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QTLs for iron concentration in seeds of the cultivated lentil (*Lens culinaris* Medic.) via genotyping by sequencing

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Abstract: Lentil, *Lens culinaris* Medic., is an important cool season grain legume because of the high level of iron (Fe) in its seeds since Fe deficiency is widespread and causes anemia. Thus, identifying genes controlling Fe concentration in the seed was needed for mapping in the lentil genome. The objectives of this study were to (i) detect phenotypic variation in Fe concentration in the seeds of a recombinant inbred line (RIL) population, (ii) construct a high-density linkage map using genotyping by sequencing (GBS), and (iii) identify localization of the quantitative trait loci (QTLs) controlling genes for Fe concentration in lentil seeds. In this work, Fe concentration in seeds of the RIL population ranged from 37.2 to 175.7 mg per kg. A linkage map was constructed covering 497.1 cM with a total of 4177 SNP markers. A total of 21 QTL regions explaining 5.9%–14.0% of the phenotypic variation were identified on six linkage groups (LG1, 2, 4, 5, 6, and 7) with LOD scores ranging from 3.00 to 4.45. This is the first report on the construction of a high-density linkage map through GBS and mapping of QTLs controlling iron uptake in lentil. Identification of these genomic regions will be useful for future biofortification studies to develop new varieties of lentil with high Fe concentrations.

Key words: GBS, DArT, SNP, QTL mapping, iron, lentil

1. Introduction

Lentil, *Lens culinaris* Medic., is an important cool season food legume in the old world. It is a self-pollinated, diploid (2n = 2x = 14) with a genome size of 4063 Mbp (Arumuganathan and Earl, 1991). The origin of cultivated lentil is the Near East Arc and Asia Minor (Zohary and Hopf, 2000; Muehlbauer and McPhee, 2005). From this region, the crop was distributed in Europe, Asia, Northern Africa and Ethiopia, the Indian subcontinent, North and South America, southern Africa, and Australia (Ford et al., 2007). Lentil is an important global crop for the human diet and daily food balance (Karakoy et al., 2012) in the case of richness with iron (Fe), selenium (Se), copper (Cu), Mn and other dietary nutrients in its seeds (Grusak, 2009). World annual lentil production was about 4.9 million tons in 2014 and per capita consumption has been increasing faster than human population growth (FAOSTAT, 2016).

Micronutrients such as Fe, Zn, Ca, Mn, and P are essential for metabolic pathways and reactions in the human body. Inadequate daily intake of these nutrients causes deficiencies resulting in diseases (Garcia-Oliveira et al., 2009). Worldwide, micronutrient deficiency is one of the most important health problems. Fe deficiency is widespread, affecting 3.7 billion people (Welch, 2002). Annually, 1.5% (0.8 million) of deaths worldwide are directly related to Fe deficiency (WHO, 2002). Fe deficiency causes Fe deficiency anemia (IDA), which leads to workforce loss and complications in childbirth (Blair et al., 2011). Approaches to solving micronutrient deficiencies through biofortification are under development; the aim is to increase the concentration and/or bioavailability of mineral elements in the parts of plants consumed by humans (White and Broadley, 2009).

Fe is an integral part of iron- and oxygen-binding proteins, including hemoglobin (Hb) and myoglobin (Mb). Fe is integral to the transport of oxygen from the lungs to all body cells and to the storage of oxygen carried to muscles. Fe is also an essential component of key cellular enzymes for energy production and metabolism (Moritz and Hornecker, 2006). Fe enzymes are involved in electron

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transport and energy metabolism (cytochrome c oxidase, cytochromes, aconitase, NADH dehydrogenase, and succinatedehydrogenase), immune system function (nitric oxide synthase, myeloperoxidase, eosinophil peroxidase, and lactoperoxidase), antioxidant function (peroxidases and catalase), and neuronal functions (tyrosine peroxidase, tyrosine hydroxylase, tryptophan hydroxylase, and xanthine oxidase) (Beard, 2001; Arrendo and Nunez, 2005; Iannotti et al., 2006; Burckhardt-Herold, 2013). Fe-containing enzymes and/or proteins are incorporated into DNA synthesis (ribonucleotide reductase) and are essential for cell growth and proliferation (Cazzola et al., 1990; Lieu et al., 2001; Adlerova et al., 2008). The recommended dietary allowance (RDA) is 8 mg per day for all age groups of men and women but it is 18 mg for premenopausal women (Institute of Medicine, 2001).

