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RESEARCH ARTICLE

Association mapping of total flavonoid content and antioxidant capacities in lentil (*Lens culinaris* Medik.)

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Abstract

Ninety-six lentil genotypes were characterized for total flavonoid content (TFC) and antioxidant capacities (AOC). Analysis of variance (ANOVA) indicated significant ($p \leq 0.01$) differences among lentil genotypes for studied traits across three locations. The average content of TFC was relatively higher in advanced breeding lines (12.21 mg QE/g) and low in Indian germplasm lines (10.68 mg QE/g). Mean performances for AOC, using the DPPH (ACD) and CUPRAC(ACC) methods, were relatively higher in advanced breeding lines (ACD-4.32 µmolTE/g and ACC-1.25 µmolTE/100 g) and least in released varieties (ACD-2.79 µmolTE/g and ACC-0.92 µmolTE/100 g). Marker trait association analyses were performed with 87 polymorphic SSR markers. The number of alleles amplified per locus ranged from 2 to 5, with an average of 2.92. The polymorphism information content (PIC) value ranged from 0.04 to 0.67, with a mean of 0.25. SSR markers, LCSSR 363 and PLC 38 were significantly associated with TFC and explained 10% of phenotypic variation. PLC 60, SSR 80, PLC 77, and PBALC 216, associated with ACD, explained 7.5 to 23% of phenotypic variation, whereas PBALC 250 and PBALC 216, associated with ACC, explained 15% of the phenotypic variation. TFC and AOC have a broader range of genetic variations that can be used in lentil breeding programs to develop varieties tailored for nutraceutical and therapeutic applications. The identified markers can be validated in biparental populations and used in lentil breeding programs to improve functional constituents and health benefits.

Keywords: Lentil, total flavonoid content, antioxidant capacity, CUPRAC, DPPH, association mapping.

Introduction

Lentil (*Lens culinaris* L. ssp. *culinaris*) is a wholesome diet to sustainably meet global food and nutritional security. Lentil grains area rich source of carbohydrates, proteins, dietary fibers, and vitamins (Grela et al. 2017). Besides this, lentils are naturally fortified with many minerals such as iron, zinc, and selenium. Globally lentil is grown onover 5 million hectares yielding 6.54 million tonnes (FAOSTAT 2020). Lentils provide a wide variety of macro- and micronutrients in addition to a high concentration of bioactive substances (Faris et al. 2013). Polyphenols, flavonoids, saponins, phytic acid, lectins, trypsin/protease inhibitors, and tannins are the main bioactive substances in lentils (Ganesan and Xu 2017). Bioactive compounds confer antioxidant, anti-inflammatory, anti-aging, anti-diabetic, and anti-carcinogenic properties (Alcázar-Valle et al. 2020; Dhalaria et al. 2020). *In vitro*, *in vivo*, and clinical results have demonstrated anti-oxidative, cardio-protective, anticancer, anti-obesity, and anti-diabetic properties of bioactive compounds in lentils (Ganesan and Xu 2017). Thus, long-term consumption of lentils can exert beneficial health effects and prevention against several human illnesses while providing adequate nutrition (Faris et al. 2013). While these bioactive compounds are health benefitial, they possess ambivalent properties reducing protein digestibility and/or mineral bioavailability (Acquah et al. 2021). However, there are various traditional (soaking, germinating, dehulling, milling, boiling) and emerging processing techniques (microwaving, micronization) that significantly lowers the levels of bioactive compounds (Acquah et al. 2021).

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Owing to anti-oxidative, anti-inflammatory, anti-carcinogenic, anti-atherosclerotic, and anti-mutagenic properties (Ciumărnean et al. 2020; Panche et al. 2016), flavonoids are a vital component in pharmaceutical and neutraceutical industries. According to Amarowicz and Pegg (2008), flavonoids are one of legumes' most prevalent phenolic chemicals, particularly lentils (Alshikh et al. 2015; Zhang et al. 2015). Nowadays, food-based nutraceuticals and medicinal compounds is picking up steam.

