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Development of a panel of unigene-derived polymorphic EST–SSR markers in lentil using public database information

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ABSTRACT

Lentil (*Lens culinaris* Medik.), a diploid ($2n = 14$) with a genome size greater than 4000 Mbp, is an important cool season food legume grown worldwide. The availability of genomic resources is limited in this crop species. The objective of this study was to develop polymorphic markers in lentil using publicly available curated expressed sequence tag information (ESTs). In this study, 9513 ESTs were downloaded from the National Center for Biotechnology Information (NCBI) database to develop unigene-based simple sequence repeat (SSR) markers. The ESTs were assembled into 4053 unigenes and then analyzed to identify 374 SSRs using the MISA microsatellite identification tool. Among the 374 SSRs, 26 compound SSRs were observed. Primer pairs for these SSRs were designed using Primer3 version 1.14. To classify the functional annotation of ESTs and EST–SSRs, BLASTx searches (using E-value 1×10^{-5}) against the public UniProt (http://www.uniprot.org/) and NCBI (http://www.ncbi.nlh.nih.gov/) databases were performed. Further functional annotation was performed using PLAZA (version 3.0) comparative genomics and GO annotation was summarized using the Plant GO slim category. Among the synthesized 312 primers, 219 successfully amplified *Lens* DNA. A diverse panel of 24 *Lens* genotypes was used to identify polymorphic markers. A polymorphic set of 57 markers successfully discriminated the test genotypes. This set of polymorphic markers with functional annotation data could be used as molecular tools in lentil breeding.

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1. Introduction

Lentil (*Lens culinaris* L. Medik.) is a nutritious food legume crop grown throughout the world. Primary production regions include Canada, Australia, northwestern USA, Turkey, Syria, and the Indian subcontinent (Nepal, India, and Bangladesh). World annual production is nearly five million tons [1]. Lentil is classified into several market classes, including extra small red, small red, large red, small green, medium green, large green, Spanish brown, zero tannin, and Puy.

Lentil originated in the Fertile Crescent (southwest Asia and Mediterranean region) and is believed to be one of the earliest domesticated food crops [2]. The cultivated lentil, *L. culinaris*, has two types, *macrosperma* and *microsperma*, based on seed and pod characteristics [3]. Like other food legumes, lentil has a narrow genetic base. Realization of potential yield is limited by various biotic and abiotic stresses such as foliar and root diseases, high or low temperature, soil pH (<5.4), and waterlogging. Optimization of crop management is also important, as weed management, water availability, and soil fertility vary among growing environments. Breeding programs worldwide are working to breed high-yielding lentil cultivars with resistance to one or more of these stresses. Many breeding programs have implemented marker-assisted selection to speed up the selection process.

Availability of molecular markers and their ease of use in large breeding programs is a priority for many crop species. However, because of the unavailability of a full genome sequence as well as the complexity of the large (4063 Mbp) genome [4], the number of available polymorphic markers is limited in lentil. Hamweigh et al. [5] developed 14 microsatellite markers from a genomic library developed from the lentil cultivar "ILL5588". The genetic diversity index calculated from the number of alleles amplified was high and markers could accurately discriminate cultivated from wild types. In another study, Kaur et al. [6] developed expressed sequence tag–simple sequence repeat (EST–SSR) markers by transcriptome sequencing of lentil and validated 79 polymorphic EST–SSRs among 13 lentil genotypes including one *Lens nigricans* accession. Verma et al. [7] developed EST–SSRs by transcriptome sequencing of the lentil genotype "Precoz" [8] and validated 54 polymorphic EST–SSRs among 22 lentil genotypes including one *L. culinaris* subsp. *orientalis* and two *Lens lamottei* genotypes.

The total number of lentil ESTs (9513) in the National Center for Biological Information (NCBI) database has remained constant. Development of genomic or transcriptome libraries is expensive and time-consuming. Researchers working in various crop species including rice [9], wheat [9], maize [10], chickpea [10], faba bean [11], pea [12], tea tree [13], and *Medicago* [14] have developed polymorphic markers using sequence information available in public databases.