The Fe concentration in edible seeds has been studied in staple food crops such as rice (*Oryza sativa* L.) (Gregorio et al., 2000), common bean (*Phaseolus vulgaris* L.) (Islam et al., 2002), maize (*Zea mays* L.) (Oikeh et al., 2003), wheat (*Triticum aestivum* L.) (Garvin et al., 2006), chickpea (*Cicer arietinum* L.) (Zia-Ul-Haq et al., 2007), and soybean [*Soja max* (L.) Merill.] (Ramamurthy et al., 2014). Lentil seed contains Fe between 73 and 90 mg per kg (Thavarajah et al., 2011). The concentration of Fe in lentil seed is higher than in wheat grain (Ates et al., 2016). Studies of quantitative trait loci (QTLs) that control Fe concentration in seeds have been conducted for thale cress [*Arabidopsis thaliana* (L.) Heynh.] (Vreugdenhil et al., 2004; Waters and Grusak, 2008), rapeseed (*Brassica rapa* L.) (Wu et al., 2008), canola (*Brassica napus* L.) (Ding et al., 2010), rice (*Oryza sativa* L.) (Stangoulis et al., 2007; Lu et al., 2008; Garcia-Oliveira et al., 2009; Norton et al., 2010; Anuradha et al., 2012), *Lotus japonicus* L. (Klein and Grusak, 2009), barrelclover (*Medicago truncatula* Gaertn.) (Sankaran et al., 2009), common bean (*Phaseolus vulgaris* L.) (Cichy et al., 2009; Blair et al., 2009, 2010, 2011), wheat (*Triticum* sp.) (Peleg et al., 2009; Tiwari et al., 2009; Xu et al., 2012; Pu et al., 2014; Srinivasa et al., 2014), maize (*Zea mays* L.) (Simic et al., 2012; Jin et al., 2013), and soybean (Ramamurthy et al., 2014). These studies identified QTLs linked to the genes controlling Fe accumulation in seed.

Single nucleotide polymorphism (SNP) markers are the most abundant genetic markers spread over the whole genome (Agarwal et al., 2008). Recently, a new approach known as genotyping- by-sequencing (GBS) has been used for SNP discovery and genotyping (Kujur et al., 2015). In this technology, a mapping population is created using selected two parents. Using NGS technologies, the parents and their lines are sequenced to identify SNPs. Sequences obtained from sequencing are used to establish allelic diversity per individual. Based on parental data, genotypes are assigned. After that, recombination maps are created for each RIL (Deschamps et al., 2012). GBS is used for population studies, characterization, breeding, and trait mapping in diverse organisms and is based on high-throughput, next-generation sequencing of genomic fragments derived by digesting the genome of each individual in a population with restriction enzymes (Elshire et al., 2011; Kumar et al., 2012). This type of high-throughput genotyping technology allows for the development of high-density applications in QTL characterization (Deschamps et al., 2012). For example, QTLs controlling genes for drought tolerance in chickpea were identified using GBS (Jaganathan et al., 2015). Due to cost efficiencies, GBS is a preferred approach when constructing linkage maps of mapping populations with high-density SNP markers (He et al., 2014). Many such SNP-based maps and/or QTL maps have been constructed in plant species such as lentil (Sharpe et al., 2013; Gujaria-Verma et al., 2014; Ates et al., 2016), maize (Chen et al., 2014; Li et al., 2015), maize and barley (Elshire et al., 2011), wheat and barley (Poland et al., 2012), chickpea (Jaganathan et al., 2015), cotton (*Gossypium hirsutum* L.) (Gore et al., 2014), potato (*Solanum tuberosum* L.) (Uitdewilligen et al., 2013), raspberry (*Rubus idaeus* L.) (Ward et al., 2013), wheat (Saintenac et al., 2013), apple (*Malus* spp.) (Gardner et al., 2014), barley (Mascher et al., 2013; Liu et al., 2014), canola (Raman et al., 2014), cassava (*Manihot esculenta* Crantz) (Rabbi et al., 2014), oat (*Avena sativa* L.) (Huang et al., 2014), and soybean (Sonah et al., 2013; Bastien et al., 2014). Before the current study, there was no study on QTL mapping of genes controlling Fe concentration in seeds of the cultivated lentil. Thus, the objectives of this study were to: (i) detect phenotypic variation in Fe concentration in a recombinant inbred line (RIL) population, (ii) construct a high-density linkage map using GBS technology, and (iii) localize the QTL(s) controlling genes for Fe concentration in lentil seed.

2. Materials and methods

2.1. Plant materials and field trials

Lentil RIL population LR11 was supplied from the Department of Plant Sciences/Crop Development Centre (CDC), University of Saskatchewan (UofS), Saskatoon, Canada. The LR11 population of 118 RILs was derived from the cross "ILL 8006" × "CDC Milestone". "ILL 8006" is also known as Barimasur-4, a lentil cultivar released in Bangladesh (Sarker et al., 1999). "CDC Milestone" was developed at the CDC (Vandenberg et al., 2001). The parents were contrasting for Fe concentration in their seeds based on an initial field evaluation near Saskatoon, Canada. The population was advanced by single seed descent until the F_7 generation. All 118 LR11 RILs and their parents ("ILL 8006" and "CDC Milestone") were sown at three field locations including Ege University in

İzmir (27°09′E, 38°25′N and 0 m sea level), Çukurova University in Adana (35°18′E, 37°01′N and 0 m sea level), and Harran University in Şanlıurfa (38°46′E, 37°08′N and 477 m sea level) in Turkey (during the 2012 and 2013 growing seasons. The field experiments were designed according to randomized complete block design (RCBD) with three replications. The plot size was 1.2 × 2.0 m and fertilizer was not applied in either year. In all locations, seeds for each RIL were increased in the first year; the increased RIL seeds were sown in 2012 and the seeds from 2012 were sown in 2013.