Accumulation of bioactive compounds in seed and seed coats is a quantitatively-inherited complex trait significantly influenced by environmental factors (García-Díaz et al. 2018). QTL mapping and association mapping (AM) are complementary strategies for dissecting complex traits into component loci (Xu et al. 2017). QTL mapping relies on recombination during the development of the biparental mapping population, whereas AM takes advantage of historic recombinations and mutations accumulated in the population over generations to decipher significant associations between molecular markers/QTL and traits of interest. AM is resource-effective and offers comparatively higher mapping resolution than the conventional QTL mapping approach (Ibrahim et al. 2020; Shi et al. 2017). The inclusion of diverse genotypes in an association mapping panel allows for surveying multiple alleles and allele mining.

SSR markers featuring multi-allelic nature are powerful alternate tools to SNPs in gene/QTL mapping (Chen et al. 2021). SSRs are the preferred marker for conducting association mapping in crop species with large genome sizes (Zhao et al. 2017). With this background, the present study was undertaken to characterize 96 diverse lentil genotypes for total flavonoid content (TFC) and antioxidant capacities (AOC) and identify associated genomic- and EST-SSRs with these traits through association mapping approach.

Materials and methods

Genetic material and field experimentation

Ninety-six diverse lentil accessions were investigated for total flavonoid content (TFC) and antioxidant capacities (AOC). Genetic material included exotic (Mediterranean landraces and germplasm lines from International Center for Agricultural Research in the Dry Areas) and Indian (germplasm lines, lentil varieties released for commercial cultivation, advanced breeding lines developed at various lentil breeding centres) lentil lines (Table 1). The experiment was carried out at three geographical sites: (i) Delhi (North-Western Plain Zone; 28° 38′23′′N, 77° 09′27′′E, 228 meters above mean sea level [masl]), (ii) Sagar (Central Zone; 30.9°N, 75.85°E, 244 masl) and (iii) Sehore (Central Zone; 23.06° N, 77.05° E, 498.77 masl)in randomized block design with two replications (3 rows of 5 m length) per entry. Distance maintained between plants and between rows was 5 x 25 cm. Recommended package of practice was followed to procure healthy grains. A working sample of 15 g was used for further extraction and analysis.

Table 1. A list of genotypes used in the study

Origin	Category of genotypes	Source	Genotype	Number of genotypes
India	Indian germplasm lines	NBPGR, New Delhi	IC 201704, IC 208326, IC 262839, IC 267663, IC 268248, IC 560135, IC 560169, IC 560181, IC 560206, IC 560212, IC 560333, IC 560372, IC 560812	13
	Advanced breeding lines	AICRP MULLaRP, IIPR, Kanpur	L 11-243, L 11-273, L 11-279, L 11-282, L 11-289, L 11-291, L 11-294, L 11-297	08
		CCS,HAU, Hisar	LH 90-57	01
		GBPUAT, Pantnagar	PL 117, PL 24, PL 77-12, PL 97	04
		IARI, New Delhi	L 5253, L 7818, L 7903, L 7916, L 7920, LC 282-1444, LC 282-1485, LC 282896, LC 282907, L 4076s, LC 300-15, LC 300-16, LC 300-17, LC 300-19	14
		PAU, Ludhiana	LL 1122, LL 147, LL 461, LL 649	04
	Released variety	IARI, New Delhi	L 4076	01
		GBPUAT, Pantnagar	PL 02, PL 04, PL 05, PL 06, PL 07, PL 08, PL 406	07
Exotic	Mediterranean landraces	ICARDA, Aleppo, Syria	IG 111996, IG 112078, IG 112128, IG 112131, IG 115, IG 129214, IG 129291, IG 129302, IG 129304, IG 129317, IG 130033, IG 195, IG 49, IG 5320, IG 569608, IG 70230, IG 73798, IG 73920, IG 73933, IG 9, ILL 10832, ILL 108331, ILL 147, ILL 2581, ILL 7663	25
	ICARDA Nursery selection	ICARDA, Aleppo, Syria	P 13108, P 13129, P 13135, P 13138, P 13142, P 13143, P 15104, P 15121, P 15127, P 16214, P 2113, P 2116, P 2118, P 2125, P 2127, P 3233, P 3234, P 8112, P 8115	19

Extract preparation for phenotyping

Grains were dried and ground into fine powder using an electric grinder. Five grams of powdered samples were percolated with n-hexane for 8 h to remove fatty substances. Two grams of the de-fatted sample of each genotype was extracted using 30 mL of 80% ethanol for 3 h at room temperature. Extracts were collected after centrifugation at 3000 rpm for 15 min and stored at 5°C for analysis.

