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Characterization of grain iron and zinc in lentil (*Lens culinaris* Medikus *culinaris*) and analysis of their genetic diversity using SSR markers

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Abstract

Forty-one elite lentil lines were studied for stability of grain Fe and Zn concentration across three locations (New Delhi, Ludhiana and Pantnagar) in India. The stability analysis was carried using Eberhart and Russel's stability model. Pooled analysis of variance over locations revealed highly significant differences between genotypes, locations and genotype × location interaction. The maximum mean for grain Fe concentration over the locations was obtained for L 4704 (136.91 mg/kg grain), while for grain Zn concentration was highest for VL 141 (81.542 mg/kg grain). The highest mean grain Fe and Zn was recorded at Indian Agricultural Research Institute, New Delhi (Fe-87.30 mg/kg and Zn-68.602 mg/kg). Although both micronutrients were influenced by environment, grain Fe showed more G × E interaction in comparison to grain Zn concentration. Six lentil genotypes exhibited stability for grain Fe concentration and fifteen genotypes for grain Zn concentration were identified. The studied genotypes with diverse micronutrients concentration were analysed for molecular diversity using 32 polymorphic SSR markers. These markers amplified a total of 130 bands with PIC value ranging from 0.138 to 0.798. Based on SSR allelic diversity, genotypes were grouped in two major clusters. The clustering pattern indicated variability in the studied lines. On the basis of genetic diversity for micronutrient concentration, ten crosses are suggested for hybridization purpose to obtain the transgressive segregants. The combined analysis of multi-location phenotyping and genetic diversity further suggests six potential crosses for developing micronutrient rich varieties for the future.

Keywords: Lentil, grain micronutrients, stability, molecular characterization.
Abbreviations: IVT_Initial Varietal Trail, bi_Regression Coefficient, S^2Di_ Deviation from Regression line, SSR_Simple Sequence Repeat, Tm℃_Annealing temperature, % PB_% of polymorphic bands TB_Total number of scorable bands, PB_Number of polymorphic bands, PIC_Polymorphism Information content.

Introduction

Lentil (*Lens culinaris* Medikus. *culinaris*) is a diploid (2n = 2x = 14), self pollinated and annual cool season grain legume with genome size of 4,063 Mpb (Arumuganathan and Earle, 1991). Globally lentil was grown in 4.16 million hectares with production of 4.41 million tons during 2011. The important lentil producing countries are Canada, India, Turkey, Nepal, Australia, China, Iran, the United States, Syria, and Ethiopia (Harish *et al*., 2012). In India lentil was grown in about 1.48 m ha with production of 1.03 m ton during 2010-11 (FAOSTAT, 2011). Lentil seeds are valued source of food for quality protein and fiber (Tickoo et al., 2005). Lentil is important source of energy, protein, carbohydrate, fibre, mineral, vitamin and antioxidant compound. Lentil is good source of vitamin A, vitamin B complex potassium, zinc and iron. Lentil has an excellent macro and micronutrient profile and favourable levels of mineral bioavailability enhancing factors. Lentil straw is equally valuable animal feed and fodder. In India lentil is mainly grown as rainfed crop in Uttar Pradesh, Madhya Pradesh, Jharkhand, Bihar and West Bengal. The population density is high in these lentil growing regions in India so is the malnutrition and micronutrient deficiency (Harish et al., 2012). Micronutrient malnutrition caused due to non/poor availability of minerals is a major problem in developing countries. At global level over 3 billion people (UNSCN, 2004) are affected. In India 230 million are affected (Lodha et al., 2005) by micronutrient malnutrition. Iron deficiency results in anaemia affecting cognitive development, growth, reproductive performance and work productivity (Bouis, 2002), whereas the Zinc deficiency causes anorexia, depression, impaired growth and development, altered reproductive biology, gastro-intestinal problems and impaired immunity (Solomons, 2003). Dietary diversification, supplements and fortified foods are the possible means to reduce micronutrient malnutrition. Biofortification involves increasing the levels of specific, limiting micronutrients in edible tissues of crops by combining crop management, breeding, and genetic approaches (Banziger and Long, 2000; Pfeiffer and McClafferty, 2007). Screening of available lentil germplasm for grain Fe and Zn concentration is prerequisite for their utilization in breeding programmes for enhancing of these traits in commercial cultivars. DNA markers provide an opportunity for precise characterization of genotypes and measurement of genetic relationships over morphological and