The use of genic SSR markers or EST–SSRs is important from a breeding point of view. Despite recent advances in molecular markers such as single-nucleotide polymorphisms (SNPs) or DNA array-based markers, SSRs hold promise as breeder-friendly markers involving limited technical or operating difficulties. SSR markers are reproducible and PCR-based, resulting in easy application in breeding programs for marker-assisted selection or prediction of breeding values. Public databases such as NCBI NR (http://www.ncbi.nlm.nih.gov/), UniProt (http://www.uniprot.org/), and TAIR (http://www.arabidopsis.org/) can be used to functionally annotate the ESTs or EST–SSRs. This algorithm-based or alignment-based prediction of gene functions can be verified in trait-specific cases. The synchronization between functional annotation and wet-lab validation depends largely on the standard of draft sequence available. Functional annotation of the SSRs provides an opportunity for expression analysis of specific genes.

The objectives of this study were to (1) develop polymorphic SSR markers in lentil using EST sequences, (2) validate polymorphic EST–SSR markers in a diverse panel of *Lens* genotypes including wild lentil species, and (3) functionally annotate the EST–SSRs using public protein databases.

2. Materials and methods

2.1. EST sequence assembly, SSR detection, and functional annotation

Microsatellite or SSRs were developed from 9513 expressed sequence tags (ESTs) downloaded from the NCBI database using the queries "*Lens culinaris*" [Organism] OR *Lens culinaris* [All Fields] AND "*Lens culinaris*" [porgn] (Table 1). ESTs representing ("*Lens culinaris*/*Colletotrichum truncatum* mixed EST library" [porgn:__txid880151]) were excluded and only *L. culinaris*-specific ESTs were used for the analysis. The downloaded ESTs were cleaned of contamination using UniVec (http://www.ncbi.nlm.nih.gov/tools/vecscreen/univec). Cleaned ESTs were assembled using the Overlap-Layout-Consensus assembler MIRA (parameters: job = denovo, est, accurate, 454 using the -notraceinfo option) [15]. Following the MIRA assembly, unigenes were created using CAP3 [16] with parameters -p 95, -o 49, and -t 10,000 as previously described [17–19]. In addition to the parameters described in Zheng et al. [18], the parameter –t was assigned a value of 10,000, a choice that can substantially improve the quality of the assembly using the maximum available memory. This decision avoided the misassembly of the ESTs and formation of counterfeit long assemblies, as previously suggested [18,19]. The assembled unigenes were searched for SSRs using MISA [20] (http://pgrc.ipk-gatersleben.de/misa/). SSRs were defined by a minimum repeat sequence of 10 nucleotides as mono-, a sequence of six consecutive repeat units as di-, and a sequence of five repeat units for tri-, tetra-, penta- and hexanucleotide sequences. To identify and classify compound repeats, the minimum distance

Table 1 – Summary of data mining of unigene sequences of *Lens culinaris*.

Parameter	Number
Total ESTs	9513
Total size of examined sequences (bases)	2,574,487
Total unigene sequences	4054
Total SSRs detected	374
Sequences with more than one SSR	32
Total compound SSRs	26
Total ESTs with SSRs	348

between two repetitive units was kept at ≤100 bp as suggested in MISA [20]. Open reading frames were extracted from the assembled unigenes using the getorf function of the EMBOSS package (http://emboss.sourceforge.net/).

2.2. Database mining

2.2.1. Development of EST–SSRs and primer design

Following the identification of SSRs, primer pairs were designed using Primer3 version 1.1.4 (http://primer3.sourceforge.net/) with minimum and maximum amplicon size 100–300 bp, primer size (minimum, optimum, and maximum) 18–27 bp, primer T_m (minimum, optimum, and maximum) 57–63 °C, primer GC content 30–70%, CG clamp 0, maximum end stability 250, maximum T_m difference 2, maximum self-complementarity 6, maximum 39 self-complementarity 3, maximum Ns accepted 0, and maximum poly-X 5.