Determination of total flavonoid content (TFC)

TFC of the sample extract was estimated following aluminum chloride colorimetric assay according to Patel et al. (2010). The absorbance value of the sample and blank was measured at 510 nm. TFC was expressed as mg quercetin equivalents per gram of dry mass (mg QE/ g).

Antioxidant capacity (AOC) measurement

Antioxidant determination was done using two methods: Antioxidant capacity using 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay (ACD) and antioxidant capacity using cupric reducing antioxidant capacity (CUPRAC) method (ACC).

Antioxidant capacities of lentil extracts were gauged using DPPH free radical scavenging assay according to Brand-Williams et al. (1995). Free radical scavenging activity of samples was expressed as IC_{50}(50% decrease in absorbance by DPPH radical compared to control) calculated as % inhibition of DPPH. Lower IC_{50} values indicate higher DPPH radical scavenging activity (Maisuthisakul et al. 2007). The percentage of scavenged DPPH was calculated as:

$$\%DPPHsc = \{(A_{cont} - A_{samp})/A_{cont}\} \times 100$$

where, A_{cont} is absorbance of the control, A_{samp} is absorbance of sample.

Results were expressed as Trolox equivalent per gram of the sample (µmol TE/g).

Antioxidant capacity using CUPRAC method was estimated as per Apak et al. (2008). The absorbance of the blank and sample was read at 450 nm following incubation for half an hour at room temperature. Ascorbic acid was used as a reference for the estimation of antioxidants in the samples. Results were expressed as Trolox equivalent per 100 grams of the sample (µmol TE/100g).

Genomic DNA isolation and amplification

DNA has been extracted from twenty days old seedlings following the cetyl trimethyl ammonium bromide (CTAB) method as per Murray and Thompson (1980). A total of 299 EST-SSRs and 86 genomic SSRs were used for genotyping 96 diverse lentil accessions. A total of 87 SSR markers (62 EST-SSRs and 25 genomic SSRs) exhibiting polymorphism were selected for association mapping studies.

Genetic diversity analysis and population structure

All 96 genotypes were scored for the presence/absence of amplified bands. Informativeness of SSR markers, based on polymorphism information content, was determined using the formula: PIC = 1-ΣPi –ΣΣPiPj, where I being the total number of alleles generated for a given SSR marker, Pi being the frequency of allele 'i' obtained in the panel of ninety-six genotypes and j being i+1(Botstein et al. 1980).

The number of subgroups present within the population was deduced by the STRUCTURE 2.3.4 program, assuming k values from 1 to 10 using the Bayesian clustering approach (Pritchard et al. 2000). The optimum *k* value was estimated by plotting the Ln P (D) value against the given *k* value using the software Structure harvester v 6.92. The highest plateau at delta *k* = 3 inferred number of sub-populations to be three for further analysis.

Marker-trait association (MTA) analysis

Association analysis between TFC, AOC, and 87 polymorphic SSRs was established using Trait Analysis by association, Evolution, and Linkage (TASSEL) ver 3.01. Linkage disequilibrium (LD) between each pair of polymorphic loci was determined by calculating the square of the correlation coefficient (r^2). General Linear Model (GLM) and the Q matrix were employed to check spurious associations. Manhattan plots have illustrated markers significantly associated with the traits.

The mean value reported for all the determinations was assayed in triplicate for each sample. The significance of differences between the mean values ($p<0.05$) was tested using one-way ANOVA and F-test.