isozyme markers (Soller and Beckmann, 1983; Kumar et al., 2011). Different DNA based molecular markers have been used by different workers to study the genetic diversity in Lens. The studied markers include restriction fragment length polymorphism (RFLP) analysis of nuclear DNA (Havey and Muehlbauer, 1989) and chloroplast DNA (Muench et al., 1991; Mayer and Soltis, 1994) amplified fragment length polymorphism (AFLP) analysis (Sharma et al., 1996, Fiocchetti et al., 2004), inter simple sequence repeat (Sonnante and Pignone, 2001) and random amplified polymorphic DNA (RAPD) analysis (Abo-Elwafa et al., 1995; Sharma et al., 1995; Ford et al., 1997) sequence-tagged microsatellite (Agrawal and Katiyar, 2008) and simple sequence repeats (Babayera et al., 2009; Bacchi et al., 2010; Massimo et al., 2012). However, Simple Sequence Repeats (SSRs) offer several advantages for population genetic studies over other markers such as locus specificity, which are often multi-allelic because of their high mutation rate, co-dominance and high repeatability. Since improved cultivars are frequently used as parental materials in hybridization programmes, an understanding of the genetic diversity among them would be of immense value for their utilization for further enhancement of productivity of lentil genotypes. The present investigations were conducted to identify grain Fe and Zn rich stable lentil genotypes and analyze genetic diversity in elite lines of lentil using SSR markers. This is first report on estimation of Fe and Zn concentration and its stability across locations in lentil from South Asia.

Results and Discussion

Fe and Zn studies over locations

The ANOVA indicated that the genotypes under study differed significantly ($P \leq 0.01$) among themselves for both grain Fe and Zn concentrations at all the three locations. The ranges for grain Fe concentration were 53.73-131.53 mg/kg (mean 87.30±5.20 mg/kg) at Delhi, 40.13-160.66 mg/kg (mean 72.00±2.97 mg/kg) at Ludhiana, and 40.63-141.42 mg/kg (mean 68.99±3.68 mg/kg) at Pantnagar, while the range across all the three locations was 50.85-136.91 mg/kg (mean 76.11±10.48 mg/kg). The range for grain Zn concentration was 37.60-102.68 mg/kg (mean 68.60±3.21 mg/kg) at Delhi, while the ranges for Ludhiana and Pantnagar were 47.73-91.15 mg/kg (mean 64.630±3.05 mg/kg) and 22.53- 66.60 mg/kg (mean 45.004±3.67 mg/kg), respectively (Table 1).The range across the three environments was found to be 40.20-80.57 mg/kg (mean 59.41±5.96mg/kg). The environmental index was negative at Ludhiana and Pantnagar (-4.10 & -7.10) for grain Fe concentration and at Pantnagar (-14.40) for grain Zn concentration.

Stability analysis

The pooled analysis of variance (Table 2) was carried out after ascertaining the homogeneity of error variance using the Bartlett's test. G × E interactions were found to be significant (at $P \leq 0.01$) for both the micronutrients. Although both micronutrients are influenced by environment grain Fe expressed higher G × E interaction in comparison to grain Zn concentration. Similar finding was reported in maize (Chakraborti et al., 2011).

The ANOVA for stability using Eberhart Russel model revealed variation due to G × E (linear) significant for grain Fe and non significant for grain Zn. However environment (linear) was significant for both grain Zn and Fe (Table 3). The grain micronutrient concentration depends upon factors like soil type, soil fertility status, soil moisture, G × E, genotypic variation, soil profile, crop management practice and interaction among nutrients (Pfeiffer and McClafferty, 2007; Mandal et al., 2009). Soil Fe and Zn concentration at three locations were as follows, 5.01 mg/kg Fe and 1.68 mg/kg Zn at IARI New Delhi, 4.2 mg/kg Fe and 0.62 mg/kg Zn at Ludhiana, 4.5 mg/kg Fe and 0.985 mg/kg Zn at Pantnagar. The soil status exhibited variation for Fe and Zn concentration at the studied locations. In addition to this factors like microclimatic effects and meteorological parameters play important role. Significant effects of genotype × location and genotype × year interaction for kernel Fe; and genotype × location × year interaction for kernel Zn were earlier reported in maize (Oikeh et al., 2004; Chakraborti et al., 2011). Despite various factors affecting grain micronutrient status including differential behaviour of genotypes in locations, this study was successful in identifying promising lentil genotypes based on mean value of genotypes, regression values and deviations from regression. Six lentil genotypes viz. L4589, VL140, PL104, LH07-28, KLB 102, LL1190 were identified as stable genotype for grain Fe concentration and VL140, RLG109, HPCL649, PL063, L4588, KSL314, LL114, DL10-3, VL141, LH07-26, LL1190, IPL406, PL099, IPL320, L4704 were identified as stable genotype for grain Zn concentration (Table 4). The 41 genotypes are classified for high, medium and low Fe & Zn concentration (Table 5). The study indicated higher sensitivity of grain Fe to environmental fluctuations. The present study revealed no correlation between grain Fe and Zn concentrations at all the three locations in the analysed genotypes. This indicated the possibility of genetically improving the two target traits independent of each other using this specific set, including the most promising genotypes.

