at 0.1% significance level ($P \leq 0.001$). Four SSR markers namely Lu151-2, Lua68-2, Lu138-3 and Lu143-1 were associated with 1000 seed weight. SSR markers Lu236-2, Lu144a-1, Lu151-2, and Lu143-2 were significantly ($P \leq 0.001$) associated with seed yield per plant. Lub14-4 and Lu143-1 were associated with oil content in seeds (Table: 4).

Table 3. SSR marker profile across linseed genotypes

Primer code	Number of alleles	Number of polymorphic alleles	Polymorphism (%)	PIC
Lub4	2	2	100	0.34
Lub9	2	2	100	0.47
Lub11	3	2	66.66	0.36
Lub13	2	2	100	0.35
Lub14	4	3	75	0.48
Lua25	2	2	100	0.50
Lua32	2	2	100	0.39
Lua37	2	2	100	0.30
Lua41	2	2	100	0.27
Lua46	3	3	100	0.62
Lua47	3	2	66.66	0.58
Lua49B	2	2	100	0.18
Lua52	2	2	100	0.56
Lua56	2	2	100	0.45
Lua58	2	2	100	0.59
Lua60	2	2	100	0.45
Lua61	2	2	100	0.49
Lua64	2	2	100	0.59
Lua68	2	2	100	0.19
Lua69	2	2	100	0.34
Lua81	2	2	100	0.21
Lua83B	2	2	100	0.29
Lua91	3	3	100	0.59
Lua104	2	2	100	0.48
Lua105	2	2	100	0.44
Lua106	2	2	100	0.41
Lua113	2	2	100	0.33
Lua114	2	2	100	0.48
Lua123	2	2	100	0.49
Lua125a	3	3	100	0.48
Lua128	2	2	100	0.34
Lua129	2	2	100	0.62
Lua130	2	2	100	0.22
Lua133	2	2	100	0.15
Lua137	3	3	100	0.65
Lu139	2	2	100	0.45
Lu140	2	2	100	0.36
Lu143	2	2	100	0.38
Lu144a	2	2	100	0.14
Lu146	3	2	66.66	0.63
Lu151	3	3	100	0.65
Lu154	2	2	100	0.15
Lu158	2	2	100	0.22
Lu236	3	3	100	0.77
Lu273	4	3	75	0.81
Lu442	3	3	100	0.58
NCL_3	2	2	100	0.62
NCL_4	2	2	100	0.63
NCL_26	2	2	100	0.75

Routinely, molecular markers associated with QTL/genes are being identified in crops using planned populations such as F_2, RIL and DH populations. However, this requires mapping populations, tight linkage map and lot of time to develop such planned populations. Germplasm based regression association



Primer - Lub4

Plate 2: Amplified products of primer-Lub4 on gel indicating polymorphism between linseed genotypes

M	100 bp ladder	**13**	A-459	**26**	EC-561
1	A-23-1-1	**14**	ARNY	**27**	EC-564
2	A-49	**15**	BAU-111-1	**28**	EC-569
3	A-170	**16**	BAULK	**29**	EC-589
4	A-180	**17**	BR-1	**30**	EC-1187
5	A-199	**18**	BR-14	**31**	EC-1389
6	A-210	**19**	BR-3-62	**32**	EC-1410
7	A-375	**20**	BS-2	**33**	EC-1433
8	A-385	**21**	BENGAL-23	**34**	EC-1434
9	A-388	**22**	BENGAL-62	**35**	EC-1529 B
10	A-396A	**23**	BENGAL-70	**36**	EC-9832
11	A-404	**24**	BEHAMPUR	**37**	EC-99080
12	A-449	**25**	BIJAPUR LOCAL	**38**	EC-12077 B

Supplementary information

Appendix-I. List of genotypes used in the present study for molecular analysis

Sl No.	Genotypes	Sl No.	Genotypes	Sl No.	Genotypes	Sl No.	Genotypes
1	A-23-1-1	26	EC-561	51	EC-99007	76	KANPUR LOCAL
2	A-49	27	EC-564	52	EC-99009	77	KARAM BANDA
3	A-170	28	EC-569	53	EC-99025	78	L-4
4	A-180	29	EC-589	54	EC-99029	79	L-14
5	A-199	30	EC-1187	55	EC-115178	80	L-18
6	A-210	31	EC-1389	56	ES-1534	81	L-21
7	A-375	32	EC-1410	57	ES-16381	82	L-43
8	A-385	33	EC-1433	58	EX-6-3	83	L-48
9	A-388	34	EC-1434	59	EX-131-10	84	L-53
10	A-396A	35	EC-1529 B	60	PB-3 No. 3	85	L-108
11	A-404	36	EC-9832	61	FR-11	86	LC-1044
12	A-449	37	EC-99080	62	GLC-1-2	87	LCK-152
13	A-459	38	EC-12077 B	63	GS-121	88	LCK-254
14	ARNY	39	EC-16390	64	GS-134	89	LCK-3532
15	BAU-111-1	40	EC-22583	65	GS-178	90	LCK-87312
16	BAULK	41	EC-22684	66	GS-183	91	LCK-88311
17	BR-1	42	EC-22688	67	GS-194	92	LMH-379
18	BR-14	43	EC-22848	68	GS-204	93	LS-1
19	BR-3-62	44	EC-22850	69	GS-206	94	No. 3
20	BS-2	45	EC-23592	70	GS-219	95	No. 11
21	BENGAL-23	46	EC-41561	71	GS-280	96	NO. 18 X RR 9
22	BENGAL-62	47	EC-41577	72	GS-337	97	NO-55
23	BENGAL-70	48	EC-41627	73	GS-401	98	S-36
24	BEHAMPUR	49	EC-41656	74	GUNAWAL LOCAL	99	Indira Alsi
25	BIJAPUR LOCAL	50	EC-41704	75	Gangroochi	100	NL115

method overcomes these limitations, where molecular markers for traits of interest have been identified using germplasm collections (Virk *et al.,* 1996;; Maccaferri *et al.*, 2005; Breseghello *et al.,* 2005; Skot *et al.*, 2005).

The present study involved a set of 170 genotypes, which constitute an important and diverse elite germplasm of linseed, exhibiting moderate to high genetic variability. The association analysis has been performed for the 15 morphological and biochemical traits using 111 alleles following multiple regressions. The regression analysis was capable of identifying markers which shows a strong association with morphological traits. Twenty one alleles out of 111 showed significant association with eight of the 15 morphological traits. Alpha linolenic acid content was associated with three SSR markers. Seed yield per plant found to have four associated markers.

GRC marker-trait association identification has many advantages such as this allows the detection of QTL that varies across a wide spectrum of biodiversity, QTL for any quantitative trait can be studied in the same investigation and this requires fewer inputs of time, labor and financial resources, compared to the linkage-based QTL identification. Hence the GRC marker-trait association identification will play an import role in plant MAS/QTL breeding programs, especially in orphan crops such as linseed when no other genetic information such as linkage maps are available.

Conclusion

Above association studies could provide valuable information to screen the germplasm accessions for the specific traits of interest using the linked SSR markers. However, presence of false positives and/or the use of structured populations may lead to spurious associations. As the SSR markers used in the present study are not mapped, it is difficult to study population structure at this stage. Therefore, markers identified during the present study need to be validated before using for marker assisted selection for the development of nutraceutically improved, high yielding linseed varieties.

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