Journal of Genetics (2019) 98:66
https://doi.org/10.1007/s12041-019-1112-3

RESEARCH ARTICLE

Genetic dissection of grain iron and zinc concentrations in lentil (*Lens culinaris* Medik.)

HARISH KUMAR[1,2], AKANKSHA SINGH[1,3], H. K. DIKSHIT[1]*, GYAN P. MISHRA[1], M. ASKI[1], MAHESH C. MEENA[4] and SHIV KUMAR[5]

[1] *Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi 110 012, India*
[2] *Punjab Agriculture University, RRS, Faridkot, Punjab 151 203, India*
[3] *Amity Institute of Organic Agriculture, Amity University, Noida 201 303, India*
[4] *Division of Soil Science and Agricultural Chemistry, ICAR-Indian Agricultural Research Institute, New Delhi 110 012, India*
[5] *Biodiversity and Integrated Gene Management Program, International Centre for Agricultural Research in the Dry Areas, Rabat 10106, Morocco*
*For correspondence. E-mail: harshgeneticsiari@gmail.com.

Received 17 May 2018; revised 16 March 2019; accepted 1 April 2019; published online 28 June 2019

Abstract. Iron (Fe) and zinc (Zn) deficiencies are wide spread in South Asia and Africa. Biofortification of food crops is a viable means of addressing micronutrient deficiencies. Lentil is an important pulse crop that provides affordable source of proteins, minerals, fibre and carbohydrates for micronutrient deficient countries. An association mapping (AM) panel of 96 diverse lentil genotypes from India and Mediterranean region was evaluated for three seasons and genotyped using 80 polymorphic simple-sequence repeat (SSR) markers for identification of the markers associated with grain Fe and Zn concentrations. A Bayesian model based clustering identified five subpopulations, adequately explaining the genetic structure of the AM panel. The linkage disequilibrium (LD) analysis using mixed linear model (MLM) identified two SSR markers, GLLC 106 and GLLC 108, associated with grain Fe concentration explaining 17% and 6% phenotypic variation, respectively and three SSR markers (PBALC 364, PBALC 92 and GLLC592) associated with grain Zn concentration, explaining 6%, 8% and 13% phenotypic variation, respectively. The identified SSRs exhibited consistent performance across three seasons and have potential for utilization in lentil molecular breeding programme.

Keywords. association mapping; biofortification; DNA markers; lentil; population structure; mixed linear model.

Introduction

Lentil (*Lens culinaris* sub sp. *culinaris*) is an annual cool season grain legume with an estimated haploid genome size of 4063 Mbp (Arumuganathan and Earle 1991). This crop is important for both human and animal health (Sarker and Kumar 2011), as its grains are important dietary source of proteins, minerals, fibre and carbohydrates (Kumar *et al.* 2015). Lentil grains are rich in iron (Fe), selenium (Se), copper (Cu), manganese (Mn) and other dietary nutrients (Grusak 2009). This crop is highly recommended for consumption for the people suffering from diabetes, cardiovascular diseases and obesity (Srivastava and Vasishtha 2012). Lentil is grown in South Asia, North America, west Asia and North Africa with a current global production of 4.83 metric tons from 4.52 mha area (FAOSTAT 2017, http://www.fao.org/faostat/en/#data/QC).

Grain Fe and Zn concentration is a complex quantitative trait influenced by environmental conditions (Diapari *et al.* 2015; Aldemir *et al.* 2017). Traditional quantitative trait locus (QTL) mapping and association mapping (AM)

Electronic supplementary material: The online version of this article (https://doi.org/10.1007/s12041-019-1112-3) contains supplementary material, which is available to authorized users.

are being utilized for dissection of complex traits exhibiting quantitative inheritance. AM also known as linkage disequilibrium (LD) mapping involves larger genetic variation with likelihood for higher resolution mapping. AM eliminates the main drawback of traditional QTL mapping, low resolution and simultaneous evaluation of only a few alleles. Additionally, it allows the exploitation of those recombination events that had taken place through multiple generations in any natural populations. The AM originated in human genetics, but now it is being widely utilized for the mapping of various traits, including grain quality parameters in different crops such as *Brassica* (Körber *et al.* 2016), rice (Anandan *et al.* 2016), and pearl millet (Anuradha *et al.* 2017). On lentil, two reports (Khazaei *et al.* 2017; Singh *et al.* 2017) on AM for grain Fe and Zn have been published. Khazaei *et al.* (2017) reported two SNP markers linked to grain Fe and Zn concentration and Singh *et al.* (2017) identified three SSRs (PBALC 13, PBALC 206, and GLLC 563) associated with grain Fe concentration and four SSRs (PBALC 353, SSR 317-1, PLC 62 and PBALC 217) associated with grain Zn concentration. SSR markers detect greater level of LD in comparison to SNP and are more efficient over SNP for tracking population structure (Flint-Garcia *et al.* 2003). The study by Singh *et al.* (2017) was based on general linear model incorporating population structure which can result in spurious marker-trait association (MTA).

The present study is based on mixed linear model (MLM) which can account for multiple level of relatedness simultaneously with improved control of both type I and type II error rates (Yu *et al.* 2005). The objectives of this study were (i) to measure the variation for Fe and Zn concentrations in a set of 96 diverse Indian and Mediterranean lentil genotypes, (ii) to study the genetic diversity and population structure using both genomic and EST-SSR markers, and (iii) to analyse the association of SSR markers with natural variations for grain Fe and Zn concentrations in AM panel.

Materials and methods

Plant materials and phenotyping

The AM panel consisted of 96 diverse lentil genotypes including advanced breeding lines and released varieties from India and Mediterranean landraces, and global collection of lentil conserved at ICARDA GenBank (table 1). The AM panel of 96 accessions (48 having high grain Fe (>75 mg/kg grain) and grain Zn (>60 mg/kg grain) concentration and 48 having low grain Fe (<60 mg/kg grain) and grain Zn (<50 mg/kg grain) concentration), was constituted from a set of 195 accessions which was previously assessed for its Fe and Zn concentration, by growing them at Indian Agricultural Research Institute, New Delhi, India (28°63′24′°N, 77°15′14′°E, 218 m amsl). The evaluation of panel genotypes was also done over three seasons (2011–12 to 2013–14) at same location (Indian Agricultural Research Institute, New Delhi, India). The panel was raised in a randomized complete block design with two replication during the winter season for three consecutive years (2011–12, 2012–13 and 2013–14). The plot size was three rows of 4 m with 5 cm spacing between plants and 30 cm between rows. Standard agronomic practices were followed for successful crop cultivation (Kumar *et al.* 2018).

A total number of 10 representative plants were selected from each test genotype. Pods were harvested and threshed avoiding the contamination with dust and metal particles. The collected seeds were washed with double distilled water and oven dried at 60°C for 18h before grinding. The grounded powder of each genotype were kept overnight in an oven at 60°C. Seed powder (0.5 gm) was digested with appropriate blanks following the modified diacid protocol (Singh *et al.* 2005) in a microwave digestion system (Multiwave ECO, Anton Paar, les Ulis, France). Grain Fe and Zn concentrations (in mg/kg) were estimated for each genotype in two replications for all the three years (2011–12 to 2013–14), using an automatic sampling protocol in ICP-MS system (Perkin Elmer, model: NexION 300 ICP-MS, USA). Summary statistics, including mean, range, standard deviation, variance and coefficient of variation were estimated using SPSS v14.0 (http://www.ibm.com/software/analytics/spss). The variance components were analysed to find the effect of genotypes, and genotypes × year interactions for both Fe and Zn concentrations, individually. Probability was accepted at $P \leq 0.01$ and $P \leq 0.05$ levels.

SSR genotyping

Young leaves from each of the studied accessions were collected and stored at –20°C. The total genomic DNA was extracted using CTAB extraction procedure (Murray and Thompson 1980) following method reported by Singh *et al.* (2017). The isolated DNA was quantified on NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific) and its quality was checked by running on 0.8% agarose gel. The DNA of all the samples were diluted to a uniform concentration of 20 ng/μL for further utilization in PCR reactions.

A total of 309 DNA markers, including 245 EST-SSRs (Kaur *et al.* 2011; Jain *et al.* 2013) and 64 genomic SSRs (Hamwieh *et al.* 2005, 2009; Saha *et al.* 2010), were surveyed for genetic polymorphism by using protocol described by Singh *et al.* (2017). The EST-SSR and genomic SSR primers were synthesized from the Eurofins Genomics, India (table 1 in electronic supplementary at http://www.ias.ac.in/jgenet) and the PCR reagents were procured from Sigma-Aldrich, St Louis, USA. Three percent Metaphor agarose gel (Lonza, Rockland, USA) was

Table 1. List of 96 diverse lentil genotypes, collection sources and grain Fe and Zn concentration.