Microsatellite marker polymorphism

The genetic variation was detected among forty one lentil genotypes using 32 SSR primers (Table 6). The 32 markers produced a total of 130 amplified products. The SSR marker PLC 46 amplified the maximum number of 7 bands and SSR primers PLC 16 and PBALC 207 amplified a minimum of 2 bands. The PIC value ranged from 0.13 (PBALC 219) to 0.79 (PLC 42). Hamweih et al. (2009) also reported large variation among microsatellite markers for both allele numbers and gene diversity among lentil species. In total 8 rare alleles (present in less than 5% frequency) and 7 unique alleles (present in one genotype only) were observed at 7 SSR loci. Higher number of rare and unique alleles suggests that genotypes containing these alleles are more diverse or less utilized in the breeding programme.

Genetic relationships

The estimates of genetic similarity based on Jaccards' coefficient observed during the investigation ranged from 0.20 to 0.86 with an average of 0.53, which indicates the existence of high degree of variability among the studied lentil genotypes. The studied genotypes were grouped in two major clusters (Fig 1). Cluster 1 comprised of eighteen genotypes which were grouped in two sub clusters. Sub cluster 1a comprised of seventeen genotypes with both microsperma (2-6 mm diameter grain size) and macrosperma (6-9 mm diameter grain size) genotypes. This sub cluster is further divided into two groups. Group 1a (I) comprising of 4 genotypes LH 84-8, DL 10-1, KSL 107, IPL 406. LH 84-8

Table 1. Details of grain source, pedigree, mean Fe and Zn concentration of studied lentil genotypes.

S. No	Entry	Grain source	Pedigree	Grain Fe mean (mg/kg grain)	Grain Zn mean (mg/kg grain)
1	L 4589	IARI, New Delhi	L 4603 x PKVL 1	69.04	64.61
2	DL 10-2	TCA, Dholi	ARUN x PL 406	73.34	65.02
3	VL 140	VPKAS, Almora	VL 501 x SEHORE 74-3	97.07	73.96
4	PL 406	GBPUAT, Pantnagar	Selection from P495	73.17	57.35
5	IPL 219	IIPR, Kanpur	ILL 7657 x DPL 61	80.28	60.31
6	RLG 109	ARS, Durgapura	RLG 14 x L 4076	82.81	59.33
7	PL 4	GBPUAT, Pantnagar	UPL 175 (PL 184 x P 288)	65.20	40.26
8	HPCL 649	HPKVV, Palampur	L 4148 x VIPASHA	84.68	48.03
9	PL 101	GBPUAT, Pantnagar	PL 5 x FLIP 9671	69.24	59.84
10	PL 063	GBPUAT, Pantnagar	DPL 59 x IPL 105	76.25	52.28
11	L 4588	IARI, New Delhi	L 4147 x PL 4	64.84	51.82
12	KSL 314	CSAU, Kanpur	DPL 62 x LG 6O	66.38	58.55
13	DL 10-1	TCA, Dholi	L 9-12 x PL 639	64.71	59.48
14	PL 104	GBPUAT, Pantnagar	PL 5 x DPL 58	59.31	54.77
15	L 4147	IARI, New Delhi	(L 3875 x P 4) PKVL 1	75.62	60.57
16	LL 1114	PAU, Ludhiana	LL 699 x LL 773	62.16	59.61
17	HPCL 617	HPKVV, Palampur	VIPASHA x PL 639	72.32	60.75
18	DL 10-3	TCA, Dholi	Not available	70.00	55.45
19	SL2-28	Shillongani	ILL 7617 x ILL 2573	81.37	54.57
20	VL 141	VPKAS, Almora	VL4 x VL 501	101.8	81.54
21	KSL 107	CSAU, Kanpur	KLS 224 x KLS 233	60.41	50.42
22	IPL 322	IIPR, Kanpur	(DPL44 x DPL62) x DPL 58	63.35	56.80
23	RVL 32	MPKVV, Sehore	SL 94-14 x JL 3	59.86	62.93
24	LH 07-28	CCS HAU Hisar	LH 84-8 x IPL 138 (L 9-104	60.46	60.86
25	DPL 62	IIPR, Kanpur	JL 1 x LG 171	112.9	72.09
26	KLB 102	CSAU, Kanpur	L 4076 x Precoz	68.94	61.42
27	LL 1161	PAU, Ludhiana	IPL 70 LL 811	72.21	62.88
28	LH 07-26	CCS HAU Hisar	LH 90-54 x L 4641	102.4	66.34
29	LH 84-8	CCS, HAU Hisar	L 9-12 x JLS 2	109.2	64.00
30	VL 520	VPKAS, Almora	DPL 15 x SEHORE 74-3	109.8	75.25
31	IPL 321	IIPR, Kanpur	K 75 x DPL 62	80.68	51.66
32	PL 024	GBPUAT, Pantnagar	L 4076 x DPL 15	74.73	59.17
33	KLB 104	CSAU, Kanpur	K 75 x KLB 137	53.21	49.11
34	L 4705	IARI,New Delhi	Precoz x LC 74-1-5-1	50.85	56.84
35	DPL 15	IIPR, Kanpur	PL 406 x L 4076	53.60	52.17
36	PL 100	GBPUAT, Pantnagar	PL 5 x DPL 15	68.50	53.84
37	LL 1190	PAU, Ludhiana	LL 699 x IPL 124	75.14	58.16
38	IPL 406	IIPR, Kanpur	DPL 35 x EC 157634 / 382	70.05	48.77
39	PL 099	GBPUAT, Pantnagar	PL 5 x L 4603	82.85	57.37
40	IPL 320	IIPR, Kanpur	ILL 6002 JL 1	64.60	66.91
41	L 4704	IARI, New Delhi	L 4149 x L 4076	136.9	71.69
Locations					
New Delhi				87.30±5.20	68.60±3.21
Ludhiana				72.00±2.97	64.63±3.05
Pantnagar				68.99±3.68	45.00±3.67
Over all mean across location				76.11 ±10.48	59.41 ±5.96