Results

Descriptive statistics of ninety-six lentil genotypes are presented in Table 2. Analysis of variance (ANOVA) indicated a significant ($p \leq 0.01$) difference among lentil genotypes for TFC and AOA across locations. Pooled ANOVA indicated that genotype x environment interaction was also significant ($p<0.001$) for these traits, which allowed marker-trait association analyses to be investigated separately for each location/environment. Mean performances for TFC were relatively higher in advanced breeding lines (12.21 mg QE/g) and low in Indian germplasm lines (10.68 mg QE/g) (Table 2). Mean performances for AOC, using the DPPH (ACD) and CUPRAC (ACC) methods, were relatively higher in advanced breeding lines (ACD-4.32 µmol TE/g and ACC-1.25 µmol TE/100g) and least in released varieties (ACD-2.79 µmol TE/g and ACC-0.92 µmol TE/100g) (Table 2). Genotypes exhibiting high AOC were: L 11-282 (10.04 µmole TE/g), P 3233 (9.46 µmole TE/g), P 2125 (9.15 µmole TE/g), LC 74151 (8.14 µmole TE/g) and LC 300-17 (8.13 µmole TE/g) using DPPH method while P 3233 (2.31 µmole TE/100g), L 11-282 (2.27 µmole TE/100g), LC 74151 (2.19 µmole TE/100g), LC 300-17 (1.91 µmole TE/100g), P 2125 (1.89 µmole TE/100g) using CUPRAC method. Genotypes exhibiting high antioxidant capacity using DPPH method were also identified as having high AOC with CUPRAC method, though with a difference in

Table 2. Descriptive statistics of ninety-six lentil genotypes for TFC, ACD, and ACC across three locations

Variable	Type	Mean	SE	SD	Min	Max
TFC (mg QE/g)	IG	10.68	1.23	4.45	2.97	15.37
	ABL	12.21	0.86	4.48	3.52	20.27
	RV	11.81	1.24	4.32	4.62	17.26
	EL	12.13	0.79	5.26	2.65	23.48
	Combined	11.91	0.49	4.79	2.65	23.48
ACD (µmol TE/g)	IG	3.23	0.29	1.05	1.67	4.97
	ABL	4.32	0.42	2.20	1.22	10.04
	RV	2.79	0.22	0.77	1.49	3.6
	EL	3.82	0.29	1.93	0.46	9.46
	Combined	3.75	0.19	1.87	0.46	10.04
ACC (µmol TE/100 g)	IG	0.99	0.08	0.30	0.54	1.51
	ABL	1.25	0.09	0.47	0.38	2.27
	RV	0.92	0.11	0.39	0.53	1.74
	EL	1.18	0.07	0.45	0.36	2.31
	Combined	1.14	0.04	0.44	0.36	2.31

IG = Indian germplasm, ABL = Advances breeding lines, RV = Released varieties, EL = Exotic lines

their ranking. Mean performances for TFC and AOC across locations were relatively higher in advanced breeding lines, while mean performance for TFC was low in Indian germplasm lines and for antioxidant capacities, released varieties exhibited the least mean values (Table 2).

Analysis of genetic diversity

Polymorphism information content (PIC) and number of alleles are genetic diversity parameters that indicate level of diversity among sampled genotypes. The PIC value indicates the worth of markers for linkage analysis, germplasm characterization, genotypic discrimination, and gene tagging. Resolving power (R_p) signifies the discriminatory power of a marker. The size of PCR products ranged from 109 to 389 basepairs exhibiting a high level of genetic diversity among studied genotypes. A total of 87 polymorphic SSRs detected 254 alleles. The number of alleles (N_a) amplified per locus varied from 2 to 5 (Table 3). The average number of alleles amplified per locus was 2.92. The highest N_a (5) was amplified with SSRs PLC 104, PBALC 92, PBALC 273, PBALC 373, GLLC 614, SSR 19, and SSR 156. The set of SSR markers generated informative loci having PIC values ranging from 0.04 (PLC 17, PBALC 90, PBALC 652) to 0.67 (PBALC 273), with a mean of 0.25 (Table 3). Out of 87, 30 polymorphic SSRs had PIC values >0.29. Genomic SSRs generated a higher average number of alleles (3.04) as well as a greater PIC value (0.269) compared to EST-SSRs (average N_a-2.87, PIC value-2.38). SSR markers' resolving power (Rp) varied from 2 to 3.17 with a mean value of 2.18 (Table 3).