2.2.2. Functional annotation of unigenes and EST–SSRs

Functional annotation and gene ontology of the ESTs and EST–SSRs were performed using BLASTx searches (E-value, 1×10^{-3}) against GenBank (http://www.ncbi.nlm.nih.gov/), UniProt (http://www.uniprot.org/), and TAIR (https://www.arabidopsis.org/) databases. Additional functional annotation and gene ontology were obtained using FastAnnotator [21]. GO annotations obtained were further analyzed using GO-SLIM (Plant) (http://www.geneontology.org/ontology/subsets/goslim_plant.obo) and functional GO-SLIM categories were defined.

2.3. Plant materials and DNA extraction

Four *Lens* genotypes were used for initial screening of 312 primers, including three *L. culinaris* (Red Chief, ILL669, and WA8649041) and one *L. nigricans* (PI 572340) genotype. A diverse panel of 22 *Lens* genotypes, consisting of *L. culinaris* advanced breeding lines, parents of mapping populations, wild taxa, and genotypes of *L. nigricans*, *L. culinaris* ssp. *orientalis*, and *L. lamottei* was used to identify polymorphic markers among primers amplifying *Lens* DNA (Table 2). DNA samples were extracted from individual plant leaf tissue (100 mg) when seedlings were two weeks old using a DNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA). The DNA concentrations of the extracted samples were recorded using a Nanodrop 2000c spectrophotometer (Nanodrop, Wilmington, DE USA). The extracted DNA samples were diluted to a uniform concentration of 20 $\mu g\ \mu L^{-1}$ for successful PCR amplification.

Table 2 – Details of plant materials used.

Genotype	Species	Pedigree	Reference
Red Chief	*L. culinaris* Medik. subsp. *culinaris*	Cultivar in USA, RIL parent, PI 181886/PI 329171	[37]
WA8649041	*L. culinaris* Medik.	Pure-line selection from bulk of 8 PI lines from Turkey, RIL parent	[38]
ILL669	*L. culinaris* Medik.	RIL parent	[38]
WA8649090	*L. culinaris* Medik. subsp. *culinaris*	Pure-line selection from bulk of 8 PI lines from Turkey, RIL parent	[38]
Precoz	*L. culinaris* Medik. subsp. *culinaris*	Cultivar, donated from Argentina; Synonym = ILL 1405, RIL parent	[39]
Pennell	*L. culinaris* Medik. subsp. *culinaris*	Cultivar in Northern Plains, F_6 selection from the cross of LC660194/Brewer	[40]
Brewer	*L. culinaris* Medik. subsp. *culinaris*	Cultivar in USA, RIL Parent	[41]
Barimasur 4	*L. culinaris* Medik. subsp. *culinaris*	Cultivar in Bangladesh. ILL588/FLIP-84-112L (ILL5782)	[42]
Emerald II	*L. culinaris* Medik. subsp. *culinaris*	Cultivar in USA	[41]
Morton	*L. culinaris* Medik. subsp. *culinaris*	Cultivar in USA, autumn-sown, winter-hardy	[43]
Morena	*L. culinaris* Medik. subsp. *culinaris*	Cultivar in USA, PI 297754	Jerry Robinson, pers. comm.
PI 320937/ILL 505	*L. culinaris* Medik. subsp. *culinaris*	Germplasm collected in Germany	https://www.genesys-pgr.org/acn/id/46329
Barimasur 2	*L. culinaris* Medik. subsp. *culinaris*	Cultivar in Bangladesh, cross between ILL4353/ILL353	[44]
CDC Redberry	*L. culinaris* Medik. subsp. *culinaris*	Cross between $1049F_3$/819-5R; line $1049F_3$ was derived from the cross between 567-16/545-8; line 819-5R was derived from the cross between 86-360/[458-258G(458-122/C8L27-RC//Precoz)F_2] F_1	[45]
Barimasur 3	*L. culinaris* Medik. subsp. *culinaris*	Cultivar in Bangladesh	[46]
Pardina	*L. culinaris* Medik. subsp. *culinaris*	Cultivar in USA	Jerry Robinson, pers. comm.
Shasta	*L. culinaris* Medik. subsp. *culinaris*	Cultivar in USA. LC960027/3/PI 345635/Palouse//Brewer	Jerry Robinson, pers. comm.
Avondale	*L. culinaris* Medik. subsp. *culinaris*	Cultivar in USA	Jerry Robinson, pers. comm.
Lo4	*L. culinaris* subsp. *orientalis* (Boiss.) Penert	RIL parent	[37]
Lo56	*L. culinaris* Medik. subsp. *orientalis* (Boiss.) Penert	RIL parent	[37]
IG 72618	*L. lamottei*	Germplasm from Turkey	https://www.genesys-pgr.org/acn/id/648625
PI 572340	*L. nigricans* (M. Bieb.) Webb & Berth	Germplasm	CJ Coyne, pers. comm.