Soil samples obtained from the three experimental fields were analyzed for structural and chemical properties including pH (Black, 1965), total soluble salts (Richards, 1954), organic matter (Black, 1965), $CaCO_3$ (Schlicting and Blume, 1966), and macro- and micronutrients (Bingham, 1949; Pratt, 1965; Linsday, 1978). Texture analysis was conducted according to Bouyoucos (1955). All soil analyses were performed at the Department of Plant and Soil Science at Ege University in Turkey.

2.2. Fe concentration analysis in seeds

Fe concentrations in the seeds were determined using the method of Kacar (1972). First, all harvested lentil seeds of each individual RIL and their parents were washed with tap water and then with distilled water. After washing, the samples were dried at 65 °C and then ground using an analytical mill. One gram of ground seed sample was placed in a 150-mL Erlenmeyer flask. Acid was added (12 mL of nitric:perchloric acid of 4:1 ratio per 1 g of ground sample) and then wet decomposition was performed. The total Fe concentration of the prepared extract was measured by atomic absorption spectrophotometry (AAS) (Kacar, 1972; Kacar and Inal, 2008). Seed Fe concentration was detected as mg/kg. Standard Fe solutions of different concentrations (1, 2, 4, 6, 8, and 10 ppm) were prepared and measured in the AAS to prepare a calibration curve (linear; $r^2 = 0.999$). Each analysis was repeated three times.

2.3. DNA extraction

Young leaves from 4–6-week-old seedlings of the RILs and parents from İzmir in 2012 were harvested and placed in aluminum foil, labelled with RIL number, and then placed in liquid nitrogen before transfer to a –86 °C freezer. Each leaf sample was ground using a tissue lyser. Total genomic DNA of the RILs and parents was isolated using a QIAGEN isolation kit (Catalog No. 69181) according to the manufacturer's instructions. Purity of DNA was checked on 1% agarose gel by visual inspection. The purified DNA was quantified with a Qubit 2.0 Fluorometer.

2.4. Genotyping by sequencing analysis

Genotyping by sequencing analysis was carried out at DArT PLT (Diversity Arrays Technology Pty. Ltd., Canberra, Australia) following the protocol of Raman et al. (2014). DArTseq takes advantage of a DArT complexity reduction method and combines it with next generation sequencing platforms, providing a less complex representation of sequencing (Altshuler et al., 2000; Kilian et al., 2012; Courtois et al., 2013; Cruz et al., 2013; Ward et al., 2013). Similarly to DArT methods based on array hybridization, the technology was optimized for each organism and application by selecting for the most appropriate complexity reduction method (both the size of the representation and the fraction of a genome selected for assays). Four methods of complexity reduction were tested in olives (data not presented) and the *Pst*I-*Mse*I method was selected. DNA samples were processed in digestion/ligation reactions principally as described by Kilian et al. (2012), but instead of one single *Pst*I-compatible adaptor, two different adaptors were chosen to create two different restriction enzyme (RE) overhangs. In parallel to Elshire et al.'s (2011) previously reported sequence, the first adapter was designed as a *Pst*I- compatible adapter, and it consisted of a sequencing primer sequence, Illumina flowcell attachment sequence, and a barcode region that varies in length. The second adaptor was chosen with an *Mse*I-compatible overhang sequence as well as a flowcell attachment region. "Mixed fragments" containing *Pst*I-*Mse*I sites were exclusively amplified by PCR as follows: 94 °C for 1 min; 30 cycles of 94 °C for 20 s, 58 °C for 30 s, and 72 °C for 45 s; and then 72 °C for 7 min.

Next, amplification products containing equal amounts of DNA in the 96-well microtiter plate were mixed together and applied to c-Bot (Illumina) bridge PCR, followed by a sequencing (single read) step that was carried out with 77 cycles using Illumina Hiseq2500. Each generated sequence was processed by using DArT analytical pipelines as advised. The primary pipeline was used on the fastq files to eliminate the poor quality results. In this way, "barcode split" steps became more reliable with the addition of more strict criteria. In marker calling, for each barcode/sample, around 2,000,000 (±7%) sequences were used. As the last step, "fastqcall files" were created using identical sequences and were applied to the secondary pipeline for DArT PL's proprietary SNP and SilicoDArT (presence/absence of restriction fragments in representation) calling algorithms (DArTsoft14).

2.5. Linkage mapping and QTL analysis

The linkage map was constructed from genotype data using JoinMap 4.0 software (Van Ooijen, 2006) with a minimum LOD (logarithm of the odds) of 3.0–10.0 and a recombination fraction of 30 cM. Recombination frequencies were converted into map distances using the Kosambi mapping function (Kosambi, 1943). The positions of QTLs for seed Fe concentration were determined following simple interval mapping (SIM)

(Lander and Botstein, 1989) using MapQTL6 (Van Ooijen, 2009). The genome-wide LOD score threshold for QTL detection was determined using the permutation test (1000 repetitions) at a P value of 0.01 and 0.05 for normally distributed data (Churchill and Doerge, 1994). The LOD score threshold was set to three to declare the presence of a QTL. The amount of variation explained by each locus or combination of loci was calculated by multiplying the coefficient of phenotypic determination (r^2 value) by 100.