Linkage disequilibrium (LD) and marker-trait association analysis

Phenotypic variations for TFC and AOC among 96 genotypes were used to perform association analyses using eighty-seven SSR markers. The LD patterns of total 3552 pairwise combinations of 87 SSR markers were assessed using the software TASSEL. The LD based on the squared allele frequency correlations (r^2 value) ranged from 0.0 to 0.47. The present study employed GLM with the Q model to decipher associated markers with TFC and AOC. A total of eight markers (two each for TFC, ACC, and four for ACD) were identified to be significantly associated (-log10 *p* value >2) with studied traits (table 4). Six markers out of eight exhibited consistent association across all three locations. The SSRs LCSSR 363 and PLC 38, associated with TFC accounted for nearly 10% of phenotypic variation. PLC 60, SSR 80, PLC 77, and PBALC 216, associated with ACD accounted for 7.5, 10.8, 21.5, and 23% of phenotypic variation, respectively. PBALC 250 and PBALC 216 were associated with ACC explaining 15% of the phenotypic variation. For TFC, SSR markers LCSSR 363 and PLC 38, and for ACC, SSR markers PBALC 250 and PBALC 216 were consistently identified in all three datasets. Similarly, for ACD, SSR markers PLC 77 and PBALC 216 were consistently identified in all three datasets, while PLC 60 (Sagar) and SSR 80 (Delhi) were identified in a single dataset (Table 4). Manhattan plots have been used to display environment-wise SSRs that are significantly (-log10 *p* value >2) linked with the attributes (Figs. 1 to 3).

Discussion

In the current study, we investigated genetic variation for TFC and AOC in grains of Indian and exotic lentil accessions. Wide variance for the traits under investigation shows that

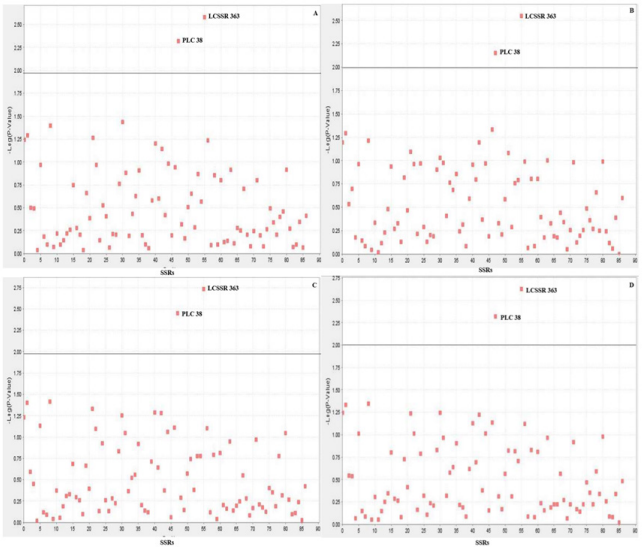

Fig. 1. Manhattan plot depicting association of SSR markers with total flavonoid content at different locations. A. Delhi, B. Sagar, C. Sehore and D. Combined of A, B and C

Table 3. Determination of polymorphism information content (PIC) values, number of alleles (N_a) amplified, and resolving power (R_p)