Table 3 – T_m, allele size, and polymorphism information content (PIC) of expressed sequenced tagged–simple sequence repeat (EST–SSRs) polymorphic primers; putative functions were assigned based on BLASTx search against the nr protein database (NCBI).

Sl. no.	Marker	Transcript/ unigene I.D.	SSR type	Putative function	Forward primer		Reverse primer		Allele size (bp)	PIC
					Sequence (5′–3′)	T_m (°C)	Sequence (5′–3′)	T_m (°C)		
1	PUT99	PUT187 aLensculinaris99	(AG)10	Histidine-containing phosphotransfer protein [*Medicago truncatula*]	GCGACCACTGTGTTGTTTGT	60	ATTTGAAGTCGGTGAGGTCG	60	316–322	0.65
2	PUT668	PUT187 aLensculinaris668	(AG)9	PHD1 protein [*Medicago truncatula*]	TTTTGCAGAGACGAGAGAGAAA	60	TCAGGATCGCATTGGTTGTA	60	147–149	0.40
3	PUT1105	PUT187 aLensculinaris1105	(TTG)6	Unknown protein [*Medicago truncatula*]	AGGAGGAGGAGGATGTTGCT	60	CGCACTTCCAGACAAGTTCA	60	123–129	0.54
4	PUT1231 (PBA_LC_0335)[a]	PUT187 aLensculinaris1231	(ACC)5	Proline rich protein [*Medicago truncatula*]	TGTGGTACATGCACACCAAAT	60	GGTGGTAGCAGTGGTGGAGT	60	228–244	0.49
5	PUT1263 (PBA_LC_1831)[a]	PUT187 aLensculinaris1263	(TGG)5	Aspartic proteinase nepenthesin-2 [*Medicago truncatula*]	TCACTACCGGGAGAAAGTGG	60	CTACCCACCACCTCCTCAAA	60	130–136	0.10
6	PUT1271 (PBA_LC_2023)[a]	PUT187 aLensculinaris1271	(AG)6	BEL1-like homeodomain protein [*Medicago truncatula*]	GGAGAGAAAGAGACGACAGGAG	60	TCGTTTTCTCTTCTGCGGTT	60	234–237	0.35
7	PUT2033	PUT187 aLensculinaris2033	(CCA)8	Low-temperature inducible protein [*Medicago truncatula*]	ACAATCAGGTTTCGGACCAG	60	GCATCATCGATTTTGTGGTG	60	257–266	0.64
8	PUT2096 (PBA_LC_0995)[a]	PUT187 aLensculinaris2096	(ATC)5	BHLH transcription factor [*Medicago truncatula*]	TTGCATGTATGAAACCGCAT	60	ATGGAGAAGCTAAGGGGGAA	60	267–288	0.50
9	PUT2104 (PBA_LC_2291)[a]	PUT187 aLensculinaris2104	(AAC)5	Chaperone protein dnaJ [Medicago truncatula]	ATTGCAGCCAGAGTGGAATC	60	AGAACGGCGTAAGCAGAAAA	60	195–201	0.37
10	PUT2213 (PBA_LC_2242)[a]	PUT187 aLensculinaris2213	(AAC)5	Unknown protein	CGACCTTCAGAAAGCTTGATTC	60	CAACGCAGACAACAACACAG	59	270–299	0.62
11	UN3.1	UN0003	(A)12	Acyl carrier protein [*Medicago truncatula*]	TGTGTGTTTGGAGCAATGCT	59	GATGAGGACCTGGACCTCCT	60	198–204	0.37
12	UN32	UN0032	(AT)6	Eukaryotic aspartyl protease family protein [*Medicago truncatula*]	TGTTGGTGCTGGTAAGATAGGT	59	CCCTAACCAGCCCAAAGCAT	60	272–276	0.51
13	UN33.1	UN0033	(A)10	Early nodulin-like protein [*Medicago truncatula*]	CCCAAGCCAACCATTTTTGC	59	GCATCAGGTTTGCCACCAAG	60	177–182	0.30
14	UN46	UN0046	(TTC)6	Phospholipid hydroperoxide glutathione peroxidase [*Medicago truncatula*]	TCAACTCGCATCCTCTTCACA	59	TGATTGGGGGTTTGATGGGG	60	231–238	0.47
15	UN3776	UN3776	(TATT)5	PHD finger alfin-like protein [*Medicago truncatula*]	TCCAGGTAAACGAGAAGTTGAAGA	60	AGTGTGTGAATTCGTGCCCA	60	125–313	0.96
16	UN3302	UN3302	(CCT)5	Hypothetical protein MTR_2g010790 [*Medicago truncatula*]	TGGCACCACCAAAGAGACTC	60	TGGGGTTCGAGATTGGGGTA	60	114–266	0.90
17	UN3176	UN3176	(T)10	Protein nuclear fusion defective 6, chloroplastic/mitochondrial isoform X1 [*Cicer arietinum*]	TTTGCTTTTAGGCCGCCAAG	60	TCCCAGAATGAAGGGTTAACCA	59	211–264	0.66
18	UN3814.1	UN3814	(A)11	Cyclin [*Medicago truncatula*]	TCGGTAGCTGCTAGTGTCAC	59	CTTCCACCACCACCTTGACA	60	231–373	0.75
19	UN3814.2	UN3814	(T)13	Cyclin [*Medicago truncatula*]	TTGTGCAGGGTCGACCTTAC	60	GTCGATGTCCCAGATCAGCC	60	234–315	0.78
20	UN3720	UN3720	(A)10	Structural maintenance of chromosomes domain protein [*Medicago truncatula*]	CTCACTCACCCGAGAAACTCA	59	CTTCTGCGACGCAATGCTTT	60	230–387	0.69