2.6. Variance analysis

Analysis of variance (ANOVA) was used to determine variation for Fe concentration of the RILs population grown for 2 years at three locations using TOTEMSTAT software (Acikgoz et al., 2004). Probability was accepted at $P \leq 0.01$ and $P \leq 0.05$ levels.

3. Results

3.1. Soil properties

Soil analyses showed that the three locations had very loamy, nonsaline, and slightly alkaline soils (Table 1). Organic matter content of the soil samples was low. Available Fe was at sufficient levels in all three locations.

3.2. Fe concentration in seeds

In 2012, seed Fe concentration of the RILs ranged from 37.2 to 238.5 mg/kg at İzmir, from 37.5 to 144.0 mg/kg at Adana, and from 35.5 to 149.0 mg/kg at Şanlıurfa. Mean seed Fe concentrations at İzmir, Adana, and Şanlıurfa in 2012 were 66.4, 62.5, and 64.5 mg/kg, respectively. In 2013, Fe concentration of the RILs ranged from 37.1 to 146.8 mg/kg at İzmir, from 39.2 to 151.9 mg/kg at Adana, and from 36.9 to 223.8 mg/kg at Şanlıurfa. Again, mean seed Fe concentrations in 2013 at İzmir, Adana, and Şanlıurfa were similar to the data in 2012, i.e. 62.9, 66.3, and 66.3 mg/kg, respectively (Table 2). Seed Fe concentrations of the parents, "CDC Milestone" and "ILL 8006", were 40.5 and 114.3 mg/kg, respectively, when averaged over the two years and three locations. Two lines of RILs (LR11-17 and LR11-133) had much higher seed Fe concentrations (223.8–238.5 mg/kg, respectively) than that of the high-Fe parent "ILL 8006" (Figure 1). To confirm these data showing high Fe concentrations in specific RILs, the same amounts of seeds of these individuals grown at İzmir were reanalyzed (data not shown). Heritability for Fe concentrations was determined as 0.95 and 0.96 for 2012 and 2013, respectively (Table 2).

Table 1. Chemical and structural properties of experimental field soils.

Soil properties	İzmir	Adana	Şanlıurfa
pH	4.8	4.7	4.7
Total salt (%)	0.040	0.031	0.043
$CaCO_3$ (%)	29.4	48.0	34.5
Organic matter (%)	1.8	1.2	1.9
Fine sand (%)	50.2	44.2	44.2
Silt (%)	28.0	26.0	32.0
Clay (%)	21.7	29.7	23.7
Texture	Loamy	Loamy clay	Loamy
Total N (%)	0.062	0.056	0.050
Available P (mg/kg)	3.2	2.6	1.8
Available K (mg/kg)	417	116	485
Available Ca (mg/kg)	6.272	6.762	7.252
Available Mg (mg/kg)	554	170	430
Available Na (mg/kg)	220	307	20
Available Fe (mg/kg)	4.3	5.0	6.5
Available Zn (mg/kg)	0.7	0.4	1.0
Available Cu (mg/kg)	1.1	0.1	0.6
Available Mn (mg/kg)	5.3	4.6	8.0

Table 2. Minimum, maximum, and mean for Fe concentration in lentil seeds (mg/kg) of parents and RILs at İzmir, Adana, and Şanlıurfa in 2012 and 2013.

Location	İzmir		Adana		Şanlıurfa		Overall mean
Years	2012	2013	2012	2013	2012	2013	
CDC Milestone	39.0	42.9	38.0	44.1	41.0	38.2	40.5
ILL 8006	112.5	123.8	100.5	126.1	116.5	106.5	114.3
Minimum	37.2	37.1	37.5	39.2	35.5	36.9	37.2
Maximum	238.5	146.8	144.0	151.9	149.0	223.8	175.7
Mean	66.4	62.9	62.5	66.3	64.5	66.3	64.8
Heritability	2012	2013					
	0.95	0.96					

Analysis of variance indicated that genotypes grown at different locations were not significantly different for seed Fe concentration (Table 3). The variance analyses showed that Genotype (G), Year × Location (Y × L), Location × Genotype (L × G), and Year × Location × Genotype (Y × L × G) interactions were significant whereas Year × Genotype (Y × G) was not. The histogram obtained from the means of seed Fe concentrations at İzmir, Adana, and Şanlıurfa in 2012 and 2013 showed nearly normal distribution (Figure 1). Transgressive segregants of RIL for Fe concentration were observed in the histogram (Figure 1).