	Genotype	Variety/breeding line/exotic collection	Collection source	Iron (mg/kg) (mean over environments)	Superiority over check L4147 (%)	Zinc (mg/kg) (mean over environments)	Superiority over check L4147 (%)
1	Precoz	Variety	ICARDA, Allepo	55.53	−33.86	67.02	−5.71
2	L4076	Variety	IARI, New Delhi	86.01	2.46	69.07	−2.82
3	L4147	Variety	IARI, New Delhi	83.95	0	71.07	0
4	L4149	Variety	IARI, New Delhi	103.37	23.13	71.17	0.13
5	L4594	Variety	IARI, New Delhi	84.21	0.31	60.85	−14.39
6	L7916	Variety	IARI, New Delhi	53.52	−36.24	63.15	−11.16
7	ILL7920	Exotic germplasm	ICARDA, Allepo	52.86	−37.03	63.3	−10.93
8	PL07	Variety	GBPUAT, Pantnagar	51.96	−38.11	72.07	1.4
9	PL08	Variety	GBPUAT, Pantnagar	75.79	−9.72	67.2	−5.45
10	PL97	Variety	GBPUAT, Pantnagar	58.78	−29.98	65.12	−8.37
11	ILL10821	Exotic germplasm	ICARDA, Allepo	92.13	9.75	66.07	−7.04
12	ILL10832	Exotic germplasm	ICARDA, Allepo	83.49	−0.55	64.23	−9.63
13	LC282-907	Breeding line	IARI, New Delhi	55.57	−33.8	48.32	−32.01
14	LC282-1077	Breeding line	IARI, New Delhi	56.42	−32.8	43.47	−38.83
15	LC292-1485	Breeding line	IARI, New Delhi	57.75	−31.21	60.26	−15.21
16	LC300-16	Breeding line	IARI, New Delhi	49.62	−40.9	70.04	−1.45
17	LC300-17	Breeding line	IARI, New Delhi	58.52	−30.29	60.11	−15.43
18	L-11-217	Breeding line	IARI, New Delhi	43.75	−47.89	48.21	−32.17
19	L-11-218	Breeding line	IARI, New Delhi	46.96	−44.06	40.17	−43.48
20	L-11-219	Breeding line	IARI, New Delhi	55.32	−34.1	60.69	−14.62
21	L-11-223	Breeding line	IARI, New Delhi	43.72	−47.93	42.65	−39.99
22	L-11-230	Breeding line	IARI, New Delhi	57.69	−31.28	49.37	−30.53
23	L-11-285	Breeding line	IARI, New Delhi	57.17	−31.9	44.08	−37.98
24	P2103	Exotic germplasm	ICARDA, Allepo	58.73	−30.04	68.21	−4.03
25	P2105	Exotic germplasm	ICARDA, Allepo	54.11	−35.54	66.07	−7.03
26	P2218	Exotic germplasm	ICARDA, Allepo	82.58	−1.63	69.61	−2.06
27	P3131	Exotic germplasm	ICARDA, Allepo	93.21	11.03	72.01	1.32
28	P3205	Exotic germplasm	ICARDA, Allepo	43.83	−47.79	40.95	−42.39
29	P3207	Exotic germplasm	ICARDA, Allepo	55.54	−33.84	42.75	−39.85
30	P3216	Exotic germplasm	ICARDA, Allepo	56.33	−32.9	48.24	−32.13
31	P3236	Exotic germplasm	ICARDA, Allepo	54.68	−34.87	62.71	−11.77
32	P4102	Exotic germplasm	ICARDA, Allepo	77.76	−7.38	75.77	6.6
33	P13108	Exotic germplasm	ICARDA, Allepo	93.87	11.81	79.04	11.21
34	P13109	Exotic germplasm	ICARDA, Allepo	98.68	17.55	81.69	14.93
35	P13124	Exotic germplasm	ICARDA, Allepo	85.43	1.76	77.63	9.22
36	P13132	Exotic germplasm	ICARDA, Allepo	58.91	−29.82	41.23	−41.99
37	P13133	Exotic germplasm	ICARDA, Allepo	58.96	−29.77	65.45	−7.91
38	P13142	Exotic germplasm	ICARDA, Allepo	78.34	−6.68	68.82	−3.17
39	P14205	Exotic germplasm	ICARDA, Allepo	55.73	−33.61	60.2	−15.29
40	P14224	Exotic germplasm	ICARDA, Allepo	50.91	−39.35	62.19	−12.5
41	P15124	Exotic germplasm	ICARDA, Allepo	89.73	6.89	74.08	4.23
42	P15201	Exotic germplasm	ICARDA, Allepo	87.77	4.55	78.15	9.96
43	P16207	Exotic germplasm	ICARDA, Allepo	102.47	22.06	76.77	8.01
44	IC27986	Indian Germplasm	NBPGR, New Delhi	57.84	−31.11	49.95	−29.73
45	IC201704	Indian Germplasm	NBPGR, New Delhi	42.8	−49.02	44.36	−37.59
46	IC208326	Indian Germplasm	NBPGR, New Delhi	52.81	−37.1	46.46	−34.63
47	IC212676	Indian Germplasm	NBPGR, New Delhi	59.43	−29.21	66.01	−7.13
48	IC241783	Indian Germplasm	NBPGR, New Delhi	48.71	−41.98	63.59	−10.53
49	IC226800	Indian Germplasm	NBPGR, New Delhi	53.86	−35.84	65.4	−7.98
50	IC268238	Indian Germplasm	NBPGR, New Delhi	53.33	−36.47	39.44	−44.5
51	IC262839	Indian Germplasm	NBPGR, New Delhi	59.5	−29.13	42.88	−39.66
52	IC267666	Indian Germplasm	NBPGR, New Delhi	56.67	−32.5	45.92	−35.39
53	IC268248	Indian Germplasm	NBPGR, New Delhi	45.68	−45.58	64.27	−9.57
54	IC318881	Indian Germplasm	NBPGR, New Delhi	59.89	−28.66	46.86	−34.07
55	IC321808-1	Indian Germplasm	NBPGR, New Delhi	59.17	−29.52	44.93	−36.79

Table 1 (*contd*)

	Genotype	Variety/breeding line/exotic collection	Collection source	Iron (mg/kg) (mean over environments)	Superiority over check L4147 (%)	Zinc (mg/kg) (mean over environments)	Superiority over check L4147 (%)
56	IC521442	Indian Germplasm	NBPGR, New Delhi	88.08	4.92	62.15	−12.56
57	IC560148	Indian Germplasm	NBPGR, New Delhi	96.62	15.09	61.73	−13.14
58	IC560181	Indian Germplasm	NBPGR, New Delhi	52.23	−37.79	43.57	−38.7
59	IC560297	Indian Germplasm	NBPGR, New Delhi	87.46	4.18	49.87	−29.83
60	IC560333	Indian Germplasm	NBPGR, New Delhi	104.3	24.24	42.06	−40.82
61	IC560331	Indian Germplasm	NBPGR, New Delhi	108.83	29.64	45.96	−35.34
62	IC560350	Indian Germplasm	NBPGR, New Delhi	45.79	−45.45	45.68	−35.72
63	IC560372	Indian Germplasm	NBPGR, New Delhi	50.77	−39.52	41.98	−40.93
64	IC564169	Indian Germplasm	NBPGR, New Delhi	55.36	−34.06	42.56	−40.12
65	IC567318	Indian Germplasm	NBPGR, New Delhi	56.27	−32.97	38.19	−46.27
66	IC560812	Indian Germplasm	NBPGR, New Delhi	58.8	−29.95	43.76	−38.43
67	IG7	Exotic germplasm	Jordan	107.48	28.02	70.37	−0.98
68	IG53	Exotic germplasm	Iraq	97.71	16.39	61.29	−13.76
69	IG161	Exotic germplasm	Turkey	107.74	28.34	39.14	−44.93
70	IG5069	Exotic germplasm	Jordan	91.71	9.25	40.63	−42.83
71	IG5360	Exotic germplasm	Jordan	80.51	−4.09	40.79	−42.61
72	IG69568	Exotic germplasm	Ethiopia	49.85	−40.62	43.46	−38.85
73	IG70223	Exotic germplasm	Turkey	83.93	−0.02	71.45	0.53
74	IG71352	Exotic germplasm	Iran	95.07	13.25	73.13	2.89
75	IG71432	Exotic germplasm	Morocco	59.92	−28.62	47.21	−33.58
76	IG71451	Exotic germplasm	Morocco	108.17	28.85	40.84	−42.55
77	IG71487	Exotic germplasm	Egypt	106.88	27.32	41.98	−40.94
78	IG71505	Exotic germplasm	Syria	85.27	1.58	39.94	−43.8
79	IG71519	Exotic germplasm	Syria	80.46	−4.15	44.94	−36.76
80	IG73920	Exotic germplasm	Syria	94.93	13.08	62.26	−12.41
81	IG73933	Exotic germplasm	Syria	99	17.93	43.44	−38.88
82	IG109039	Exotic germplasm	Turkey	110.63	31.78	70.13	−1.33
83	IG129185	Exotic germplasm	Egypt	59.66	−28.93	61.38	−13.64
84	IG129313	Exotic germplasm	Egypt	79.72	−5.03	40.71	−42.72
85	IG134342	Exotic germplasm	Iran	100.11	19.25	47.61	−33.01
86	PL4	Variety	GBPUAT, Pantnagar	79.11	−5.76	43.31	−39.07
87	IPL406	Variety	IIPR, Kanpur	86.4	2.92	41.89	−41.07
88	PL234	Variety	GBPUAT, Pantnagar	76.84	−8.47	41.28	−41.92
89	VL-4	Variety	VPKAS, Almora	81.74	−2.63	43.13	−39.32
90	LH84-8	Variety	HAU, Hisar	91.17	8.6	43.73	−38.47
91	LL56	Variety	PAU, Ludhiana	56.98	−32.13	43.95	−38.17
92	JL-7	Variety	JNKVV, Jabalpur	86.15	2.62	49.44	−30.45
93	NDC-1	Variety	NDUAT, Faizabad	105.24	25.36	46.41	−34.7
94	PL77-12	Variety	GBPUAT, Pantnagar	96.44	14.87	64.11	−9.8
95	DPL62	Variety	IIPR, Kanpur	98.21	16.99	61.04	−14.11
96	Subratha	Variety	BCKV, Kalyani	88.63	5.57	68.22	−4.02