21	UN3519	UN3519	(T)10[b]	UDP-D-glucuronate 4-epimerase [*Medicago truncatula*]	TCCCTTTTCTTCTTGACCGAGA	59	GTTCCGTTTACGCATGCGAA	60	284–291	0.83
22	UN3311	UN3311	(GAT)6	Hypothetical protein MTR_1g069440 [*Medicago truncatula*]	ACATGCCTGTGGTGGTTGAT	60	AGTGACACCATTTTCAGGGTCA	60	290–305	0.79
23	UN3728	UN3728	(CAA)5	DCD (development and cell death) domain protein [*Medicago truncatula*]	ACTCGTCCACCAAAAATGAACG	60	GCACCACCAAACTTAACTCCC	59	233–295	0.87
24	UN3652 (PBA_LC_2312)[a]	UN3652	(AAC)5	Growth-regulating factor-like protein [*Medicago truncatula*]	CCGTTCAAGAAAGCCTGTGG	59	TCCAGATGATGCTGATGACCT	59	231–362	0.73
25	UN3321	UN3321	(CAC)5	Protein PHR1-LIKE 1-like isoform X1 [*Cicer arietinum*]	ACGACTCTGTTTCTTCCGCA	60	CCCTCCGGAAACTTCTTTGC	59	146–417	0.90
26	UN3548	UN3548	(A)19	Unknown [*Medicago truncatula*]	GCGGTGGCAAACGTTAAGTA	59	AAGCAGAACCGAGCCAAGTT	60	178–542	0.90
27	UN3414	UN3414	(TTC)6	myb-like transcription factor family protein [*Medicago truncatula*]	CTCCTTCCATTTCTCTTTCTGCA	59	GACAAGGGTCAGCAAGGTGA	60	216–226	0.54
28	UN3326	UN3326	(A)10	UV radiation resistance-associated-like protein [*Medicago truncatula*]	GGAGTTTCATGCGCCAAGTT	59	GGGCCCCGTCAAATGTAACA	61	147–202	0.84
29	UN3849	UN3849	(AG)7	Defender against cell death [*Medicago truncatula*]	GACGACTTCAGTTGAAACAGCT	59	TACCTGAAGGAGAGCGGTGA	60	298–347	0.78
30	UN3573	UN3573	(GT)11	Unknown [*Medicago truncatula*]	AGGCGTCCTTTGTATGCACA	60	AACAGTCAACATAAACAACAGCGA	60	109–120	0.79
31	UN3291 (PBA_LC_0663)[a]	UN3291	(CAAC)5	Vacuolar proton-inorganic pyrophos-phatase [*Medicago truncatula*]	CAACCCATGGTGGTCTCCTC	60	CACGCGGAAAAGATTCAGCC	60	227–242	0.68
32	UN0079.2 (PBA_LC_1284)[a]	UN0079	(GGC)5	Insecticidal lentil peptide, partial [*Lens culinaris* subsp. *culinaris*]	TCGGGTGAGACCATTGTTCG	60	CAGACACCACTTGTTGCTGC	60	282–297	0.85
