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Inter-primer binding site retrotransposon and inter-simple sequence repeat diversity among wild *Lens* species

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ABSTRACT

Even though lentil has been an important food legume for centuries, genetic studies in lentil are still in their infancy. Genetic diversity and relationships among wild *Lens* species from Turkey has seldom been investigated. Additionally, a limited number of simple sequence repeat (SSR) markers have been developed for use in breeding and genetic studies of lentil crop. In this study, molecular characterization of 50 accessions mostly from Turkey, belonging to 6 wild and 1 cultivated *Lens* species, was performed using newly developed inter-primer binding site (iPBS) retrotransposons and inter-SSR (ISSR) markers. The 10 iPBS primers generated a total of 151 scorable bands, of which 150 were polymorphic (99.3%) with an average of 15.0 polymorphic fragments per primer. The 10 ISSR primers detected 138 scorable bands showing 100% polymorphism, with an average of 13.5 bands per primer. The average polymorphism information content (PIC) value for ISSR markers (0.97) was higher than that for iPBS markers (0.90). *Lens orientalis* was found to be the most diverse species, raising the possibility of wide crosses with cultivated species *Lens culinaris*. Cultivated varieties also showed high level of polymorphism, at 82.92% and 51.92% with ISSR and iPBS markers, respectively. *Lens lamottei* and *Lens tomentosus* were found as the least polymorphic species using both marker systems. The grouping of accessions and species within clusters were almost similar when iPBS and ISSR graphs were compared. Our data also suggested the role of iPBS-retrotransposons as '*a universal marker*' for molecular characterization of wild and cultivated *Lens* species.

1. Introduction

Lentil domestication in the Fertile Crescent dates back to the very beginning of agriculture (Alo et al., 2011). Even though lentil has been an important food legume for centuries, genetic studies in *Lens* species are still in their infancy. Despite several

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previous studies conducted in the *Lens* genus, genetic relationships within this complex taxon have still not been clarified. Previous studies showed that 2 inter-fertile biological groups or species exist, classified as *L. culinaris* and *L. nigricans*. The former included spp. *culinaris*, ssp. *orientalis*, and spp. *odemensis* while the later included ssp. *nigricans* and spp. *ervoides*. All *Lens* species are diploid and self-pollinated ($2n = 2x = 14$). *L. culinaris* ssp. *culinaris* is the only cultivated species belonging to the genus *Lens*, while *L. culinaris* ssp. *orientalis* is considered to be its wild ancestor (Ladizinsky et al., 1984). However, additional information now indicates that some of the proposed subspecies are species in their own right.

Understanding the extent and nature of genetic variations in crops have important implications for breeding programs as well as conservation of plant genetic resources (Cömertpay et al., 2012). Recently, more accurate determination of genetic relationships among wild and domesticated/cultivated lentils was achieved by polymerase chain reaction (PCR)-based DNA molecular markers.

Retrotransposons played an important role in the formation of genetic diversity and are also useful for marker development due to their high prevalence and genome-specific distribution (Schulman et al., 2004). Recently, Kalendar et al. (2010) described a unique PCR-based method for the rapid isolation of retrotransposon termini that could be useful as a marker system, making it ideal for "*orphan crops*" and other species with underdeveloped marker systems. The method termed "*iPBS*" amplification is based on the virtually universal presence of tRNA complement as a reverse transcriptase primer-binding site (PBS) in long terminal repeat (LTR) retrotransposons, without the need for previous knowledge of the sequence (Kalendar et al., 2010).

Turkey is considered as the center of plant diversity with rich lentil genetic resources including several wild *Lens* species (Ladizinsky, 1986). Some of the known wild species, particularly, *Lens odemensis* and *Lens tomentosus* are found only in Turkey (Ladizinsky, 1986). To date, only few studies have investigated genetic diversity among wild *Lens* species. Therefore, the objective of this study was to test the utility of iPBS retrotransposons in evaluating the genetic diversity of *Lens* as compared to the well-established inter-simple sequence repeat (ISSR) marker system. This study also gives brief information about the genetic diversity and relationship among *Lens* species in Turkey.