3.3. Genetic map construction

The linkage map of the RIL was constructed with a total of 4177 SNPs (Table 4). These mapped into seven LGs covering 497.1 cM, with an average distance between markers of 0.12 cM. The length of LGs varied from 29.0 cM (LG3) to 161.5 cM (LG4). The average number of markers per linkage group was 596. LG4 had the highest number of markers (1224) with an average distance between markers of 0.13. LG3 had the lowest number of markers (258 SNPs), with an average distance between markers of 0.11. The average distance between markers for each LG group varied from 0.08 cM (LG1, 5, and 6) to 0.17 cM (LG2).

3.4. QTL analysis

A total of 21 QTL regions were detected for seed Fe concentration using SIM (Table 5). All QTL regions were identified in 2012 and 2013 except for FeQTL1.1 and FeQTL4.1. The QTL regions explaining 5.9%–14.0% of the phenotypic variation were located on six linkage groups (LGs) (LG1, 2, 4, 5, 6, and 7) and had LOD scores ranging from 3.00 to 4.45. LG4 had the most QTL regions (6 regions) identified (FeQTL4.1, FeQTL4.2, FeQTL4.3, FeQTL4.4, FeQTL4.5, FeQTL4.6). LOD scores and percentage explaining values (% expl.) of the QTL regions

Table 3. Analysis of variance for seed Fe concentration in the RIL population.

Source of variation	Degree of freedom	Mean squares	F
Year	1	49.0	0.13 ns
Location	2	343.2	0.95 ns
Genotype	117	13,274.6	36.92**
Year × Location	2	1865.6	5.19**
Year × Genotype	117	352.3	0.98 ns
Location × Genotype	234	480.3	1.33*
Year × Location × Genotype	234	724.4	2.01**
Error	1404	359.5	

ns: not significant, *: significant at $P < 0.05$ level, **: significant at $P < 0.01$ level

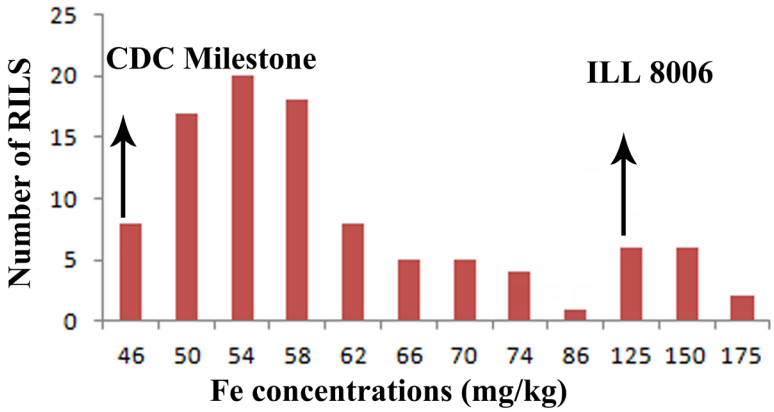

Figure 1. Frequency distribution of seed Fe concentration in the "ILL 8006" × "CDC Milestone" lentil RIL population measured at three locations in two years (2012 and 2013).

Table 4. Number and characteristics of SNP markers mapped into seven linkage groups in RIL population.

Linkage group	SNP	SNP (%)	Length (cM)	Average distance between markers (cM)
LG1	516	12.4	38.9	0.08
LG2	724	17.3	126.5	0.17
LG3	258	6.2	29.0	0.11
LG4	1224	29.3	161.5	0.13
LG5	593	14.2	48.9	0.08
LG6	523	12.5	42.5	0.08
LG7	339	8.1	49.8	0.15
TOTAL	4177		497.1	0.12

were 3.01–4.45 and 6.0–14.0, respectively. LG6 had two QTL regions. LOD scores and % expl. values of the QTL regions on LG6 (FeQTL6.1 and FeQTL6.2) varied from 3.14 to 4.12 and from 6.7 to 12.2, respectively. FeQTL5.2 had the most SNP markers (239). LOD scores and % expl. values of the QTL region were 3.03–4.07 and 6.1–12.0, respectively. FeQTL4.3 had two SNP markers. LOD scores and % expl. values of the QTL regions ranged from 3.07 to 3.38 and from 6.4 to 8.1, respectively. Additive effects of QTL regions of Fe are presented in Table 5. All QTL regions are presented in Figure 2.

4. Discussion

4.1. Fe concentration in lentil seeds

The two lines of RILs (LR11-17 and LR11-133) with much higher Fe concentrations (>200 mg/kg) than that the high-Fe parent "ILL 8006" (114.3 mg/kg) indicate transgressive segregation, which is desirable for a population due to different QTL alleles in both parents (Wu et al., 2008). For instance, transgressive segregation was detected in a RIL population of *Lotus japonicus* (Klein and Grusak, 2009). Their parent seeds had Fe concentrations of 67 and 87 mg/kg, respectively, while their RIL seed Fe concentration varied between 27 and 191 mg/kg in 2006 and 81 and 135 mg/kg in 2007 (Klein and Grusak, 2009). Moreover, transgressive segregation of Fe concentration in a wheat RIL population has been shown (Tiwari et al., 2009). Mean seed Fe concentrations were 23.8 and 40.1 mg/kg for the two parents, respectively, and for the RILs varied from 17.8 to 69.7 mg/kg.