33	UN0099	UN0099	(T)20	Transmembrane protein, putative [*Medicago truncatula*]	TACTCATCGCCGTTGGTGTT	60	TCCTTAGTTTCAAAACAGCTTTCA	57	271–292	0.81
34	UN0106 (PBA_LC_0869)[a]	UN0106	(ATA)6	Xylose isomerase [*Medicago truncatula*]	AGAAAAGGGGAAGGGGGAGA	60	CTTCCTCCCGATTCTCACCG	60	131–209	0.68
35	UN0110	UN0110	(T)11	Heat shock protein [*Medicago truncatula*]	AAGCTGATGCTGACATGCCT	60	CCATAAAAGTATGCCCAACTTGCA	60	240–243	0.40
36	UN0119	UN0119	(A)21	Spastin [*Medicago truncatula*]	ACATTTTGGTTGAAGTCTGCCT	59	AGCTGCCTTGCCTCATTTCT	60	147–399	0.88
37	UN0123	UN0123	(CT)53	NADH-quinone oxidoreductase subunit F [*Medicago truncatula*]	ACCGTCTGATTGAGCACAGT	59	TCCAAAGCCATCCAGTTCCC	60	142–288	0.91
38	UN0146 (PBA_LC_0741)[a]	UN0146	(GAT)7	Translational elongation factor 1-beta [*Medicago truncatula*]	TGACACCAAGGCCACTGAAG	60	AGTTTGGATGCGCCCCATAA	60	143–461	0.89
39	UN0225	UN0225	(A)29	40S ribosomal protein SA [*Medicago truncatula*]	ACATGTTGCAATGCTTTTAGCCT	60	TTCTTGCTTGGCGTTGAAGC	60	190–326	0.77
40	UN0230	UN0230	(T)10	Light-harvesting complex I chlorophyll A/B-binding protein [*Medicago truncatula*]	AGAGGGCTCCAACTCTGTGA	60	ACGGGCCGAATAATCATGCA	60	169–179	0.67
41	UN0281	UN0281	(A)22	Predicted: photosystem I subunit O [*Cicer arietinum*]	TGTCTGGCTTGAGCAGAAGA	59	TGTTGCCATAGCTTGCCTCA	60	120–250	0.80
42	UN0536	UN0536	(TA)6	Cysteine proteinase inhibitor [*Medicago truncatula*]	ATAGGCCTGCTTGGACCCTA	60	ACAAAGGCAATTTCCAAACGT	57	114–123	0.63
43	UN0538	UN0538	(T)12	myb transcription factor [*Medicago truncatula*]	GCAAAGAGCTCGTGTGTGTT	59	AGCAGTTAGATCACAGCTACCA	59	130–178	0.82
44	UN0575	UN0575	(T)12	Predicted: arabinogalactan peptide 16-like [*Cicer arietinum*]	CGCTCAATCTCCTTCCCCTG	60	CCTCCTCCGCGTTCTACAAA	60	139–433	0.88
45	UN0748	UN0748	(A)10	Acylamino-acid-releasing enzyme [*Medicago truncatula*]	CATTGCTGCGTGGTTCAACA	60	TCAAATATTCAGTGTCATGTTCTACTT	57	120–240	0.82