2. Materials and methods

2.1. Plant material and DNA extraction

A total of 50 accessions, consisting of 9 *L. culinaris*, 8 *Lens orientalis*, 8 *L. odemensis*, 8 *L. nigricans*, 8 *Lens ervoides*, 5 *L. tomentosus*, and 4 *Lens lamottei* samples, were used in this study. Wild *Lens* species were obtained from ICARDA (International Center of Agricultural Research in Dry Areas) gene bank. The identification number and collection site for all *Lens* accessions is provided in Table S1. Genomic DNA from each accession was extracted as previously described by Doyle and Doyle (1990) with minor modifications (Ozkan et al., 2005).

2.2. Inter-primer binding site (iPBS) retrotransposon marker analysis

Initially, 73 iPBS primers were tested on 8 lentil genotypes, including 2 lentil cultivars and 1 accession from each wild *Lens* species, to identify the primer that produced a sharp and clear banding profile. Primers names, their annealing temperature, and banding pattern are summarized in Table S2. Ten primers with good/excellent PCR products were selected for genotyping all *Lens* accessions. PCR amplifications were performed in an Eppendorf DNA thermal master gradient cycler (Eppendorf Netheler Hinz, Hamburg, Germany) using 25 μL reaction mixture containing 25 ng template DNA, 1X Dream *Taq* Green Buffer (Fermentas), 0.2 mM dNTP (Fermentas), 1 μM primer for 12–13 nucleotide (nt) primers or 0.6 μM for 18 nt primers, 1.5 U Dream *Taq* DNA polymerase (Fermentas), and 0.04 U Pfu DNA polymerase (Fermentas). The PCR thermal cycling profile was as follows: initial denaturation at 95 °C for 3 min; 30 cycles of 95 °C for 15 s, 50–65 °C (annealing temperature depending upon primer; for details see Table S2) for 1 min, and 68 °C for 1 min; and final extension at 72 °C for 5 min (Kalendar et al., 2010). All PCR products were separated using 1.7% (w/v) agarose gel electrophoresis with 0.5× TBE buffer for 2 h, stained with ethidium bromide and visualized by a UV transilluminator (Infinity ST5, Vilber Lourmat, France).

2.3. ISSR marker analysis

Inter-simple sequence repeat (ISSR; University of British Columbia, Vancouver) amplification was carried out as previously described by Zietkiewicz et al. (1994) with some modifications as described by Baloch et al. (2010). A total of 30 ISSR primers were tested for ISSR amplification on the same 8 lentil genotypes, which were used for iPBS screening. Out of the 30 ISSR primers tested, 10 primers that provided a sharp and clear banding profile were used for genotyping of all lentil accessions and cultivars. The amplification reaction was carried out in a final volume of 25 μl containing 1× Dream *Taq* Green Buffer (Fermentas), 0.2 μM primer, 100 μM each of dATP, dGTP, dCTP, and dTTP, 1.5 U Dream *Taq* DNA polymerase, and 20 ng genomic DNA. The PCR reaction program consisted of: 1 cycle at 94 °C for 2 min; 40 cycles of 94 °C for 1 min, 50–52 °C for 1 min (annealing temperature depending upon primer), 72 °C for 2 min, and a final extension step of 72 °C for 10 min. All PCR products were separated on 2% agarose gel electrophoresis with 0.5× TBE buffer for 2.5 h, stained with ethidium bromide, and gel images were visualized by a UV transilluminator (Infinity ST5, Vilber Lourmat, France).

2.4. Data analysis

The iPBS and ISSR bands were manually scored as present (1) versus absent (0) by at least 2 independent persons. Only clear and strong bands were recorded and used for further analysis. Faint bands were not scored in order to avoid scoring of artificial bands. Gene diversity (h), genetic similarity, and genetic distance were calculated by POPGENE statistical software version 1.32 (Yeh et al., 2000). The polymorphism information content (PIC) was calculated by the formula previously reported by De Riek et al. (2001);

$$PIC_i = 2f_i(1 - f_i)$$

where f_i is the frequency of the amplified allele (band present) and $(1 - f_i)$ is the frequency of the null allele (band absent) of the marker. Neighbor-Net (N-Net) planar graphs of both iPBS and ISSR Nei distances (1987) between individuals were constructed using Splits Tree version 4.11 software (Huson and Bryant, 2006).