Fe concentrations of the RIL population ranged from 37.2 to 175.7 mg/kg when averaged over the two years and three locations. The range of Fe concentration is much greater than those of earlier studies by Thavarajah et al. (2011) (73–90 mg/kg) and Alghamdi et al. (2014) (65.7–85.7 mg/kg). Fe concentration was reported between 49.4 and 69.9 mg/kg in cultivars and 49.0 and 81.4 mg/kg in lentil landraces (Karakoy et al., 2012). Seed Fe

Table 5. Significant QTL regions for seed Fe concentration in RIL grown at İzmir, Adana and Şanlıurfa in 2012 and 2013.

Name of QTL	LG	Position (cM)	Number of SNPs on the QTL region	% Explanation	LOD	Additive effects	Year/Location
FeQTL1.1	1	2.36	5	6.3	3.06	–	2012/Adana
FeQTL1.2	1	14.16	15	6.7–9.9	3.14–3.70	–	2012/İzmir, Adana, and Şanlıurfa; 2013/ İzmir, Adana, and Şanlıurfa
FeQTL1.3	1	21.15	25	6.1–7.1	3.02–3.20	–	2012/İzmir, Adana, and Şanlıurfa; 2013/ İzmir and Şanlıurfa
FeQTL2.1	2	0	18	7.6–10.4	3.28–3.79	+	2012/İzmir, Adana, and Şanlıurfa; 2013/ İzmir, Adana, and Şanlıurfa
FeQTL2.2	2	43.52–53.05	81	6.1–13.5	3.02–4.36	+	2012/İzmir, Adana, and Şanlıurfa; 2013/ İzmir, Adana, and Şanlıurfa
FeQTL2.3	2	72.39	20	6.0–7.7	3.00–3.31	–	2012/İzmir, Adana, and Şanlıurfa; 2013/ İzmir, Adana, and Şanlıurfa
FeQTL4.1	4	15.61	10	6.0	3.01	–	2013/Adana
FeQTL4.2	4	28.21	40	11.9–14.0	4.07–4.45	+	2012/İzmir, Adana, and Şanlıurfa; 2013/ İzmir, Adana, and Şanlıurfa
FeQTL4.3	4	98.26	2	6.4–8.1	3.07–3.38	–	2012/İzmir and Adana; 2013/İzmir, Adana, and Şanlıurfa
FeQTL4.4	4	106.44	5	6.4	3.07–3.09	–	2012/ Adana; 2013/İzmir
FeQTL4.5	4	129.69	18	6.1–7.5	3.03–3.27	–	2012/İzmir, Adana, and Şanlıurfa; 2013/ İzmir, Adana, and Şanlıurfa
FeQTL4.6	4	139.72	70	7.5–9.7	3.28–3.66	–	2012/İzmir, Adana, and Şanlıurfa; 2013/ İzmir, Adana, and Şanlıurfa
FeQTL5.1	5	13.06	9	6–7.3	3.01–3.23	–	2012/İzmir, Adana, and Şanlıurfa; 2013/ İzmir, Adana, and Şanlıurfa
FeQTL5.2	5	23.26–26.75	239	6.1–12	3.03–4.07	+	2012/İzmir, Adana, and Şanlıurfa; 2013/ İzmir, Adana, and Şanlıurfa
FeQTL5.3	5	38.97	11	5.9–9.4	3.00–3.62	+	2012/İzmir, Adana, and Şanlıurfa; 2013/ İzmir, Adana, and Şanlıurfa
FeQTL5.4	5	47.64	6	6.0–6.2	3.00–3.03	–	2012/ Şanlıurfa; 2013/İzmir and Şanlıurfa
FeQTL6.1	6	9.55–11.35	34	6.7–9.9	3.14–3.70	–	2012/İzmir, Adana, and Şanlıurfa; 2013/ İzmir, Adana, and Şanlıurfa
FeQTL6.2	6	15.51	4	10.4–12.2	3.78–4.12	+	2012/İzmir, Adana, and Şanlıurfa; 2013/ İzmir, Adana, and Şanlıurfa
FeQTL7.1	7	0	12	8.2–10.7	3.40–3.84	–	2012/İzmir, Adana, and Şanlıurfa; 2013/ İzmir, Adana, and Şanlıurfa
FeQTL7.2	7	3.60	9	7.1–8.9	3.19–3.52	–	2012/İzmir, Adana, and Şanlıurfa; 2013/ İzmir, Adana, and Şanlıurfa
FeQTL7.3	7	13.43	101	6.3–9.1	3.05–3.56	–	2012/İzmir, Adana, and Şanlıurfa; 2013/ İzmir, Adana, and Şanlıurfa