and DL 10-1 exhibited a very high similarity coefficient 0.71, were high in Zn concentration and evolved from common ancestor L 9-12. Based on Jaccards' coefficient value IPL 406 was identified as most diverse genotype in this group. Group 1a (II) comprised of thirteen genotypes exhibiting high level of micronutrients. Among these, eight genotypes were high in Fe concentration and seven were high for Zn concentration. The microsperma type RLG109 and L4588 were highly similar with similarity coefficient of 0.67.Sub cluster Ib included only one genotype i.e. IPL 322. Cluster 2 comprised of twenty three genotypes grouped in two sub clusters. Sub cluster 2a included six genotypes depicting that the clustering pattern was based exclusively on grain size because PL104, VL141 and SL2-28 were grouped together which are microsperma type and IPL321 and PL100 were grouped together which are macrosperma type. Microsperma types PL 104 and VL 141 exhibited greater(0.65) similarity while the small seeded exotic derivative SL 228 exhibited 54 % similarity with PL 104 and VL 141. Sub cluster 2b comprised of seventeen genotypes and both microsperma and macrosperma genotypes were included in this sub cluster. This is further divided into two groups. Group 2b (I) comprised of eight genotypes most of these were low in micronutrients concentration. Microsperma types HPCL 617 and PL 4 revealed highest similarity (0.59) and both were low in Fe concentration. Group 2b (II) included nine genotypes of which four were macrosperma and five were microsperma types. Six and seven genotypes of these were high in Fe