3. Results

3.1. iPBS diversity and relationship

A total of 73 iPBS primers were tested in 7 lentil species for initial screening. Of these, 13 iPBS primers did not produce any PCR product, 26 primers produced poor banding profiles, 29 primers showed good band profiles, and 5 primers gave excellent PCR fragments (Table S2). Of these, 10 iPBS primers yielding strong and reproducible polymorphic bands were selected for further genotyping of the complete set of *Lens* accessions. These 10 iPBS primers generated a total of 151 scorable bands. Out of these 151 bands, 150 were polymorphic (99.3%), with an average of 15.0 polymorphic fragments per primer (Table 1). The number of bands per primer ranged from 12 (iPBS2075) to 20 (iPBS2230), with an average of 15.1 bands per primer (Table 2). Gene diversity per iPBS primer ranged from 0.18 (iPBS2229) to 0.38 (iPBS2257) with an average of 0.26. The mean polymorphism information content (PIC) was 0.90, ranging from 0.84 (iPBS2374) to 0.97 (iPBS2377) (Table 2).

Gene diversity among *Lens* species varied from 0.010 to 0.110, with a mean value of 0.258 (Table 1). iPBS analysis results revealed that the highest genetic variation was shown by *L. orientalis*, closely followed by *L. ervoides*, with a total of 58 and 57 bands, of which, 43 and 42 were polymorphic among 8 accessions, with a mean gene diversity of 0.101 and 0.110, respectively. On the other hand, lowest genetic variation was detected within *L. lamottei*, in which only 4 out of 35 bands (11.42%) showed polymorphism (Table 1).

The highest genetic similarity (0.95) was observed between *L. culinaris* and *L. orientalis* (Table 3), while the lowest genetic similarity (0.68) was found between *L. culinaris* and *L. lamottei* (Table 3). The Neighbor-Net (N-Net) planar graph clearly split all 50 *Lens* accessions into 6 different groups, with each cluster having accessions from the same species, with the only exception having accessions from *L. culinaris* and *L. orientalis* together (Fig. S1).

3.2. ISSR diversity and relationship

A total of 30 ISSR primers were screened among 8 genotypes from all *Lens* species and 10 primers producing strong and clear bands were employed to genotype the complete set of accessions. A total of 138 scorable bands were detected using 10 ISSR primers from all 50 accessions, belonging to 6 wild *Lens* accessions and 9 Turkish commercially produced lentil cultivars (Table 1). All 138 bands detected by ISSR primers were found to be polymorphic (100%). The number of bands per primer ranged from 8 (UBC844) to 23 (UBC811), with an average of 13.8 bands per primer. Gene diversity (h) among species varied from 0.010 to 0.085 with a mean value of 0.169. Gene diversity per ISSR primer ranged from 0.13 (UBC854) to 0.29 (UBC841), with an average value of 0.17. The highest PIC value was 0.98 for UBC823, UBC844, and UBC853 primers, whereas the lowest PIC value was 0.95 for UBC811 (Table 2).

Table 1
Genetic diversity within and among *Lens* species based on iPBS and ISSR primers.

Species	NA	iPBS					ISSR				
		TB	NPL	PAL%	PWS %	GD	TB	NPL	PAL %	PWS %	GD
L. culinaris	9	52	27	18	51.92	0.064	41	34	24.63	82.92	0.085
L. orientalis	8	58	43	28.66	74.13	0.101	38	37	26.81	97.36	0.084
L. odemensis	8	45	26	17.33	57.77	0.053	25	19	13.76	76	0.045
L. nigricans	8	39	12	8	30.76	0.030	30	21	15.21	70	0.056
L. ervoides	8	57	42	28	73.68	0.110	33	30	21.73	90.90	0.080
L. tomentosus	5	39	12	8	30.76	0.026	21	3	2.17	14.28	0.008
L. lamottei	4	35	4	2.66	11.42	0.010	17	4	2.89	23.52	0.010
For all accessions	50	151*	150**	99.33	–	0.258	138*	138**	100	–	0.169

NA number of accession, TB total bands within each species, NPL number of polymorphic bands with each species, PAL% polymorphic locus percentage with other species, PWS% polymorphism percentage within species, GD gene diversity, * total scorable bands for all species, ** polymorphic bands for all species.

Table 2
Information about the iPBS and ISSR primers used in this study, including their sequence, PIC value, number of total and polymorphic bands.