(continued on next page)

Table 3 (*continued*)

Sl. no.	Marker	Transcript/ unigene I.D.	SSR type	Putative function	Forward primer		Reverse primer		Allele size (bp)	PIC
					Sequence (5′–3′)	T_m (°C)	Sequence (5′–3′)	T_m (°C)		
46	UN0755 (PBA_LC_0335)[a]	UN0755	(ACC)5	Proline rich protein [*Medicago truncatula*]	CATGCACACCAAATCCACCA	59	TATCGGTGGCACGACAACAA	60	146–148	0.32
47	UN0861	UN0861	(GAA)10	Peroxidase [*Medicago truncatula*]	ACAACACCATGATGAGCCTTG	59	TGTGTCATCCATGGACCACA	59	271–359	0.78
48	UN0931	UN0931	(A)16	Snakin-1 [*Medicago truncatula*]	AGGGACAAGGAAAATGCCCT	59	AGCCCTGTACATCACCCAAA	59	127–158	0.72
49	UN0953	UN0953	(A)11	Legumin [*Medicago truncatula*]	ACCTCGCAGCCATGAGATTC	60	GCTCTCGCGAATCTTTGCAG	60	204–211	0.67
50	UN0982	UN0982	(A)18	Non-specific lipid-transfer protein 3 [*Lens culinaris*]	TGATGGTGCGGTTTCAAGGT	60	CCTACTCCCCCATCCAGGTT	60	206–421	0.77
51	UN1014	UN1014	(A)19	Histone H3 [*Medicago truncatula*]	AGCTACCTGGCTACCCATTT	58	GGATTTGCGAGCGGTTTGTT	60	130–467	0.84
52	UN1128	UN1128	(A)10	Predicted: membrane-anchored ubiquitin-fold protein 3 [*Cicer arietinum*]	CACCAACAACAACAGCAGCA	60	CCAACTCCTCTTCCGGCATT	60	313–325	0.38
53	UN1583	UN1583	(TAT)5	Unknown protein	CTTCCCGATCGTCGTATCGT	59	TCAATTTTCTGCATCATGAACCT	57	177–319	0.41
54	UN1952	UN1952	(TAT)8	1-Aminocyclopropane-1-carboxylate oxidase [*Medicago truncatula*]	AGGACAAGTGTTGGTGTGGG	60	CAGTTCTAAATCACTGCATCGCA	60	264–285	0.70
55	UN2594	UN2594	(A)11	Wound-responsive family protein [*Medicago truncatula*]	TTCTTCTTCTCAATTCAGATCAACTT	57	GTACCTAAGCTGCTGGGGTC	60	215–251	0.82
56	UN2787	UN2787	(CAC)7	Adenylate kinase [*Medicago truncatula*]	GCTACAAAAAGCGCGTTTGC	60	TCATAACACGTAGCGGCTCC	60	105–211	0.49
57	UN2827 (PBA_LC_0368)[a]	UN2827	(TAA)5	Hypothetical protein MTR_1g084000 [*Medicago truncatula*]	AGCAGAAAGCACATTGCACA	59	CAAAGGCTGGGAAGGCAAAG	60	285–293	0.41