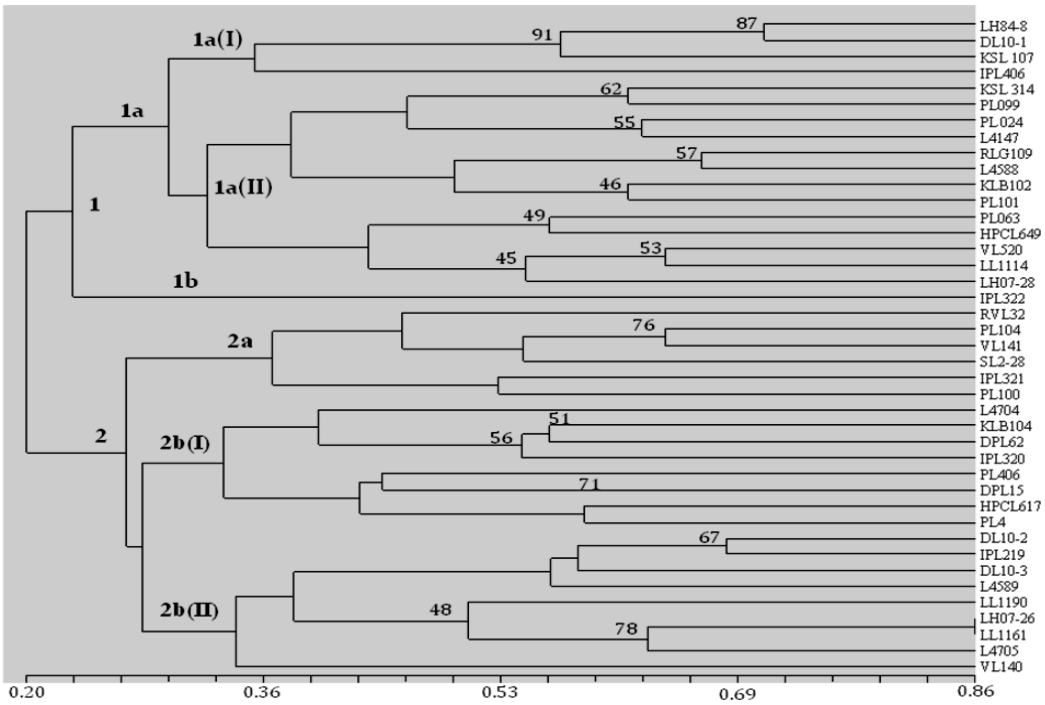

Fig 1. Dendrogram obtained by UPGMA analysis of SSR marker from 41 genotypes of lentil.

concentration and Zn concentration respectively. Macrosperma types LH 07-26 and LL 1161 exhibited high similarity possessed high Fe and Zn concentration. Microsperma types DL 10-2 and IPL 219 also exhibited high micronutrients concentration. Based on Jaccards' coefficient value VL 140 was most diverse genotype type in this group.

Iron deficiency anemia is the most common nutritional disorder in the world. More than 40% of women and preschool children in Asia and Africa are estimated to have low hemoglobin levels mainly due to Fe deficiency (World Health Organization, 2010). Zinc, another important trace element, deficiency was recognized in the early 1900s, is responsible for stunting, lower respiratory tract infections, malaria, and diarrhoeal disease. Moreover, approximately 1.4% (0.8 million) worldwide deaths were attributable to Zn deficiency (World Health Organization, 2010). Therefore, Fe and Zn are two essential nutrients for human nutrition. The nutritional benefit of pulses as a part of the diet has long been known. However, recent work carried out on lentil within global mineral biofortification efforts has highlighted its superior nutritional profiles. Lentils are a rich source of highly bioavailable minerals possibly due to their favorable food matrix factors; high in carotene and prebiotics and low in phytates (Tickoo et al., 2005).Ten potential crosses namely, PL099 × VL140, L4147 × VL140, RLC109 × VL140, KLB102 × VL140, IPL322 × VL140, LH84-8 × VL140, DL10-1 × SL2-28, KSL314 × SL2-28, PL024 × PL100, VL520 × L4705 were identified on the basis of molecular diversity for micronutrient concentration. Six crosses VL 520 × L 4705, L 4704 × L 4588, LH 84-8 × PL 4, PL 024 × PL4, LH 07-26 × KSL 107, LH 07-26 × IPL 406, were identified on the basis of combined analysis of molecular diversity and genotypic diversity in multi-location trials. The parents identified in the six crosses were highly dissimilar exhibiting a total similarity range of 0.68 to 0.86 for micronutrients concentration. Study on iron and zinc analysis identified donors which can be used to study of mode of inheritance, development of mapping population for QTL mapping. This analysis in combination with molecular characterization resulted in selection of diverse donor parents which can be included in recombination breeding for development of iron and zinc rich varieties. During last twenty years lot of effort were made to incorporate earliness and bold size in the traditional pilosae type using ICARDA germplasms (Asghar et al., 2010). In the recent years emphasis has been on earliness and bold seed size in Indian lentil breeding programmes. These traits are being transferred from Mediterranean lentil germplasm required from ICARDA. For broadening the genetic base wide crosses are required involving Mediterranean land races and wild species (Tickoo et al., 2005). ICARDA has identified sources of resistance to vascular wilt and ascochyta blight, and cold tolerance in the crossable wild progenitor *L. culinaris* spp. *orientalis* (Ferguson et al., 2000). These traits can be transferred to cultivated lentil from this species. Further broadening of the genetic base intra-specific hybridization involving early maturing Mediterranean germplasm lines and indigenous agronomic bases and inter-specific hybridization are warranted for successful lentil improvement programme (Erskine et al., 1998).