S. No	Primer	T_a°C	N_a	PIC	R_p	S. No	Primer	T_a°C	N_a	PIC	R_p
1	PLC 5	60	3	0.14	3.08	45	PBALC 317	56	2	0.19	2
2	PLC 10	59	4	0.13	2.17	46	PBALC 327	55	2	0.10	2
3	PLC 16	59	2	0.06	2	47	PBALC 333	54	4	0.06	2.27
4	PLC 17	59	3	0.04	2.04	48	PBALC 353	59	3	0.11	2.21
5	PLC 21	58	2	0.22	2.02	49	PBALC 364	59	2	0.29	2
6	PLC 30	61	3	0.24	2.27	50	PBALC 373	59	5	0.46	2.1
7	PLC 38	59	2	0.44	2	51	PBALC 376	55	4	0.34	2.21
8	PLC 40	60	2	0.13	2.19	52	PBALC 377	55	2	0.25	2
9	PLC 46	60	3	0.12	3.17	53	PBALC 383	55	3	0.13	2.12
10	PLC 60	60	2	0.15	2	54	PBALC 404	56	4	0.53	2
11	PLC 62	58	2	0.06	2	55	PBALC 408	56	2	0.46	2.01
12	PLC 63	60	3	0.09	2.31	56	PBALC 652	55	3	0.04	2.31
13	PLC 74	61	2	0.17	2	57	PBALC 742	55	3	0.37	2
14	PLC 77	56	4	0.17	2.94	58	PBALC 1351	56	3	0.37	2
15	PLC 80	58	4	0.25	2.17	59	PBALC 1375	56	4	0.41	2
16	PLC 81	60	3	0.13	2.27	60	PBALC 1526	56	3	0.28	2
17	PLC 83	60	2	0.12	2	61	PBALC 1551	56	4	0.48	2.01
18	PLC 100	60	4	0.61	2.46	62	PBALC 1698	56	3	0.32	2
19	PLC 104	59	5	0.29	2.48	63	GLLC 106	56	4	0.22	2.29
20	PBALC 2	59	2	0.10	2	64	GLLC 108	56	3	0.26	2.27
21	PBALC 10		2	0.30	2	65	GLLC 511	56	2	0.08	2.02
22	PBALC 13	60	2	0.30	2.02	66	GLLC 527	56	3	0.46	2.04
23	PBALC 18	60	2	0.09	2.25	67	GLLC 548	56	3	0.12	2.71
24	PBALC 21	60	2	0.32	2	68	GLLC 559	56	4	0.26	2.1
25	PBALC 25	60	2	0.29	2.02	69	GLLC 562	55	4	0.30	2
26	PBALC 90	58	4	0.04	2.33	70	GLLC 607	56	2	0.21	2
27	PBALC 92	58	5	0.42	2.06	71	GLLC 614	55	5	0.53	2.04
28	PBALC 117	55	3	0.51	2.02	72	SSR 19	58	5	0.51	2.15
29	PBALC 118	54	2	0.33	2	73	SSR 33	56	4	0.39	2.04
30	PBALC 205	59	2	0.15	2	74	SSR 80	56	2	0.18	2.02
31	PBALC 207	59	2	0.12	2.62	75	SSR 132 RN	52	3	0.19	2.1
32	PBALC 209	55	2	0.32	2	76	SSR 156	53	5	0.46	2.75
33	PBALC 216	60	3	0.10	2.02	77	SSR 212-1	50	3	0.53	2.21
34	PBALC 218	58	3	0.32	2.06	78	SSR 233	52	4	0.15	3.06
35	PBALC 219	59	2	0.23	2	79	SSR 317-1	53	2	0.19	2.54
36	PBALC 221	59	2	0.15	2	80	LCSSR 153	56	2	0.40	2
37	PBALC 222	59	3	0.06	2.02	81	LCSSR 161	58	3	0.48	2.12
38	PBALC 250	60	3	0.39	2.67	82	LCSSR 164	56	3	0.22	2
39	PBALC 254	60	3	0.13	2.1	83	LCSSR 166	56	2	0.23	2
40	PBALC 260	60	3	0.18	2.21	84	LCSSR 226	58	2	0.11	2.12
41	PBALC 265	59	4	0.20	2.65	85	LCSSR 363	58	2	0.14	2.62
42	PBALC 273	56	5	0.67	2	86	LCSSR 371	56	2	0.06	2
43	PBALC 278	56	2	0.12	2.65	87	LCSSR 386	58	2	0.06	2
44	PBALC 301	56	2	0.17	2						

Table 4. A list of significantly associated markers with TFC, ACC, and ACD

SSR Marker	Location	p-value	R^2 value	SSR Marker	Location	p-value	R^2 value
A. Total Flavonoid Content							
	Delhi	0.0026	9.22		Sagar	0.00096	16.11
	Sagar	0.0028	9.08		Sehore	0.0018	14.90
	Sehore	0.0019	9.84		All locations	0.00170	15.08
LCSSR 363	All locations	0.0024	9.40	C. ACD			
	Delhi	0.0048	10.85	SSR 80	Delhi	0.0048	10.83
	Sagar	0.0071	10.11	PLC 60	Sagar	0.0069	7.50
	Sehore	0.0036	11.41		Delhi	0.00178	20.69
PLC 38	All locations	0.0047	10.84		Sagar	0.00086	22.18
B. ACC					Sehore	0.00131	21.33
	Delhi	0.00214	14.64	PLC 77	All locations	0.00122	21.47
	Sagar	0.00449	13.18		Delhi	0.00002	23.45
	Sehore	0.0038	13.48		Sagar	0.00001	24.42
PBALC 216	All locations	0.0025	14.28				
PBALC 250	Delhi	0.00181	14.97	PBALC 216	All locations	0.00002	23.12