used to separate the PCR products using electrophoresis for 3 h at 100 V in 1x TBE buffer. Ethidium bromide was used to stain the gel and the amplified products were photographed using a gel documentation system (Alpha Imager) at 260 nm.

SSR diversity statistics and population structure analysis

A Bayesian model-based clustering method (Pritchard *et al.* 2000) was carried out. Structure 2.3 was utilized to determine population structure (Q) and division of accessions into subpopulation. The burn-in period and the Markov chain Monte Carlo (MCMC) replications were set at 200,000 for each run, using an admixture and allele frequency correlated model. The optimum k value was determined by plotting the Ln P (D) value against the given k value. Structure harvester v6.92 (Earl and Von-Holdt 2012) was used for obtaining the optimum k value determined by plotting the Ln P (D) value against k. The highest plateau was observed at delta k = 5, hence, the number of inferred populations was assumed to be five for further analysis. The allele frequency, allele number, heterozygosity and polymorphism information content (PIC)

Table 2. Combined analysss of variance for Fe and Zn concentration across three environments.

Source of variation	DF	Mean squares	
		Fe (mg/kg)	Zn (mg/kg)
Genotype	95	2567.35**	956.682**
Environment	2	144.756*	55.6171*
Genotype × environment	190	120.885**	51.6575**

*Significant at $P \leq 0.05$; **significant at $P \leq 0.01$.

were calculated to estimate the genetic variation within the 96 accessions. *F*-statistics was used to assess the genetic differentiation among various lentil subpopulations (Weir and Hill 2002).

MTA analysis

TASSEL 3.0 was used to calculate LD between each pair of polymorphic loci using standardized disequilibrium coefficient, D′ (Farnir *et al.* 2000) and the correlation coefficient between the alleles of two loci, r^2 (Hill and Robertson 1968). The LD coefficient significance was estimated using 1000 permutations (Bradbury *et al.* 2007) and an LD plot with P and r^2 values was generated to reveal the overall LD among the whole SSR set. Average r^2 and per cent of observations at $P < 0.01$ significance level was estimated over all the pair-wise comparisons. The MTA was studied by incorporating TASSEL v3 (http://www.maizegenetics.net) with MLM (Yu and Buckler 2006) as given by Tadesse *et al.* (2015) and Anuradha *et al.* (2017). The equation for the model is $y = Xa + Qb + Zu + e$, where y stands for phenotype vector, a is marker vector with fixed effects, b is fixed effects vector, u is for the vector of random effects (the kinship matrix), and e is the vector of residuals. X reflects the genotypes at the marker; Q is the Q-matrix derived from the Structure software and Z denotes an identity matrix. Distributions of P values of all the polymorphic SSRs were generated using Manhattan plots implemented in TASSEL (Bradbury *et al.* 2007). Quantile–quantile (QQ) of the expected –log 10 (P value) and observed –log 10 (P value) were plotted to evaluate the adequacy of controlling type I error.

Result

Phenotypic variation for Fe and Zn concentration

Lentil grain Fe and Zn concentration exhibited wide variation in all three studied environments (tables 1 and 2) grain Fe (grand mean) concentration ranged from 42.8 to 110.63 mg/kg, while Zn concentrations varied from 38.18 to 81.68 mg/kg. The highest mean grain Fe concentration above 100 mg/kg was recorded in IG 134342, P 16207, NDC-1, IG 161, IC 560331, IG 7, IG 71487, L4149, IC 560333, IG 71451, IG 109039 and lowest, below 50 mg/kg was recorded in genotypes IC 201704, L-11-223, L-11-217, P 3205, IC 268248, IC 560350, L-11-218, IC 241783, LC 300-16, IG 69568. The highest mean grain Zn concentration above 70 mg/kg was recorded in L4147, L4149, IG 70223, P 3131, PL07, IG 71352, P 15124, P 4102, P 16207, P 13124, P 15201, P 13108 and P 13109; and lowest, below 40 mg/kg was recorded in genotypes IC 567318, IG 161, IC 268238 and IG 71505. Biofortified lentil variety L 4147 was used as check variety and evaluated genotypes were compared with this variety. For grain Fe concentration, 35 genotypes exhibited superiority over the check variety. The highest superiority was recorded for IG 109039 (31.78%) followed by IG 7 (32.00%). Twelve genotypes exhibited higher grain Zn concentration in comparison to L 4147. Highest superiority was recorded for P13108 (11.21%) and P13109 (14.93%). High broad sense heritability was recorded for grain Fe and Zn concentration in all the studied environments (table 3). The absolute values of skewness and kurtosis for grain Fe and Zn concentrations in different years were <1.0 (table 3).

Genetic diversity and population structure

Eighty polymorphic SSR markers that exhibit polymorphism were used to characterize lentil association panel for assessing the genetic relationship. A total of 270 alleles were obtained from the 80 SSR loci scored for the 96 lentil genotypes, with an average of 3.4 alleles per locus varying from 2 to 7. Major allele frequency was in a range of 0.35 to 0.97 with an average of 0.76. The average gene diversity recorded using SSR was 0.35 (ranged from 0.05 to 0.75). The expected heterozygosity for individual loci ranged from 0.00 to 0.10 with an average 0.03. PIC values of 96 genotypes ranged from 0.05 to 0.72 with an average of 0.31 (table 4).

The admixture model-based simulations performed using Structure by varying the K from 2 to 10 with 5 runs for each K using all 96 lentil genotypes which showed a quite evident peak at K = 5. Although, the average LnP (D) (log-likelihood) value gradually increased; but, the most apparent inflection was recorded at K = 5 (figure 1), which

Table 3. Heritability, skewness and kurtosis of grain Fe and Zn concentration of 96 lentil genotypes across three years at IARI, New Delhi.

Traits	Environment	Heritability (%)	Skewness	Kurtosis
GIC	2011–12	97.79	0.579	−0.504
	2012–13	94.97	0.445	−0.806
	2013–14	96.16	0.393	−1.095
GZC	2011–12	96.62	0.158	−1.421
	2012–13	97.45	0.245	−1.336
	2013–14	94.62	0.334	−0.805

Table 4. Summary of genetic diversity and linkage group of 96 lentil accessions using 80 SSR markers.