Materials and Methods

Genetic material

The experimental material for the present study comprised of 41 lentil genotypes developed at different lentil breeding centres of India, under All India Coordinated Research Project on mungbean, urdbean, lentil, lathyrus, rajmash and pea Initial Varietal Trial (IVT) trials. IVT trials are conducted across the country to evaluate the yield potential. The studied elite lentil lines are the entries of IVT of this project. These

Table 2. Pooled ANOVA for grain Fe and Zn concentrations using general linear model.

Source of variation	Degree of freedom	Mean sum of squares	
		Grain Fe	Grain Zn
Genotypes	40	2990.60**	579**
Environments	2	11856.93**	19631.04**
Genotype x Environment	80	1017.08**	278.99**
Error	244	24.57	16.74

*Significant at P≤0.05; **Significant at P≤0.01

Table 3. ANOVA for stability of grain Fe and Zn concentrations using the Eberhart and Russell model.

Source of variation	Degree of freedom	Mean sum of squares	
		Grain Fe	Grain Zn
Genotypes	40	996.87**	193.21**
Environment +(Genotype × Environment)	82	427.27*	250.30**
Environment (Linear)	1	7905.06**	13089.60**
Gen. × Env. (Linear)	40	452.88*	113.05
Pooled deviation	41	219.90**	71.04**
Pooled error	240	8.25	5.52

*Significant at P≤0.05; **Significant at P≤0.01.

genotypes were analysed for grain Fe and Zn concentration (Table 1).

Field trials

The test entries were evaluated at three locations (i) Delhi (28º40'N, 77º12'E, 218 masl (meters above mean sea level) (ii) Ludhiana (30.9°N, 75.85°E 244 masl) and (iii) Pantnagar (28°58′N, 79°25′E, 344 masl) during winter season of 2010-11. The soils at these locations are deep well drained and light alluvium type. Soil Fe and Zn concentration at the experimental sites was estimated using standard procedures (Singh *et al*., 2005).The entries were planted in Randomized Complete Block Design (RCBD) with three replications per entry (4 rows per replication) with plant distance of 5cm × 30cm.

Grain micronutrient analysis

Biochemical analysis for grain Fe and Zn concentrations was carried out on triplicate grounded samples of grains by digestion with 9:4 diacid mixture (HNO_3: $HClO_4$) followed by atomic absorption spectrometry (AAS) method using ECIL AAS (Perkin Elmer) as per the protocol described by Zarcinas *et al*., (1987) and Singh *et al*., (2005).

Genomic DNA extraction

Total genomic DNA from 5 g of fresh young leaf tissue, collected from five random plants per accession, was extracted following the CTAB method as described by Murray and Thompson (1980). RNase treatment was given as suggested by Murray and Thompson (1980). The purified DNA was quantified on 0.8% agarose gel along with uncut lambda DNA (30 and 60 ng). The total genomic DNA was diluted to 20 ng/µl for PCR analysis.

Primer selection and polymerase chain reaction

Forty four SSR primers reported by (Hamwieh et al., 2005 and 2009), fifty eight SSR primers reported by (Kaur et al., 2011) and 18 EST SSR primers developed in our laboratory exhibiting polymorphism across *Lens* species were assayed for identification of polymorphic SSR primers. The primers were pre-screened and thirty two SSR primers exhibiting polymorphism were selected for genetic diversity analysis.

The PCR conditions for SSR primer were as follows: initial denaturation at 94°C for 3 min followed by 30 cycles with 30 sec at 94°C, 30 sec at 54–60°C (depending upon the primer), 1 min at 72°C with final extension at 72°C for 5 min. PCR reaction mixture consist of 20µl containing 10x buffer (100 mM Tris-Hcl, 500mM KCL, 15MM $MgCl_2$, 0.01% gelatin) 40 ng of template DNA, 0.5µM of each primer, 200µM of each dNTPs, and 1 unit Taq DNA polymerase (Sigma Aldrich) for the markers. The amplification products with microsatellite markers were resolved on 3% metaphor gel. The products were electrophoresed for 3 h at a constant voltage of 100 V in 1X TBE buffer and photographed with a CCD camera attached to a gel documentation system (Syngene). Molecular weights of the bands were estimated with 100 bp DNA ladder (Bangalore Genei) as standard.