Markers	Sequence	Number of bands			
iPBS/ISSR		Total	polymorphic	*h*[a]	PIC[a]
iPBS2075	CTCATGATGCCA	12	12	0.28	0.86
iPBS2229	CGACCTGTTCTGATACCA	19	19	0.18	0.96
iPBS2230	TCTAGGCGTCTGATACCA	20	20	0.26	0.89
iPBS2257	CTCTCAATGAAAGCACCA	13	13	0.38	0.90
iPBS2374	CCCAGCAAACCA	16	15	0.29	0.84
iPBS2377	ACGAAGGGACCA	13	13	0.20	0.97
iPBS2380	CAACCTGATCCA	14	14	0.27	0.89
iPBS2385	CCATTGGGTCCA	16	16	0.30	0.85
iPBS2395	TCCCCAGCGGAGTCGCCA	13	13	0.22	0.94
iPBS2402	TCTAAGCTCTTGATACCA	15	15	0.24	0.95
Total	–	151	150	–	–
Average	–	15.1	15	0.26	0.90
UBC808	AGAGAGAGAGAGAGAGC	15	15	0.18	0.97
UBC811	GAGAGAGAGAGAGAGAC	23	23	0.19	0.95
UBC823	TCTCTCTCTCTCTCTCC	15	15	0.18	0.98
UBC834	AGAGAGAGAGAGAGAGYT	17	17	0.16	0.97
UBC841	GAGAGAGAGAGAGAGAYC	13	13	0.29	0.97
UBC844	CTCTCTCTCTCTCTCTRC	8	8	0.14	0.98
UBC845	CTCTCTCTCTCTCTCTRG	11	11	0.16	0.97
UBC852	TCTCTCTCTCTCTCTCRA	9	9	0.18	0.96
UBC853	TCTCTCTCTCTCTCTCRT	13	13	0.14	0.98
UBC854	TCTCTCTCTCTCTCTCRG	14	14	0.13	0.97
Total	–	138	138	–	–
Average	–	13.8	13.8	0.17	0.97

[a] h: represent gene diversity, PIC: polymorphism information contents.

Genetic diversity was calculated within each *Lens* species (Table 1). ISSR analysis showed that the highest genetic variation was found in *L. orientalis*, having 37 polymorphic loci detected among 8 accessions, with a mean gene diversity of 0.084. The lowest genetic variation was detected in *L. tomentosus*, with 3 out of 21 bands showing polymorphism, at 14.28%.

Highest genetic similarity (0.968) was observed between *L. culinaris* and *L. orientalis* and lowest (0.78) between *L. nigricans* and *L. lamottei* (Table 3). In the Neighbor-Net (N-Net) planar graph, 50 accessions of different *Lens* species and cultivars were separated into 6 different groups according to their species level, except for 1 cluster, which contained 2 species, *L. culinaris* and *L. orientalis* (Fig. S2).

3.3. Combining iPBS and ISSR data

To obtain a more precise picture, 2 data sets (iPBS and ISSR) were combined and genetic similarities were calculated among 41 wild *Lens* accessions and 9 Turkish commercial lentil cultivars. The Neighbor-Net (N-Net) planar graph, combining

Table 3
Genetic similarity and genetic distance coefficient (Nei, 1987) among seven *Lens* species.

Species	iPBS markers						
	Lc	Lor	Lod	Ln	Le	Lt	Ll
L. culinaris (Lc)	–	0.9510	0.7501	0.7110	0.6966	0.7685	0.6883
L. orientalis (Lor)	0.0503	–	0.7917	0.7469	0.7435	0.7891	0.7455
L. odemensis (Lod)	0.2875	0.2335	–	0.7297	0.7298	0.7597	0.7092
L. nigricans (Ln)	0.3411	0.2919	0.3151	–	0.7482	0.7415	0.6998
L. ervoides (Le)	0.3616	0.2964	0.3150	0.2901	–	0.7571	0.7073
L. tomentosus (Lt)	0.2633	0.2369	0.2748	0.2991	0.2783	–	0.7597
L. lamottei (Ll)	0.3736	0.2937	0.3436	0.3570	0.3463	0.2748	–
	ISSR markers						
L. culinaris (Lc)	–	0.9681	0.8762	0.8260	0.8811	0.8020	0.8203
L. orientalis (Lor)	0.0324	–	0.9163	0.8720	0.9184	0.8348	0.8622
L. odemensis (Lod)	0.1322	0.0874	–	0.8609	0.8942	0.8202	0.8415
L. nigricans (Ln)	0.1911	0.1370	0.1498	–	0.8697	0.7894	0.8050
L. ervoides (Le)	0.1266	0.0851	0.1119	0.1397	–	0.8218	0.8512
L. tomentosus (Lt)	0.2206	0.1805	0.1982	0.2365	0.1962	–	0.7906
L. lamottei (Ll)	0.1981	0.1483	0.1726	0.2169	0.1611	0.2350	–

Above and below the diagonal is genetic similarity and genetic distance values.

the information from iPBS and ISSR (Fig. 1), showed main characteristics from both independent trees (Figs. S1 and S2). The analysis clearly split all 50 accessions into 6 different groups. *L. culinaris* and *L. orientalis* accessions were clustered together, while all other accessions belonging to same species were placed separately. A comparison of genetic similarity values obtained from ISSR and iPBS was performed using the Mantel test, which showed statistically significant correlation coefficient ($r = 0.91$) between both marker systems.