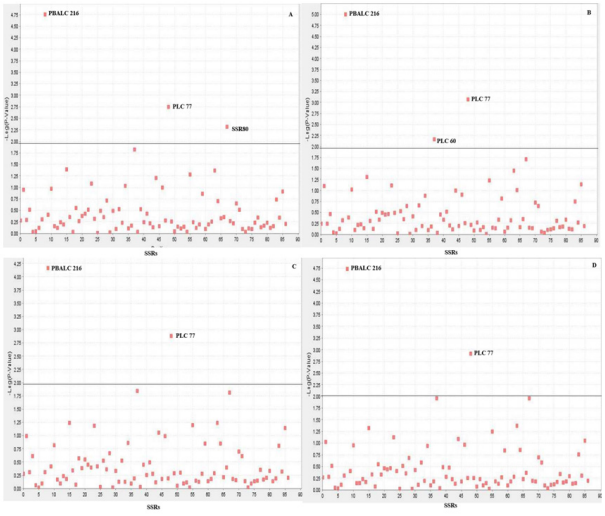

Fig. 2. Manhattan plot depicting association of SSR markers with ACD (DPPH) at different locations, A. Delhi, B. Sagar, C. Sehore and D. Combined of A, B and C

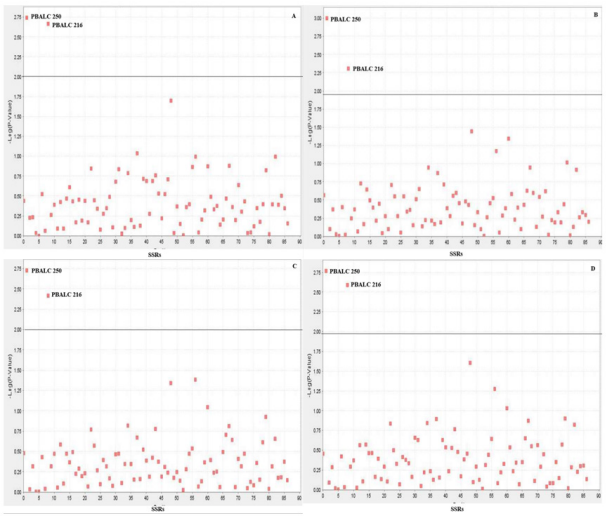

Fig. 3. Manhattan plot depicting association of SSR markers with ACC (CUPRAC) at different locations, A. Delhi, B. Sagar, C. Sehore and D. Combined of A, B and C

the interaction of genotype and environment influences the qualities. Gupta et al. (2018) reported TFC ranging between 1.89 to 22.93 mg QE/g and antioxidant capacity between 2.78 to 13.47 µmole TE/g using ferric ion-reducing antioxidant power assay in exotic lentil lines. Alghamdi et al. (2014) assessed 35 advanced breeding lines of lentils and reported that TFC varied from 4.12 to 8.92 mg QE/g, and DPPH activity varied between 10.61 to 23.26 µg/g. Irakli et al. (2021) evaluated phytochemical contents and antioxidant activities from extracts of five lentil cultivars grown in different locations. TFC ranged from 4.65 to 5.31 mg catechin equivalents/g, while DPPH ranged between 11.19 to 13.05 mgTE/g. The wide range reported in this study could be attributed to diverse genotypes, larger sample size analyzed and different extraction and determination methods.

There are two main mechanisms of antioxidant assay: hydrogen atom transfer (HAT) and electron transfer (ET). In the present study, DPPH radical scavenging and CUPRAC assays were used to estimate the antioxidant activities of lentil extracts. Of the two mechanisms, DPPH follows a mix of HAT and ET, whereas CUPRAC is based on the ET mechanism (Apak et al. 2016).