	Marker	Allele frequency	Allele no.	Gene diversity	Heterozygosity	PIC	LG	Reference for LG
1	PBALC11	0.93	2	0.14	0.00	0.13	–	–
2	PBALC13	0.71	3	0.42	0.00	0.34	LG6	Sudheesh *et al.* (2016)
3	PBALC46	0.95	2	0.10	0.00	0.09	–	–
4	PBALC51	0.82	2	0.30	0.00	0.26	–	–
5	PBALC55	0.86	2	0.24	0.00	0.21	LG4	Sudheesh *et al.* (2016)
6	PBALC79	0.93	3	0.14	0.02	0.13	–	–
7	PBALC90	0.86	3	0.24	0.02	0.21	LG4	Sudheesh *et al.* (2016)
8	PBALC92	0.79	2	0.33	0.00	0.28	–	–
9	PBALC218	0.77	4	0.37	0.04	0.32	LG1	Sudheesh *et al.* (2016)
10	PBALC219	0.84	2	0.26	0.00	0.23	LG2	Sudheesh *et al.* (2016)
11	PBALC221	0.83	2	0.28	0.00	0.24	LG3	Sudheesh *et al.* (2016)
12	PBALC222	0.82	3	0.29	0.01	0.25	–	–
13	PBALC223	0.97	2	0.06	0.07	0.06	–	–
14	PBALC233	0.80	3	0.33	0.02	0.29	LG1	Kaur *et al.* (2014)
15	PBALC250	0.70	2	0.42	0.04	0.33	LG4	Kaur *et al.* (2014)
							LG2	Sudheesh *et al.* (2016)
16	PBALC260	0.96	3	0.08	0.02	0.08	–	–
17	PBALC265	0.61	7	0.57	0.09	0.53	LG1	Kaur *et al.* (2014)
18	PBALC273	0.85	4	0.27	0.02	0.25	LG5	Sudheesh *et al.* (2016)
19	PBALC275	0.90	3	0.18	0.10	0.17	–	–
20	PBALC301	0.80	3	0.34	0.09	0.30	–	–
21	PBALC303	0.97	2	0.05	0.05	0.05	–	–
22	PBALC311	0.71	3	0.43	0.00	0.37	LG6	Kaur *et al.* (2014)
							LG3	Sudheesh *et al.* (2016)
23	PBALC323	0.76	2	0.36	0.00	0.30	LG3	Kaur *et al.* (2014)
							LG1	Sudheesh *et al.* (2016)
24	PBALC337	0.93	2	0.14	0.00	0.13	–	–
25	PBALC341	0.92	2	0.15	0.00	0.14	LG7	Sudheesh *et al.* (2016)
26	PBALC364	0.80	3	0.33	0.01	0.28	LG6	Kaur *et al.* (2014)
							LG3	Sudheesh *et al.* (2016)
27	PBALC368	0.85	3	0.26	0.01	0.23	LG4	Kaur *et al.* (2014)
							LG2	Sudheesh *et al.* (2016)
28	PBALC369	0.78	2	0.34	0.00	0.28	LG5	Kaur *et al.* (2014)
29	PBALC373	0.71	5	0.45	0.04	0.42	LG2	Kaur *et al.* (2014)
30	PBALC377	0.96	2	0.08	0.00	0.08	–	–
31	PBALC383	0.81	3	0.31	0.04	0.28	LG1	Sudheesh *et al.* (2016)
32	PLC 5	0.79	2	0.33	0.00	0.28	–	Jain *et al.* (2013)
33	PLC 17	0.76	2	0.37	0.01	0.30	–	–
34	PLC 21	0.73	3	0.40	0.00	0.33	–	–
35	PLC 31	0.75	2	0.37	0.02	0.30	–	–

Table 4 (*contd*)

	Marker	Allele frequency	Allele no.	Gene diversity	Heterozygosity	PIC	LG	Reference for LG
36	PLC 35	0.77	3	0.36	0.03	0.31	–	–
37	PLC86	0.93	2	0.14	0.00	0.13	–	–
38	PLC91	0.95	3	0.09	0.01	0.09	–	–
39	PLC93	0.92	2	0.15	0.00	0.14	–	–
40	PLC94	0.70	4	0.47	0.02	0.42	–	–
41	PLC96	0.73	4	0.44	0.06	0.40	–	–
42	PLC97	0.65	4	0.50	0.01	0.43	–	–
43	PLC101	0.84	2	0.27	0.03	0.23	–	–
44	PLC105	0.64	3	0.51	0.07	0.44	–	–
45	PLC106	0.97	3	0.06	0.00	0.06	–	–
46	GLLC106	0.72	3	0.42	0.02	0.35	LG2	Saha *et al.* (2013)
							LG9	Gupta *et al.* (2012)
47	GLLC108	0.78	3	0.35	0.02	0.29	LG2	Saha *et al.* (2013)
48	GLLC511	0.96	4	0.08	0.02	0.08	LG8	Saha *et al.* (2013)
49	GLLC527	0.74	5	0.43	0.07	0.40	LG8	Saha *et al.* (2013); Gupta *et al.* (2012)
50	GLLC541	0.54	3	0.52	0.00	0.40	LG11	Saha *et al.* (2013)
							LG2	Gupta *et al.* (2012)
51	GLLC548	0.90	2	0.19	0.02	0.17	LG1	Saha *et al.* (2013)
52	GLLC556	0.65	5	0.54	0.05	0.50	LG4	Saha *et al.* (2013)
53	GLLC559	0.71	4	0.45	0.01	0.41	LG12	Saha *et al.* (2013)
							LG9	Gupta *et al.* (2012)
54	GLLC562	0.61	7	0.59	0.09	0.57	LG2	Saha *et al.* (2013)
55	GLLC563	0.54	4	0.60	0.00	0.54	LG11	Saha *et al.* (2013)
56	GLLC591	0.79	3	0.35	0.00	0.31	LG12	Saha *et al.* (2013)
57	GLLC592	0.82	3	0.31	0.09	0.28	LG1	Saha *et al.* (2013)
58	GLLC595	0.35	5	0.72	0.02	0.67	–	–
59	GLLC598	0.74	3	0.39	0.00	0.32	LG11	Saha *et al.* (2013)
60	GLLC607	0.90	2	0.17	0.00	0.16	LG1	Saha *et al.* (2013)
61	GLLC609	0.52	4	0.63	0.06	0.57	LG2	Saha *et al.* (2013)
							LG 6	Gupta *et al.* (2012)
62	GLLC614	0.51	5	0.64	0.02	0.58	LG6	Gupta *et al.* (2012)
63	SSR13	0.86	3	0.25	0.00	0.23	LG2	Sudheesh *et al.* (2016)
							LG4	Kaur *et al.* (2014)
							LG1	Tullu *et al.* (2008); Hamwieh *et al.* (2005)
64	SSR19	0.71	4	0.45	0.06	0.41	LG6	Temel *et al.* (2015); Kaur *et al.* (2014); Hamwieh *et al.* (2005)
							LG4	Andeden *et al.* (2015)
							LG3	Sudheesh *et al.* (2016)
65	SSR130	0.51	2	0.50	0.00	0.37	LG5	www.coolseasonfoodlegume.org
							LG2	Hamwieh *et al.* (2005)
66	SSR212-1	0.69	4	0.49	0.06	0.45	LG6	Kaur *et al.* (2014); Hamwieh *et al.* (2005)

Table 4 (*contd*)

	Marker	Allele frequency	Allele no.	Gene diversity	Heterozygosity	PIC	LG	Reference for LG
67	SSR213	0.71	3	0.43	0.00	0.37	LG7	Sudheesh *et al.* (2016)
							LG3	Kaur *et al.* (2014)
							LG8	Hamwieh *et al.* (2005)
68	SSR215	0.62	4	0.56	0.05	0.51	LG2	www.coolseasonfoodlegume.org
							LG3	Hamwieh *et al.* (2005)
69	SSR317-1A	0.81	7	0.34	0.09	0.33	LG1	www.coolseasonfoodlegume.org; Hamwieh *et al.* (2005)
70	SSR317-1B	0.84	4	0.29	0.00	0.27	LG1	www.coolseasonfoodlegume.org; Hamwieh *et al.* (2005)
71	SSR317-2	0.42	7	0.75	0.08	0.72	LG2	Gupta *et al.* (2012)
							LG4	Kaur *et al.* (2014)
							LG2	Hamwieh *et al.* (2005)
72	SSR323	0.37	4	0.70	0.07	0.64	LG3	www.coolseasonfoodlegume.org
							LG5	Hamwieh *et al.* (2005)
73	SSR336	0.87	5	0.24	0.03	0.23	LG2	Sudheesh *et al.* (2016)
							LG1	Hamwieh *et al.* (2005)
74	PLC 69	0.36	7	0.75	0.06	0.71	–	–
75	PLC 72	0.74	5	0.42	0.06	0.39	–	–
76	SSR90	0.82	3	0.31	0.00	0.29	LG3	Kaur *et al.* (2014)
							LG1	Sudheesh *et al.* (2016)
77	SSR183	0.80	5	0.33	0.06	0.30	LG4	Gupta *et al.* (2012)
78	SSR191-A	0.61	6	0.59	0.01	0.56	–	–
79	SSR191-B	0.78	3	0.36	0.02	0.33	–	–
80	SSR207	0.76	4	0.39	0.04	0.36	–	–

PIC, polymorphism information content; LG, linkage group; '-' refers to LG not yet known (total = 33).