Statistical analysis

ANOVA of the grain micronutrient data from the three location trials was carried out using SAS 9.3. Stability analysis was performed following the Eberhart and Russell (1966) model, using Windostat (Version 8.0, Indostat Services) software. The sum of square due to G × E were portioned into individual genotypes (X-i), regression of environmental means (bi) and deviation from regression (S^2d). The regression coefficients (bi) and mean square deviation from regression (S^2d) were used to define genotype stability i.e. suitability of a genotype for general cultivation over a wide range of environments. The environmental mean was the mean of all genotypes in each environment. The pooled error was used to test the hypothesis that the mean square deviation did not differ significantly from 0 at 0.05 and 0.01% probability levels. Fragments amplified by primer sets were scored manually in terms of positions of the bands relative to the ladder sequentially from the smallest to the largest-sized bands. Diffused bands or bands revealing ambiguity in scoring will be considered as missing data and designated as '9'in comparison with '1' for the presence of a band and '0' for the absence of a band in the data matrix. A binary matrix was then transformed to genetic similarity (GS) matrix using Jaccard's coefficient (Jaccard, 1908). A dendrogram based on similarity coefficients was prepared by using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) using statistical software NTSYS-PC 2.02 (Rohlf, 2000). Robustness of the clusters was evaluated by bootstrap analysis using WIN BOOT software (Yap and Nelson, 1996).

Table 4. Stability parameters of forty one lentil genotypes studied across 3 locations.

Entry	Grain Fe			Grain Zn		
	Mean (mg/kg)	bi	S^2Di	Mean (mg/kg)	bi	S^2Di
L 4589	69.04	0.718	8.1629	64.61	0.055	464.5161 ***
DL 10-2	73.34	1.071	217.7458 ***	65.02	1.126	136.7532 ***
VL 140	97.07	-0.320	-0.1920	73.96	2.742	-2.6812
PL 406	73.17	0.448	25.9123 *	57.35	0.747	40.6942 **
IPL 219	80.28	2.049	629.0384 ***	60.31	1.171	44.1816 **
RLG 109	82.81	0.732	25.1547 *	59.33	1.468	-5.5436
PL 4	65.20	1.016	57.1777 **	40.26	0.574	148.7015 ***
HPCL 649	84.68	0.439	855.6990 ***	48.03	0.892	0.5029
PL 101	69.24	1.559	132.9263 ***	59.84	0.779	211.1435 ***
PL 063	76.25	3.892	119.7133 ***	52.28	-0.129	4.7530
L 4588	64.84	0.921	192.9403 ***	51.82	1.224	-4.2521
KSL 314	66.38	-0.243	321.1265 ***	58.55	0.803	-2.7571
DL 10-1	64.71	0.415	522.7945 ***	59.48	0.820	174.544 ***
PL 104	59.31	1.070	0.3849	54.77	0.710	75.1984 ***
L 4147	75.62	0.993	339.9198 ***	60.57	1.327	34.6805 **
LL 1114	62.16	1.252	32.9939 *	59.61	0.660	-1.0992
HPCL 617	72.32	5.365	122.8047 ***	60.75	0699	99.1296 ***
DL 10-3	70.00	3.872	187.4617 ***	55.45	0.044	-4.2446
SL2-28	81.37	2.961	24.5397 *	54.57	1.145	38.9383 **
VL 141	101.5	0.520	124.5879 ***	80.57	1.907	1.4826
KSL 107	60.41	0.492	63.9432 **	50.42	1.143	121.2295 ***
IPL 322	63.35	1.644	34.9536 *	56.80	0.685	64.3449 ***
RVL 32	59.86	2.060	211.3645 ***	62.93	0.612	62.1738 ***
LH 07-28	60.46	1.412	-4.6594	60.86	1.100	66.2352 ***
DPL 62	112.9	-0.999	110.1393 ***	72.09	1.149	16.3252 *
KLB 102	68.94	0.757	6.1806	61.33	1.001	50.1286 **
LL 1161	72.21	-0.445	347.6073 ***	62.88	0.331	401.5570 ***
LH 07-26	102.4	-0.294	199.7945 ***	66.34	2.219	-1.6542
LH 84-8	109.2	0.481	50.6483 **	64.00	1.701	87.1542 ***
VL 520	109.8	-1.082	31.2640 *	75.25	1.620	89.8492 ***
IPL 321	80.68	-0.138	986.4743 ***	51.66	-0.009	81.4757 ***
PL 024	74.73	-0.459	1424.87 ***	59.17	1.424	46.9530 **
KLB 104	53.21	0.237	284.5388 ***	49.11	0.833	50.8366 **
L 4705	50.85	0.246	81.3368 **	56.84	0.131	42.2122 **
DPL 15	53.60	0.912	62.1308 **	52.17	1.057	37.8502 **
PL 100	68.50	1.630	124.7327 ***	53.84	1.120	25.4574 *
LL 1190	75.14	5.016	-5.1478	58.16	1.162	-3.6224
IPL 406	70.05	1.130	145.8696 ***	48.77	1.838	-2.3634
PL 099	82.85	0.033	150.1722 ***	57.37	1.432	1.4071
IPL 320	64.60	1.947	81.4221 **	66.91	0.695	-5.5722
L 4704	136.9	-2.313	342.5436 ***	71.69	0.992	-2.2102
Mean ± SE	76.11 ± 10.48			59.41 ± 5.96		