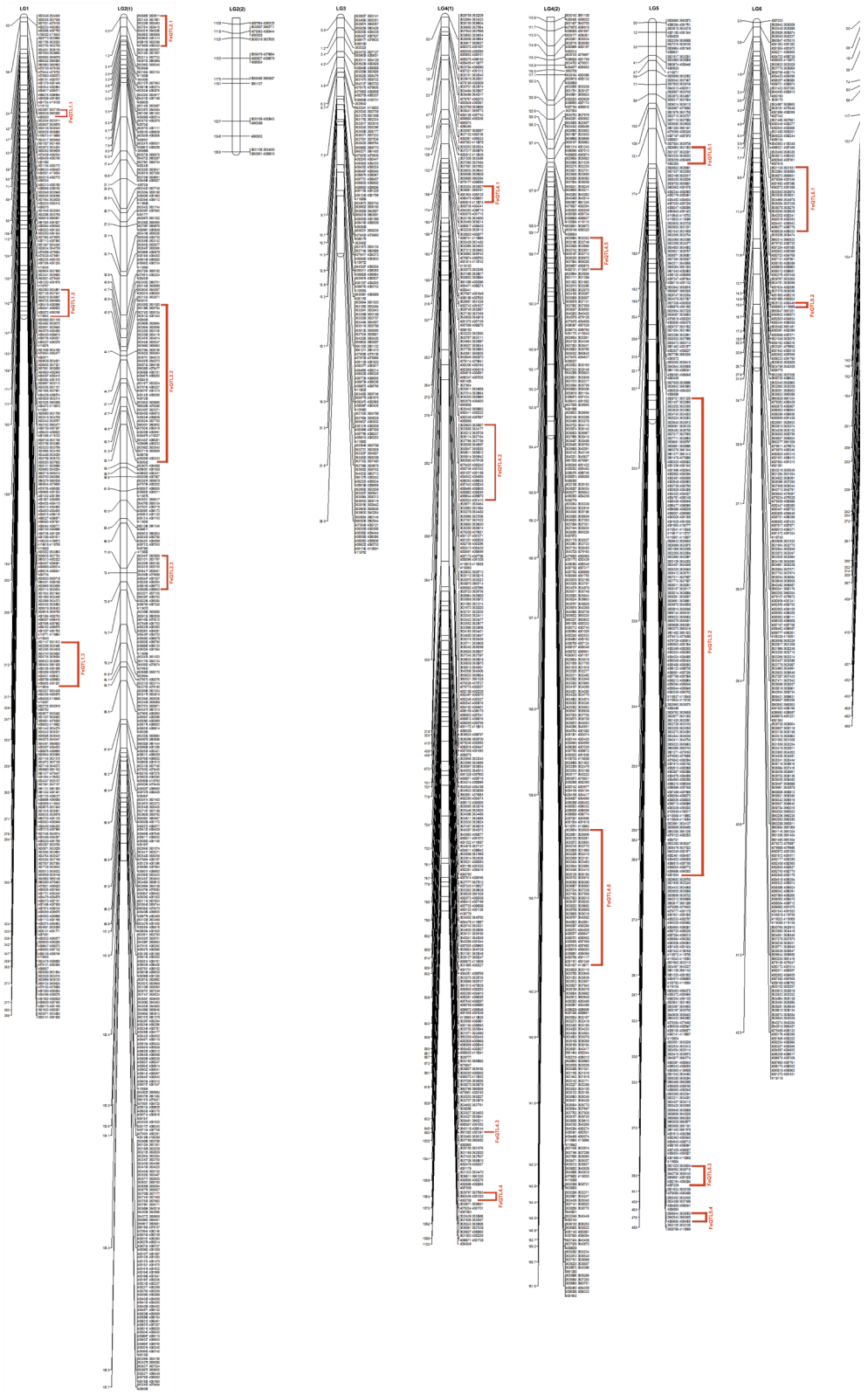

Figure 2. Linkage map of RIL based on SNPs. Left bar of linkage map is cM distance and right bar of linkage map is marker names. Red bars and vertical letters indicate QTL regions related with Fe concentration in seed. Numbers in brackets indicate continue of certain linkage map such as 2[1].

concentrations varied from 6 to 24 mg/kg in rice (Gregorio et al., 2000); 35 to 92 mg/kg (Islam et al., 2002) and 48 to 74 mg/kg (Ariza-Nieto, 2007) in common bean; 17 to 24 mg/kg in maize (Oikeh et al., 2003); 23 to 105 mg/kg in pea (Grusak and Cakmak, 2005); 24 to 43 mg/kg (Garvin et al., 2006), 20 to 45 mg/kg (Oury et al., 2006), and 25 to 56 mg/kg (Morgounov et al., 2007) in wheat; and 24 to 41 mg/kg in chickpea (Zia-Ul-Haq et al., 2007). In the current study, the two lines (LR11-17 and LR11-133) had Fe concentrations much higher than those of other staple crops in previous works.

This is the first report on Fe concentration in seeds of RILs of lentil, and also the first report on transgressive segregation for Fe concentration in lentil seeds. If seed Fe concentration above 150 mg/kg could be maintained through genetic selection, this would represent a major advance in biofortification of lentil.