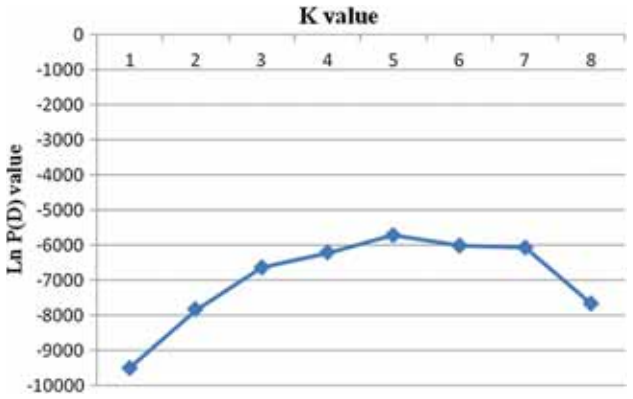

Figure 1. Optimization of number of populations (K value) varying from K = 1–10 to determine best possible population number for 96 lentil accessions using LnP(D) value derived from Structure (Pritchard *et al.* 2000).

confirms the classification of 96 lentil genotypes into five distinct populations.

The genotypes were assigned to individual subpopulation keeping in view the highest membership likelihood criterion based on *Q*-values obtained from Structure software. The genotypes were assigned to the particular sub group according to the *Q* value (more than or equal to 0.6). The five subpopulations were presented using colour codes (figure 2; figure 1 in electronic supplementary material); where each genotype is represented by a single vertical line having lengths proportional to each of the five clusters; and colour length in the line indicates the proportion of a genotype belonging to that subpopulation. Considering all these pointers, it was found that the structure in the population depicted as per the source of geographical origin. The subpopulation 1 (red colour) consisted of 12 genotypes which mainly comprises of Mediterranean landraces; whereas, subpopulation 2 (green colour) included eight genotypes, most of which are advanced breeding lines of IARI, New Delhi. Further, most of the indigenous collection grouped in subpopulation 3 (blue colour) which consisted of seven genotypes with least admixture. The subpopulation 4 (yellow colour) consisted of maximum 62 genotypes, most of which are the released cultivars used in this study. Subpopulation 5 (pink colour) contained seven genotypes which included indigenous and exotic collection. These findings clearly showed that the AM panel used had a stratified population suitable for association analyses. The F_{st} values for subpopulations were recorded as 0.36, 0.48, 0.55, 0.36 and 0.49, respectively. Further, both Pop A and Pop D clusters had lowest F_{st} (0.36). The overall F_{st} value estimated within the subpopulations was also moderate (0.45, table 5).

LD and MTA analyses

For the determination of specific critical LD value, the background LD (unlinked) generated by the population structure in the lentil genome was used and pair-wise comparisons among 3160 pair-wise combinations of the 80 SSR loci was used to evaluate the extent of genomewide LD using TASSEL3 with multiple permutations of 10,000 times. The LD calculated by squared value of Pearson correlation, R^2 varies 0 to 1 with a mean of 0.40 (figure 3). Highest R^2 value was observed between PBALC 92 and PBALC 301, GLLC 548 and GLLC 607. Moreover, 32 pair-wise combinations of the SSR markers exhibited r^2 LD values more than 0.40.

MLM was utilized for detecting associations of molecular markers with grain Fe and Zn concentration. The model identified SSR markers significantly associated with Fe (GLLC 106 and GLLC 108) (figure 4; figure 2 in electronic supplementary material) and Zn (PBALC 364, PBALC 92 and GLLC 592) concentration (figure 5; figure 3 in electronic supplementary material). Significant SSRs marker identified in more than two datasets were considered as reliable. Mean percentage of variation

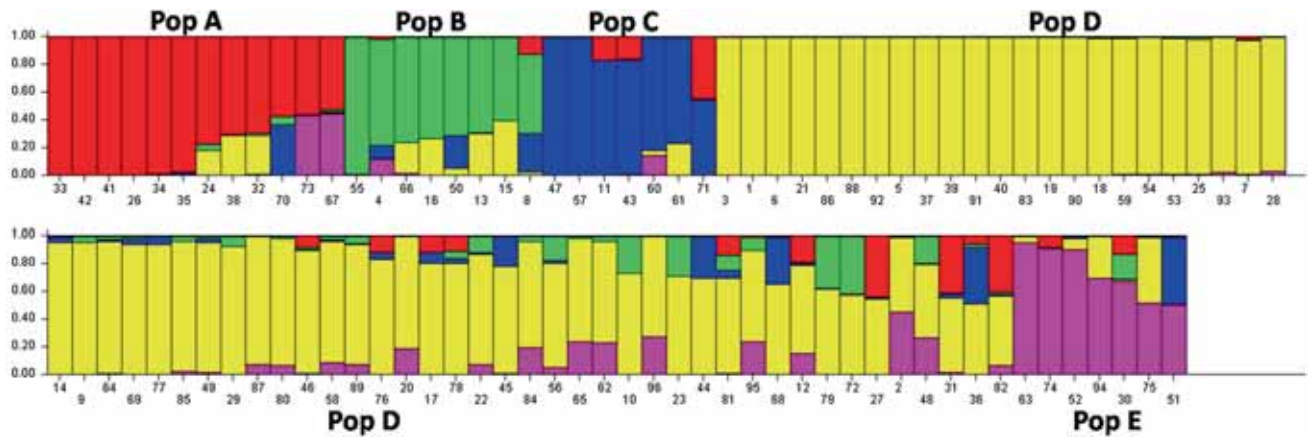

Figure 2. Admixture bar plot showing five subpopulations and 96 genotypes of diverse lentil genotypes. Each colour represents one cluster, and individual fragment represents the membership fraction for single individual in K clusters.

Table 5. Mean value of F_{st} within the population.

Population	Mean F_{st} value
Pop A	0.365
Pop B	0.485
Pop C	0.558
Pop D	0.365
Pop E	0.494

explained by the marker loci GLLC 106 and GLLC 108 for the grain Fe concentration was found 17% and 6%, respectively (table 6). Mean percentage of variation explained by the marker loci PBALC 364, PBALC 92 and GLLC 592 for the grain Zn concentration was found 6%, 13% and 8%, respectively (table 6). Hypothetical QQ plots of the MTA study for Fe and Zn concentration in the lentil grains showed normal distribution of the data (figures 4 and 5 in electronic supplementary material). Moreover, the region between expected –log10 (*P* value) from 0 to 2 seems to represent adequate differences in the allele frequencies which are more likely due to the inherent population structure than the associated markers.

Discussion

AM is a powerful tool for identifying alleles and loci responsible for economically important traits. Constitution of AM panel with diverse lines is the prerequisite for the AM study (Flint-Garcia *et al.* 2005). The AM panel comprised of 96 diverse lentil genotypes with diverse pedigrees originating from different parts of India and Mediterranean region. The studied set of genotypes exhibited wide variation for grain Fe and Zn concentration observed in different environments (table 1 in electronic supplementary material). A positive correlation was noticed between grain Fe and Zn concentrations in our study, similar results were also reported in barley (Mamo *et al.* 2014), pea (Diapari *et al.* 2014), chickpea (Diapari *et al.* 2015) and pearl millet (Anuradha *et al.* 2017).

SSR markers are used for the genetic diversity analysis, gene-tagging, and genetic mapping in a number of crops including lentil (Kumar *et al.* 2014; Dikshit *et al.* 2015). Eighty SSR primers (45 EST-SSRs and 35 genomic SSRs) exhibiting polymorphism (table 2 in electronic supplementary material) were used in this study. Sun *et al.* (2015) and Gupta *et al.* (2014) have used 90 and 50 polymorphic SSRs for the AM studies in tall fescue and foxtail millet, respectively. Based on reported linkage maps (Rubeena and Taylor 2003; Gupta *et al.* 2012; Saha *et al.* 2013; Kaur *et al.* 2014; Sudheesh *et al.* 2016), the positions of 47 studied SSRs was derived from various linkage groups. However, some SSRs such as PBALC 250, PBALC 311, GLLC 106, GLLC 541, SSR 13, SSR 90 etc. were found located on