*Significant at P≤0.05; **Significant at P≤0.01

Table 5. Classification of genotypes for high, medium and low Fe & Zn concentration.

Fe concentration	Genotypes
High (>75 mg/kg)	DL10-2, VL140, IPL219, RLG109, HPCL649, PL063, L4147, SL2-28, VL141, DPL62, LH07-26, LH84-8, VL520, IPL321, LL1190,PL099,L4704
Medium (55-75 mg/kg)	L4589, PL406, PL4, PL101, L4588, KSL314, DL10-1, PL104, LL1114, HPCL617, DL10-3,KSL107, IPL322, RVL32, LH07-28, KBL102, LL1161, PL024, PL100, IPL406, IPL320
Low (<55 mg/kg)	KBL104, L4705, DPL15
Zn concentration	Genotypes
High (>60 mg/kg)	L4589, DL10-2, VL140, IPL219, L4147, HPCL617, VL141, RVL32, LH07-28, DPL62, KBL102, LL1161, LH07-26, LH84-8, VL520, IPL320, L4704
Medium (45-60 mg/kg)	PL406, RLG109, HPCL649, PL101, PL063, L4588, KSL314, DL10-1, PL104, LL1114, DL10-3, SL2-28, KSL107, IPL322, IPL321, PL024, KBL104, L4705, DPL15, PL100, LL1190,IPL406, PL099
Low (<45 mg/kg)	PL4

Table 6. Melting temperature, Total bands, Number of polymorphic bands, Polymorphic information content produced by 32 SSR markers in 41 lentil genotypes.

S.No	Primer	Bp	Tm^0C	TB	PB	PIC	S.No	Primer	Bp	Tm^0C	TB	PB	PIC
1	PLC4	343	60	3	3	0.32	17	LC24	111	55	4	4	0.62
2	PLC5	345	59	4	4	0.59	18	LC25	261	56	4	4	0.39
3	PLC10	296	60	4	4	0.68	19	LC26	182	55	3	3	0.65
4	PLC16	272	60	2	2	0.34	20	LC30	253	54	5	5	0.71
5	PLC17	314	58	3	3	0.57	21	PBALC203	141	55	5	5	0.60
6	PLC30	242	60	4	4	0.33	22	PBALC207	141	54	2	2	0.21
7	PLC35	258	60	3	3	0.55	23	PBALC209	152	55	3	3	0.13
8	PLC38	302	60	6	6	0.75	24	PBALC219	166	55	5	5	0.31
9	PLC39	182	58	3	3	0.71	25	PBALC224	155	55	4	4	0.70
10	PLC40	158	58	4	4	0.72	26	PBALC238	155	56	3	3	0.42
11	PLC42	145	59	6	6	0.79	27	PBALC250	149	54	3	3	0.50
12	PLC45	189	58	5	5	0.77	28	PBALC260	153	54	4	4	0.61
13	PLC46	200	60	7	7	0.75	29	PBALC264	145	54	6	6	0.76
14	PLC51	121	60	3	3	0.64	30	PBALC265	155	54	5	5	0.21
15	PLC74	175	54	5	5	0.77	31	PBALC347	153	55	3	3	0.61
16	PLC82	165	54	3	3	0.64	32	PBALC353	155	55	6	6	0.74

Tm℃ - Annealing temperature, % PB- % of polymorphic bands TB – Total number of scorable bands, PB - Number of polymorphic bands, PIC – Polymorphism Information content

Polymorphism information content (PIC) values for each band was calculated based on allele frequencies according to Smith *et al*. 1997, as $PIC=1-\Sigma Pi^2$, where Pi is the band frequency of the i^{th} allele.

Conclusion

The study of forty-one elite lentil lines at three locations in India revealed significant variation for grain Fe and Zn concentration. The variation detected can be utilized for genetics studies and also for development of mapping population for identification of QTLs linked to these traits. The detection of variation indicates that the grain Fe and Zn concentration can be improved in Indian lentil cultivars using conventional and molecular technique.

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