4.2. Lentil linkage map

Next-generation sequencing has been used to discover tens of thousands SNPs and construct genetic linkage maps and conduct genome-wide association studies (Metzker, 2010). As the methodology discovers large numbers of markers in a short time and is cost effective, researchers prefer using GBS technology for genome mapping studies, such as those conducted in barley and wheat by Poland et al. (2012) and raspberry (*Rubus idaeus* L.) by Ward et al. (2013).

A total of 4177 SNPs were mapped on seven LGs. The total length of the linkage map was 497.1 cM in the current study. In previous lentil mapping studies, lengths of the linkage maps varied from 751 cM to 4060.6 cM (Eujayl et al., 1998; Rubeena et al., 2003; Duran et al., 2004; Hamwieh et al., 2005; Tanyolac et al., 2010; Gupta et al., 2012; De la Puente et al., 2013; Sharpe et al., 2013; Gujaria-Verma, 2014; Ates et al., 2016). The length of the map reported here was shorter than in these previous studies, but the value was close to the findings (751 cM) published by Hamwieh et al. (2005). The average distance between the markers in our study was 0.12 cM. Compared to the average distance between markers in previous studies, the average distance was shorter than those of findings of 2.3–19.3 cM by other authors (Eujayl et al., 1998; Rubeena et al., 2003; Duran et al., 2004; Hamwieh et al., 2005; Tanyolac et al., 2010; Gupta et al., 2012; De la Puente et al., 2013; Sharpe et al., 2013; Gujaria-Verma, 2014; Ates et al., 2016). Use of SNP-based technologies has led to increased marker frequency per linkage group (Deschamps et al., 2012). Most linkage maps of lentil have been constructed using RAPD, SSR, ISSR, and AFLP markers. The linkage maps of lentil constructed by Sharpe et al. (2013) and Ates et al. (2016) had 537 and 1780 SNP markers, respectively, which is less than the 4177 SNPs in the current study. The number of mapping markers in this study, 4177, is higher than the results from other linkage maps [e.g., 114 (Rubeena et al., 2003) to 283 (Hamwieh et al., 2005)].

4.3. QTLs for seed Fe concentration

This study is the first report on QTLs linked to genes controlling Fe concentration in lentil seeds. A total of 21 QTL regions (each 3 QTL regions in LG1, LG2, and LG7; 6 QTL regions in LG4; 4 QTL regions in LG5; and 2 QTL regions in LG6) distributed across six LGs were associated with Fe concentration in lentil seed. Previous QTL mapping studies of cereal crops identified fewer QTL(s) for seed Fe concentration. In legumes like lentil, 14 and 5 QTLs for Fe concentration in seed were detected in common bean (Blair et al., 2009) and in *Lotus japonicus* (Klein and Grusak, 2009). As for cereals, 1 QTL for Fe concentration in grain of maize was detected on LG5 (Jin et al., 2013), while 3 QTLs for Fe concentration in grain of rice were identified on LG2, 8, and 12 (Stangoulis et al., 2007). Similarly, a total of 3 QTLs (2 additional QTLs and 1 epistatic QTL) for Fe concentration were determined in wheat (Xu et al., 2012) whereas Srinivasa et al. (2014) detected 5 QTLs for Fe concentration in wheat grain on LG1A, 2A, and 3B. In the current study, more QTLs were mapped on the lentil genome compared to previous QTL mapping studies due to the construction of a high-density map using SNPs produced by GBS technology. This technology (GBS) is used to increase the density of markers for precise detection of QTLs (Bandillo et al., 2013; Chen et al., 2014; Raman et al., 2014). High marker density can enhance the resolution of genetic linkage maps (Yu et al., 2011). The high-density linkage maps obtained in this way could improve the possibility that one of the markers is localized in chromosomal fragments with nonrecombination events and the accuracy of the QTL localization (Liu et al., 2013; Stange et al., 2013).

A total of 21 QTLs for Fe concentration explained variance of 5.9%–14.0% with LOD scores ranging from 3.00 to 4.45 (Table 5). The phenotypic variation was statistically significant. The phenotypic variation for Fe concentration in the current study was close to variations reported in cabbage (Wu et al., 2008), rice (Stangoulis et al., 2007), wheat (Tiwari et al., 2009; Pu et al., 2014), and maize (Jin et al., 2013).

In conclusion, this study represents the first use of GBS as a means of identifying QTLs for Fe concentration in seeds of lentil. A wide range of phenotypic variation was detected for Fe concentration among RILs population. The results showed that Fe concentration in lentil seed was quantitatively inherited. The QTL analysis indicated that most QTLs were significant and stable at more than one location over 2 years. The presence of the same QTLs at different locations is a genotypic characteristic and not an environmental effect. These stable QTLs could be useful

for molecular breeding strategies that use marker-assisted selection to detect genotypes with high Fe concentration in seed. This would help accelerate biofortification strategies to increase and stabilize the concentration of Fe in lentil seed. By this means, new varieties of lentil with high Fe concentration could be developed in the future. A high density linkage map using SNP markers through GBS shows promise for use with lentil and may open avenues for further genetic mapping and biofortification studies.

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