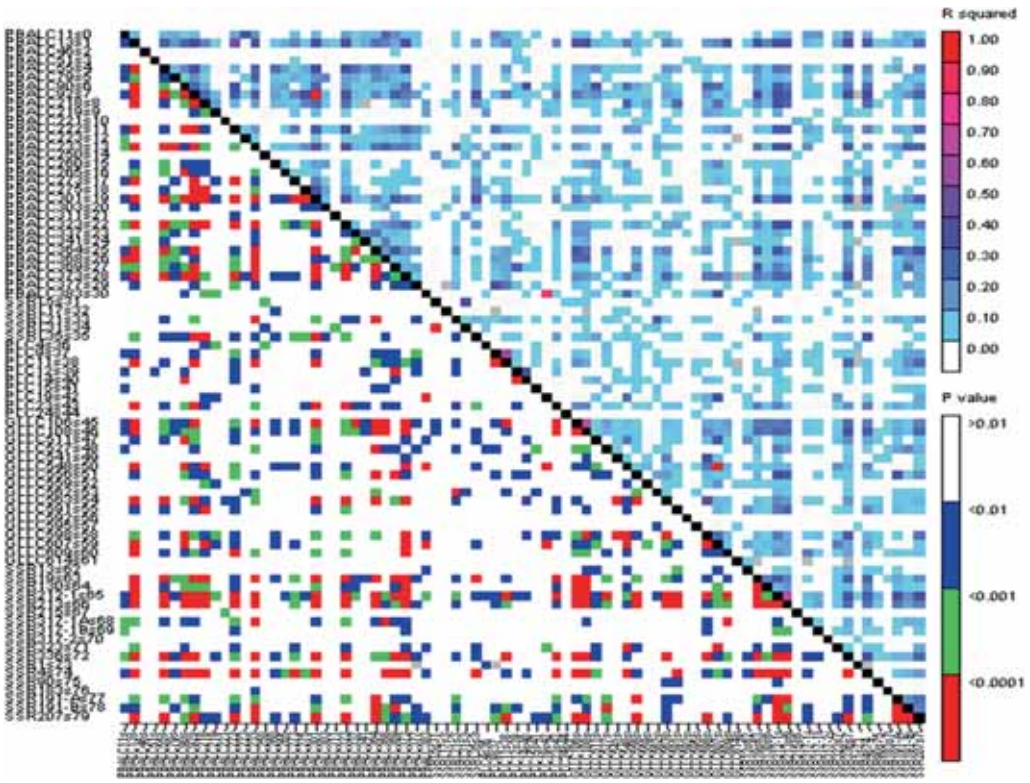

Figure 3. Linkage disequilibrium patterns among 96 diverse lentil accessions genotyped with 80 polymorphic SSR markers. The squared correlation coefficients (r^2) for each pair of markers are presented in the upper triangle and their equivalent *P* values in the lower triangle.

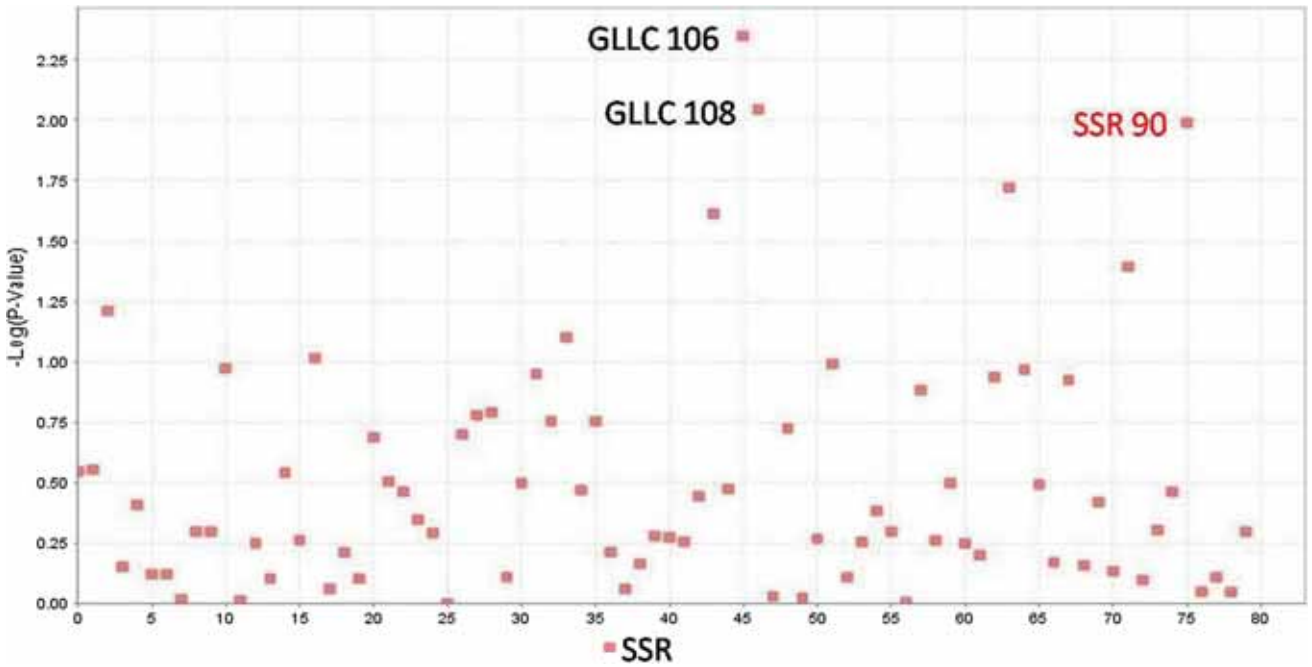

Figure 4. Manhattan plot depicting the association of SSRs with three year mean grain iron content in lentil using mixed linear model.

Table 6. MTA with *P* value and marker-trait regression coefficient derived from 80 SSR markers and 96 accessions in lentil.

Trait	Markers	MLM	
		Adjusted *P* value	R^2
Grain Fe concentration	GLLC106	0.004	0.17
	GLLC108	0.060	0.06
Grain Zn concentration	PBALC364	0.068	0.06
	PBALC92	0.137	0.08
	GLLC592	0.002	0.13

different linkage groups, by different researchers in various mapping populations (table 4). Comprehensive saturated map is required for establishing the precise location of the markers.

The average number of alleles detected in the studies are comparable to other *Lens* species and genotypes showing 2 to 7 alleles per locus for SSR markers (Kumar *et al.* 2014; Dikshit *et al.* 2015). But, very high range of 4–22 alleles per locus are also reported in cultivating lentil genotypes (Roy *et al.* 2015). Dikshit *et al.* (2015), Roy *et al.* (2015) and Khazaei *et al.* (2016) have reported variable average gene diversity. The expected heterozygosity in the study is in congruence with that obtained using SNP markers (0.0375±0.0755, Khazaei *et al.* 2016). However, somewhat higher values were reported by other studies in lentil (0.02 to 0.62, Dikshit *et al.* 2015; 0.0 to 0.4, Roy *et al.* 2015). PIC values of 96 genotypes ranged from 0.05 to 0.72 with an average of 0.31 (table 3). On the similar note, it was found ranging from 0.05 to 0.77 (Dikshit *et al.* 2015) and 0.13 to 0.74 (Kumar *et al.* 2014) for various *Lens* species for SSR markers. Even for SNP markers, nearly similar value (0.3092 ± 0.0789) is reported (Khazaei *et al.* 2016). But, higher PIC range (0.234 to 0.931) has been reported by Roy *et al.* (2015) using SSR markers. PIC can be used for identifying the SSRs for accurate assessment of diversity.

The population structure is considered to avoid false positive associations while employing the AM strategy (Yu and Buckler 2006); therefore, Bayesian clustering approach has been used in this study, since it probabilistically assigns individuals to populations based on genotype. The grouping in Structure depends on the percentage of membership of an individual to a subpopulation (Gupta *et al.* 2014). The F_{st} value of the subpopulations was in the range of 0.36 to 0.55 suggesting that these clusters are moderately uniform; but, a very tight association could not be established. In this study, all pairwise F_{st} values were significantly different from each other, therefore the populations may be considered as genetically variable from one another (Nachimuthu *et al.* 2015). The subpopulation Pop A and Pop D exhibits lowest F_{st} values of 0.36, indicating that the individuals of these two subpopulations shared their alleles. Even the overall F_{st} value estimated within the subpopulations was also moderate (0.45, table 4). Somewhat lower F_{st} values ranging from 0.17 to 0.25 with a mean of 0.21 has been reported for the population structure of foxtail millet (Gupta *et al.* 2014).