The average number of alleles detected per locus was 2.92, which are in accordance with previous studies on lentil (Verma et al. 2014; Dikshit et al. 2015; Singh et al. 2017). The polymorphism information content (PIC) value measures the informativeness of molecular markers. PIC considers the relative frequency of alleles offering a more precise assessment of diversity than the raw number of alleles (Peng and Lapitan 2005). The PIC value ranged from 0.04 (PLC 17,

PBALC 90, PBALC 652) to 0.67 (PBALC 273). The PIC value reported earlier (Andeden et al. 2015; Dikshit et al. 2015; Singh et al. 2017) also varied from 0.07 to 0.89, 0.05 to 0.77, and 0.08 to 0.68, respectively. Markers with a PIC value of more than 0.5 are high, from 0.25 to 0.5 are moderate, and less than 0.25 are considered slightly informative (Botstein et al. 1980). The average PIC value of 0.25 reported in this study indicates moderate genetic diversity for the lentil panel evaluated. Owing to the high PIC value, SSR marker PBALC 273 was the most appropriate for assessing genetic diversity in lentils. The result indicated genomic SSRs to be more polymorphic, producing a higher average number of alleles and PIC than EST-SSRs. Genomic SSRs belonging to non-transcribed genomic regions exhibit higher polymorphisms and abundance, while EST-SSRs are highly transferable across species with consistent amplification efficiency (Xia et al. 2016; Parthiban et al. 2018). Deployment of both genomic and EST-SSRs provides better genome coverage facilitating marker-aided selection.

Environmental conditions substantially impact the bioactive chemical accumulation in grains, controlled by QTL. QTL mapping and association mapping are used to analyze the genetic architecture of traits with complex inheritance. In addition to many other features, association mapping for total flavonoid concentration and antioxidant activity has been carried out in rice (Shao et al. 2011), barley (Han et al. 2018), and sorghum (Habyarimana et al. 2019). In this study, association mapping was done on 96 different lentil genotypes from the Mediterranean and Indian subcontinent. A diverse panel of lentil genotypes has previously been utilized for association mapping of grain iron and zinc concentration (Singh et al. 2017), agronomic traits (Kumar et al. 2018), grain diameter and weight (Singh et al. 2019) using SSR markers. Eight SSR markers have been reported to be significantly associated with the studied traits. The proportion of total phenotypic variance of individual traits explained ranged from 7.5% for PLC 60 to 24% for PBALC 216; both the markers were found to be associated with ACD. Earlier Kumar et al. (2018), following MLM, reported 24 SSR markers associated with nine agronomic traits in lentils, explaining 7.3 to 25.8% phenotypic variation. SSR markers LCSSR 363 and PLC 38 were associated with TFC explaining nearly 10% of phenotypic variation. PLC 60, SSR 80, PLC 77, and PBALC 216 were associated with ACD and explained 7.5 to 23% of phenotypic variation for the trait, whereas PBALC 250 and PBALC 216 were associated with ACC explaining 15% of the phenotypic variation.

To conclude, wide genetic variations observed for TFC and AOC in lentils can be utilized in lentil breeding programs for manipulating total flavonoid content and antioxidant capacity. Genotypes having high (IG 195, P 3233, IG 5320, L 11-282) and low mean TFC (P 3234, P 13108, IC 560206, IC 208326, L 7920) can be further investigated to gain insight into biochemical pathways involved. High AOC genotypes (P 3233, L 11-282, LC 74151, LC 300-17, P 2125) can be hybridized with high-yielding lines for incorporating the trait. Lentil cultivars suitable for nutraceutical purposes may be developed using advanced breeding lines with greater TFC and AOC. SSR markers identified in the present study can be validated and used in lentil breeding programs to enhance the nutritional value and health benefits.

Authors' contributions

Conceptualization (HKD, SG); Designing of the experiments (HKD, SG); Contribution of experimental materials (HKD, GPM, MA); Execution of field/lab experiments and data collection (SG); Analysis of data and interpretation (SG, MA); Preparation of the manuscript (SG, HKD, GPM). All authors have read and approved the content of the manuscript.

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