4. Discussion

In this study, the genetic diversity of *Lens* species as well as some varieties of cultivated lentil was studied using a recently developed protocol employing iPBS-retrotransposon markers and results were compared with a well-known ISSR DNA marker system. The iPBS DNA markers have previously been successfully employed to evaluate genetic diversity in *Cicer* (Andeden et al., 2013), *Saussurea esthonica* (Gailite et al., 2011) and grape (Guo et al., 2014). To date, the iPBS retrotransposon markers have not been used for genetic diversity studies in wild *Lens* species. It was observed that the number of total and polymorphic bands, as well as percentage of polymorphism obtained with iPBS markers, were considerably higher than those with ISSR markers in *L. culinaris* ssp. *orientalis*, *L. odemensis*, *L. ervoides*, and *L. tomentosus*; however, these were lower in *L. culinaris* ssp. *culinaris* and *L. nigricans* and the same in *L. lamottei*. However, percentage of polymorphism was higher with ISSR than with iPBS markers in most of the lentil species studied, except *L. tomentosus*. Our results also revealed that *L. lamottei* contains the least genetic diversity as evidenced by both marker systems; however, the small germplasm size of this species (only 4 accessions) could be the cause. Therefore, further study is required to validate this observation in a larger germplasm size of *L. lamottei*.

A Neighbor-Net planner graph for all accessions was constructed for iPBS and ISSR (Figs. S1 and S2) separately as well as in combination (iPBS + ISSR) (Fig. 1). In both, iPBS and ISSR, there was perfect discrimination at the sub-specific and specific levels. The iPBS-based grouping of 7 *Lens* species was fully consistent with that obtained using ISSR markers ($r = 0.91$). All accessions were separated into 6 main groups in both, iPBS and ISSR analyses. In all dendrograms, *L. culinaris* ssp. *culinaris* and *L. culinaris* ssp. *orientalis* were clustered under the same group and this showed that *L. culinaris* ssp. *orientalis* is the species closest to cultivated lentil (Sonnante et al., 2003). Our results also supported the idea that *L. culinaris* ssp. *orientalis* is the wild precursor of the domesticated lentil, and these are subspecies of a single species. This conclusion has been reported by various previous studies that used morphological, cytological, isozyme, restriction fragment length polymorphism (RFLP), chloroplast DNA, and DNA sequences (Ferguson et al., 2000; Alo et al., 2011). These results clearly showed that outcrossing events could have occurred between these species. Horneburg (2006) recorded a range of average outcrossing between 0.06% and 5.12% among lentil cultivars. Alo et al. (2011) reported a variation in outcrossing rate among plants within individual varieties (0–22.2%). Similar results were previously reported not only in lentil but also in the common bean (Papa et al., 2007).

Accessions from *L. nigricans* and *L. ervoides* formed separate clusters in iPBS + ISSR, ISSR, and iPBS analysis, and were found to be the most distantly related species (Fig. 1). Similar observation were reported by Sonnante et al. (2003), who reported using ITS analyses that *L. nigricans* is distantly related to all the other *Lens* species, except *L. ervoides*. Recently, Alo et al. (2011)