Several statistics have been proposed for LD measurement (Chao *et al.* 2010); but, the r^2 statistic is considered

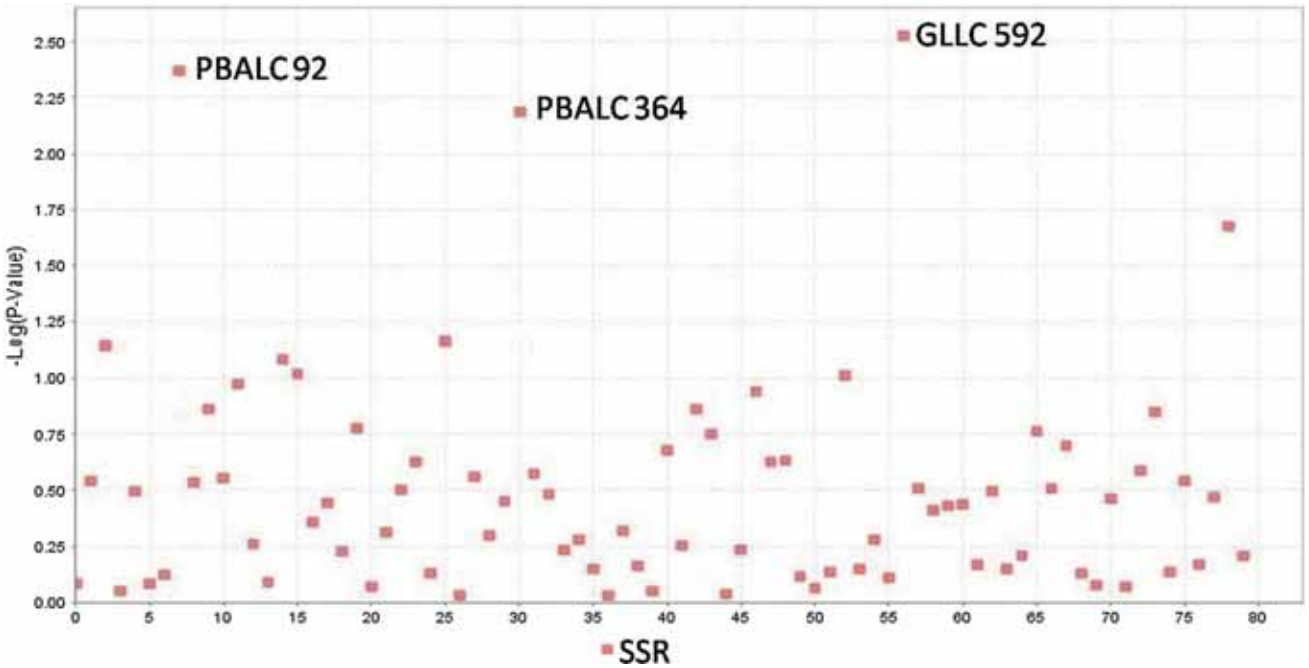

Figure 5. Manhattan plot depicting the association of SSRs with three year mean grain zinc content in lentil using MLM.

as a more accurate method and was used for the LD plot generation (figure 3, LD based on the r^2 was found ranging from 0 to 1. The highest r^2 LD value was observed between the markers PBALC 92 and PBALC 301, GLLC 548 and GLLC 607. Moreover, 32 pair-wise combinations of the SSR markers had r^2 LD values more than 0.40; which may be due to the self-pollinated nature of lentil; since such crops generally sustain high LD due to less variation (Zhu *et al.* 2008). The high value of LD may need less number of markers for population study but contrarily mapping resolution will be compromised.

AM strategy was used to search for linked/responsive marker for the Fe and Zn traits in 96 lentil genotypes. Using MLM approach, we identified two SSR markers, namely GLLC 106 and GLLC 108, associated with grain Fe at –log10 (*P*-value) >2 in all four datasets (2011–12, 2012–13, 2013–14 and mean data; figure 4; figure 2 in electronic supplementary material). Mean percentage of variation explained by the marker loci GLLC 106 and GLLC 108 for the grain Fe concentration was found 17% and 6%, respectively. Recently, in lentil, using genotyping by sequencing (GBS), a SNP marker-based map has been developed on which 21 QTL regions, explaining 5.9–14.0% of the phenotypic variation for grain Fe concentration were identified on six linkage groups (LG1, 2, 4, 5, 6, and 7; LOD: 3.00 to 4.45) (Aldemir *et al.* 2017). MTA analysis by Khazaei *et al.* (2017) detected two SNPs tightly linked to seed Fe and one linked to seed Zn concentration in the cultivated lentil genotypes. Similarly, for the grain Zn concentration, three markers, PBALC 364, PBALC 92 and GLLC 592 were found significantly associated for all three years at –log10 (*P* value) >2 (figure 5; figure 3 in electronic supplementary material). The mean percentage of variation explained by these SSR loci for grain Zn concentration was 6%, 8% and 13%, respectively. Hypothetical QQ plots of the MTA study for Fe and Zn concentration in the lentil grains showed normal distribution of the data (figures 4 and 5 in electronic supplementary material). Moreover, the region between expected –log10 (*P* value) from 0 to 2 seems to represent large differences in the allele frequencies which are more likely due to the inherent population structure than the associated markers. In addition, the QQ plots showed more consistency in the grain Fe concentration (figure 4 in electronic supplementary material) than the grain Zn concentration (figure 5 in electronic supplementary material).

Our findings on Fe and Zn associations using SSR markers will augment the QTL information and thus improvement of lentil genotypes for the specified traits like grain Fe and Zn enrichment.

Acknowledgements

We thank Head, Division of Genetics, Joint Director Research and Director, IARI, New Delhi for providing the necessary research facilities. The germplasm collections provided by NBPGR, New Delhi and ICARDA, Aleppo Syria is also gratefully acknowledged. This study was partially supported by Harvest Plus: Lentil Biofortification project by ICARDA and in-house project by ICAR-IARI, New Delhi.

References

Aldemir S., Ateş D., Temel H. Y., Yağmur B., Alsaleh A., Kahriman A. *et al*. 2017 QTLs for iron concentration in seeds of the cultivated lentil (*Lens culinaris* Medic.) via genotyping by sequencing. *Turk. J. Agric. For.* **41**, 1–13.

Anandan A., Mahender A., Pradhan S. K. and Ali J. 2016 Population structure, diversity and trait association analysis in rice (*Oryza sativa* L.) germplasm for early seedling vigor (ESV) using trait linked SSR markers. *PLoS One* **11**, e0152406.

Andeden E. E., Baloch F. S., Cakir E., Tpklu F. and Ozkan H. 2015 Development, characterization and mapping of microsatellite markers for lentil (*Lens culinaris* Medik.) Plant Breed. **134**, 589–598

Anuradha N., Satyavathi C. T., Bharadwaj C., Nepolean T., Sankar S. M., Singh S. P. *et al*. 2017 Deciphering genomic regions for high grain iron and zinc content using association mapping in pearl millet. *Front. Plant Sci*. **8**, 412.

Arumuganathan K. and Earle E. D. 1991 Nuclear DNA content of some important plant species. *Plant Mol. Biol.* **9**, 208–218.

Bradbury P. J., Zhang Z., Kroon D. E., Casstevens T. M., Ramdoss Y. and Buckler E. S. 2007 TASSEL: software for association mapping of complex traits in diverse samples. *Bioinformatics* **23**, 2633–2635.

Chao S., Dubcovsky J., Dvorak J., Luo M., Baenziger S. P., Matnyazov R. *et al.* 2010 Population and genome-specific patterns of linkage disequilibrium and SNP variation in spring and winter wheat (*Triticum aestivum* L.). *BMC Genom.* **11**, 727–744.

Diapari M., Sindhu A., Bett K., Deokar A., Warkentin T. D. and Taran B. 2014 Genetic diversity and association mapping of iron and zinc concentrations in chickpea (*Cicerarietinum* L.). *Genomics* **57**, 459–468.

Diapari M., Sindhu A., Warkentin T. D., Bett K. and Tar'an B. 2015 Population structure and marker-trait association studies of iron, zinc and selenium concentrations in seed of field pea (*Pisum sativum* L.). *Mol. Breed.* **35**, 30.

Dikshit H. K., Singh A., Singh D., Aski M. S., Prakash P., Jain N., Meena S., Kumar S. and Sarker A. 2015 Genetic diversity in *Lens* species revealed by EST and genomic simple sequence repeat analysis. *PLoS One* **10**, e0138101.

Earl D. A. and VonHoldt B. M. 2012 Structure Harvester: a website and program for visualizing structure output and implementing the Evanno method. *Conserv. Genet. Res.* **4**, 359–361.

Farnir F., Coppieters W., Arranz J. -J., Berzi P., Cambisano N., Grisart B. *et al*. 2000 *Extensive genome-wid*e linkage disequilibrium in cattle. *Genome Res.* **10**, 220–227.

Flint-Garcia S. A., Thornsberry J. M. and Buckler E. S. 2003 Structure of linkage disequilibrium in plants. *Annu. Rev. Plant Biol.* **54**, 357–374.

Flint-Garcia S. A., Thuillet A. C., Yu J., Pressoir G., Romero S. M., Mitchell S. E. *et al*. 2005 Maize association population: a high-resolution platform for quantitative trait locus dissection. *Plant J.* **44**, 1054–1064.

Grusak M. A. 2009 Nutritional and health-beneficial quality. In *The Lentil: Botany, production and uses* (ed. W. Erskine, F. Muehlbauer, A. Sarker and B. Sharma), pp. 368–390. Oxford, UK.

Gupta M., Verma B., Kumar N., Chahota R. K., Rathour R., Sharma S. K. *et al*. 2012 Construction of intersubspecific molecular genetic map of lentil based on ISSR, RAPD and SSR markers. *J. Genet.* **91**, 279–287.

Gupta S., Kumari K., Muthamilarasan M., Parida S. K. and Prasad M. 2014 Population structure and association mapping of yield contributing agronomic traits in foxtail millet. *Plant Cell Rep.* **33**, 881–893.

Hamwieh A., Udupa S. M., Choumane W., Sarker A., Dreyer F., Jung C. *et al*. 2005 A genetic linkage map of *Lens* sp. Based on microsatellite and AFLP markers and the localization of fusarium vascular wilt resistance. *Theor. Appl. Genet*. **110**, 669–677.

Hamwieh A., Udupa S. M., Sarkar A., Jung C. and Baum M. 2009 Development of new microsatellite markers and their application in the analysis of genetic diversity in lentils. *Breed. Sci.* **59**, 77–86.

Hill W. G. and Robertson A. 1968 Linkage disequilibrium in finite populations. *Theor. Appl. Genet.* **38**, 226–231.

Jain N., Dikshit H. K., Singh D., Singh A. and Kumar H. 2013 Discovery of EST-derived microsatellite primers in the legume *Lens culinaris* (*Fabaceae*). *Appl. Pl. Sci.* **1**, 1200539.

Kaur S., Cogan N. O. I., Pembleton L. W., Shinozuka M., Savin K. W., Materne M. *et al*. 2011 Transcriptome sequencing of lentil based on second-generation technology permits large-scale unigene assembly and SSR marker discovery. *BMC Genom.* **12**, 265.

Kaur S., Cogan N. O. I., Stephens A., Noy D., Butsch M., Forster J. W. *et al*. 2014 EST–SNP discovery and dense genetic mapping in lentil (*Lens culinaris* Medik.) enable candidate gene selection for boron tolerance. *Theor. Appl. Genet.* **127**, 703–713.

Khazaei H., Caron C. T., Fedoruk M., Diapari M., Vandenberg A., Coyne C. J. *et al*. 2016 Genetic diversity of cultivated lentil (*Lens culinaris* Medik.) and its relation to the world's agro-ecological zones. *Front. Plant Sci.* **7**, 1093.

Khazaei H., Podder R., Caron C. T., Kundu S. S., Diapari M., Vandenberg A. and Bett K. E. 2017 Marker–trait association analysis of iron and zinc concentration in lentil (*Lens culinaris* Medik.) seeds. *Plant Genom* **10**, 2.

Körber N., Bus A., Li J., Parkin I. A. P., Wittkop B., Snowdon R. J. *et al*. 2016 Agronomic and seed quality traits dissected by genome-wide association mapping in *Brassica napus*. *Front. Plant Sci.* **7**, 386.

Kumar H., Dikshit H. K., Singh A., Jain N., Kumari J., Singh A. M. *et al*. 2014 Characterization of grain iron and zinc in lentil (*Lens culinaris* Medikus *culinaris*) and analysis of their genetic diversity using SSR markers. *Aust. J. Crop Sci.* **8**, 1005–1012.

Kumar J., Kumar S., Sarker A. and Singh N. P. 2018 Analysis of genetic variability and genotype× environment interactions for iron and zinc content among diverse genotypes of lentil. *J. Food Sci. Tech.* **55**, 3592–3605.

Kumar S., Rajendran K., Kumar J., Hamwieh A. and Baum M. 2015 Current knowledge in lentil genomics and its application for crop improvement. *Front. Plant Sci.* **6**, 78.

Mamo B. E., Barber B. L. and Steffenson B. J. 2014 Genome-wide association mapping of zinc and iron concentration in barley landraces from Ethiopia and Eritrea. *J. Cereal Sci.* **60**, 497–506.

Murray M. and Thompson W. 1980. The isolation of high molecular weight plant DNA. *Nucleic Acids Res.* **8**, 4321–4325.

Nachimuthu V. V., Muthurajan R., Duraialaguraja S., Sivakami R., Pandian B. A., Ponniah G. *et al*. 2015 Analysis of population structure and genetic diversity in rice germplasm using SSR markers: an initiative towards association mapping of agronomic traits in *Oryza sativa*. *Rice* **8**, 1.

Pritchard J. K., Stephens M., Rosenberg N. A. and Donnelly P. 2000 Association mapping in structured populations. *Am. J. Hum. Genet.* **67**, 170–181.

Roy S., Ray B. P., Sarker A. and Das S. C. 2015. DNA fingerprinting and genetic diversity in lentil germplasm using SSR markers. *Asian J. Conserv. Biol.* **4**, 109–115.

Rubeena F. R. and Taylor P. W. J. 2003 Construction of an intraspecific linkage map of lentil (*Lens culinaris* ssp. *culinaris*). *Theor. Appl. Genet.* **107**, 910–916.

Saha G. C., Sarker A., Chen W., Vandemark G. J. and Muehlbauer F. J. 2010 Inheritance and linkage map positions of genes conferring resistance to stemphylium blight in lentil. *Crop Sci.* **50**, 1831–1839.

Saha G. C., Sarker A., Chen W., Vandemark G. J. and Muehlbauer F. J. 2013 Inheritance and linkage map positions of genes conferring agromorphological traits in *Lens culinaris* Medik. *Int. J. Agron.* 2013. Article ID 618926.

Sarker A. and Kumar S. 2011 Lentils in production and food systems in West Asia and Africa. International Center for Agricultural Research in the Dry Areas (ICARDA), Aleppo, Syria. *Grain Leg.* **57**, 46–48.

Singh A., Sharma V., Dikshit H. K., Aski M., Kumar H., Thirunavukkarasu N. *et al*. 2017 Association mapping unveils favorable alleles for grain iron and zinc concentrations in lentil (*Lens culinaris* subsp. *culinaris*). *PLoS One* **12**, e0188296.

Singh D., Chonkar P. K. and Dwivedi B. S. 2005 *Manual on soil, plant and water analysis*. Westville Publishers, New Delhi.

Srivastava R. P. and Vasishtha H. 2012 Saponins and lectins of indian chickpeas (*Cicer arietinum*) and lentils (*Lens culinaris*). *Ind. J. Agric. Biochem.* **25**, 44–47.

Sudheesh S., Rodda M. S., Davidson J., Javid M., Stephens A., Slater A. T. *et al*. 2016 SNP-based linkage mapping for validation of QTLs for resistance to ascochyta blight in lentil. *Front. Plant Sci.* **7**, 1604.

Sun X., Du Z., Ren J., Amombo E., Hu T. and Fu J. 2015 Association of SSR markers with functional traits from heat stress in diverse tall fescue accessions. *BMC Plant Biol.* **15**, 116.

Tadesse W., Ogbonnaya F. C., Jighly A., Sanchez-Garcia M., Sohail Q., Rajaram S. *et al*. 2015 Genome-wide association mapping of yield and grain quality traits in winter wheat genotypes. *PLoS One* **10**, e0141339.

Temel H. Y., Göl D., Akkale H. B. K., Kahriman A. and Tanyolac M. B. 2015 Single nucleotide polymorphism discovery through Illumina-based transcriptome sequencing and mapping in lentil. *Turk. J. Agric. For.* **39**, 470–488.

Tullu A., Tar'an B., Warkentin T. and Vandenberg A. 2008 Construction of an intraspecific linkage map and QTL analysis for earliness and plant height in lentil. *Crop Sci.* **48**, 2254–2264.

Yu J., Arbelbide M. and Bernardo R. 2005 Power of *in silico* QTL mapping from phenotypic, pedigree and marker data in a hybrid breeding program. *Theor. Appl. Genet.* **110**, 1061–1067

Weir B. S. and Hill W. G. 2002 Estimating F-statistics. *Ann. Rev. Genet.* **36**, 721–750.

Yu J. and Buckler E. S. 2006 Genetic association mapping and genome organization of maize. *Curr. Opin. Biotechnol.* **17**, 155–160.

Zhu C., Gore M., Buckler E. S. and Yu J. 2008 Status and prospects of association mapping in plants. *Plant Genomics* **1**, 5–20.

Corresponding editor: ARUN JOSHI