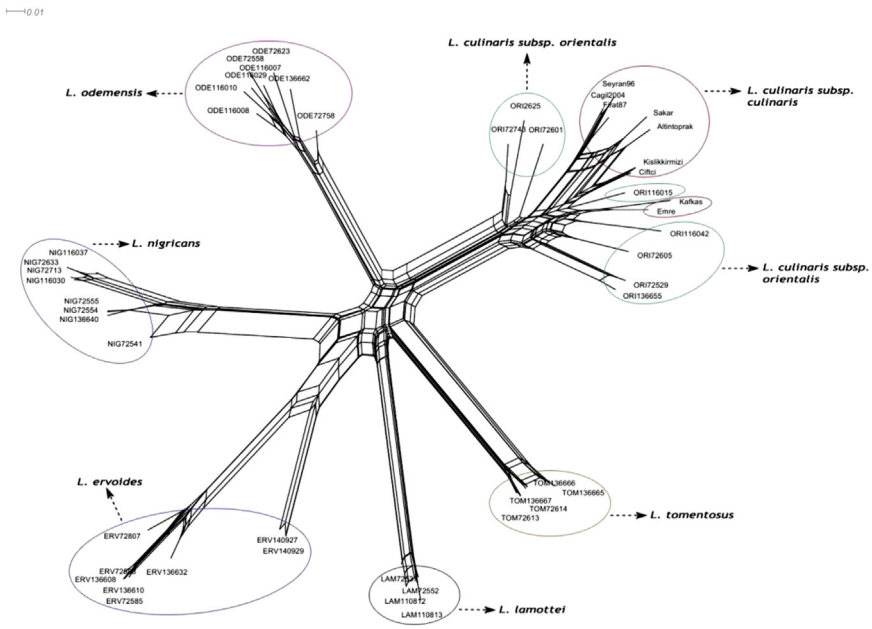

Fig. 1. Neighbor Net (NNet) planar graph of six *Lens* species consisting of 50 accessions and cultivars based on combined analysis of iPBS + ISSR markers.

reported using DNA sequences information, that *L. nigricans* and *L. ervoides* are well-separated from each other and other *Lens* species. In combined iPBS + ISSR analysis, *L. odemensis* was clearly separated from *L. nigricans*, *L. ervoides*, *L. tomentosus*, and *L. lamottei*, but it was found close to *L. culinaris* ssp. *orientalis* and *L. culinaris* ssp. *culinaris* (Fig. 1). It is well documented that *L. odemensis* readily produces hybrids with *L. culinaris* ssp. *orientalis* and *L. culinaris* ssp. *culinaris* (Ladizinsky et al., 1984). Fertility of these hybrids depends on whether or not they are heterozygous for chromosomal rearrangements (Ferguson et al., 2000). Current taxonomic status of *L. odemensis* should be revisited; either this is a single subspecies of *L. culinaris* or a separate *Lens* species altogether, as per crossability analyses (Alo et al., 2011). The results this study showed that accessions from these 3 species from Turkey have different and unique genetic profiles as compared to accessions from other countries. Therefore, further surveys are required to elucidate the genetic structure of Turkish wild lentil.

In the last decade, researchers debated whether *L. tomentosus* and *L. odemensis* are subspecies of *L. culinaris* (Ferguson et al., 2000). In the present study, *L. tomentosus* was clearly differentiated from *L. orientalis* in both; iPBS and ISSR analysis and this confirmed that *L. tomentosus* is a distinct species. Sonnante et al. (2003) reported that *L. tomentosus* and *L. orientalis* are morphologically similar, except for the hairy pods of the former, but karyotypically and genetically, they are very different. In addition to these characters, *L. tomentosus* and *L. lamottei* were also separated into different groups, in accordance with results by Sonnante et al. (2003) who described that *L. tomentosus* and *L. lamottei* could be considered as independent entities, since they showed specific automorphies. A similar conclusion was reached by Galasso (2003) using FISH karyotype analyses.

Lev-Yadun et al. (2000) proposed a "*core area*" for the origins of agriculture within the Fertile Crescent. This was based on the proposition that wild einkorn and wild emmer from this area are genetically more closely related to the domesticated crop plants than elsewhere; and that lentil, one of the oldest grain legume crops and its all wild relative particularly *L. culinaris* subsp. *orientalis* showed distribution and overlap in Southeast Turkey, particularly in the Karacadağ mountain range (Toklu et al., 2009). More detailed study is indispensable in order to collect *Lens* germplasm, particularly *L. culinaris* subsp. *orientalis* and other related species from southeastern Turkey. This would promote preservation and management of this species, as well as screening germplasm for useful agronomic and quality traits, resistance against different biotic and abiotic stress. Furthermore, it would promote utilization in future breeding in order to enhance genetic diversity of modern lentil varieties through hybridization. A large amount of genetic diversity harbored by wild accessions, particularly in *L. culinaris* subsp. *orientalis*, could be used efficiently by crossing wild and cultivar types in developing mapping population for gene/QTL tagging for insect, pest and disease resistance and introgression in cultivated varieties.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bse.2014.12.002.

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