[a] These markers were first identified by Kaur et al. [6]. In the present study, we validated these as being polymorphic markers in lentil.
[b] (T)10: ccgtattgtatttttacatccaacttaattaaaaatcctaacaaactaaaaagatatttcaaaaat (A)10; (A)11: cataatagcatctattaaaacatacatgatggacaagcaatttctcaac (A)12.

2.4. PCR amplification

Three hundred and twelve primer pairs were synthesized by Eurofins MWG Operon (Louisville, KY, USA) and used in this study. PCR reactions (25 μL volume) were conducted in an ABI 7500 (Applied Biosystems, Foster, CA, USA) thermocycler, with each reaction containing 2.5 μL of *Taq* buffer, 1.5 μL of $MgCl_2$ (25 mmol L^{-1}), 0.20 mmol L^{-1} of each dNTP (all from Promega, Madison, WI, USA), 0.50 mmol L^{-1} of each primer (Eurofins MWG Operon), 0.25 μL of Hot Start *Taq* polymerase (Promega) and 20 ng of template DNA. For initial screening of primers, touchdown PCRs were performed using DNA from four lentil genotypes and the following program: 94 °C for 3 min, followed by 18 cycles of 94 °C for 50 s, 65–55 °C for 50 s, and 72 °C for 50 s, followed by 20 cycles of 94 °C for 50 s, 55 °C for 50 s, and 72 °C for 50 s, and a final elongation of 72 °C for 7 min. The PCR products were resolved in 2% agarose gels (molecular biology grade, Sigma, USA) and bands were scored using a gel documentation system. Primers amplifying *Lens* DNA were validated on a set of 22 diverse *Lens* genotypes in an ABI 7500 thermocycler using the following program: 94 °C for 5 min, followed by 42 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min, followed by a final elongation step of 72 °C for 5 min. Forward primers were tagged with M13 sequence (5′-CACGACGTTGTAAAACGAC-3′) at the 5′ end. Four dyes were used to set up the multiplex PCR reactions. PCR products were separated using an ABI 3730xl (Applied Biosystems, Foster, CA, USA) according to the manufacturer's instructions with the addition of an ABI GeneScan LIZ500 size standard, and amplification product sizes were determined with GeneMapper v3.7 software (Applied Biosystems).

3. Results

3.1. Assembly and SSR detection

A set of 9513 ESTs were downloaded in FASTA file format from NCBI [22] and clustered by an identity of 0.95 into 4106 unigene sequences. Unigenes shorter than 100 bp were removed and the homopolymer ends of unigenes longer than 100 bp were trimmed. MIRA assembly of the 9513 EST sequences ultimately generated 4053 unigene sequences. The total length of the analyzed sequences was 2,574,487 bases. MIRA detected 374 SSR-bearing EST sequences among these unigenes. Of these EST–SSRs there were 36 sequences with more than one SSR. Also, 26 compound SSRs were observed. For further analysis, 348 EST–SSRs were chosen. Using Primer3, primer pairs were designed for these 348 EST–SSRs (Table S1). In addition, 279 primer pairs (Table S2) were designed based on the plant GDB assembly (version 187a) of *L. culinaris* (http://www.plantgdb.org/download/download.php?dir=/Sequence/ESTcontig/Lens_culinaris/current_version). These were designed based on the detected EST–SSRs, and were further e-validated using iPCRessipcress software (http://www.ebi.ac.uk/about/vertebrate-genomics/software/ipcress-manual). Primers co-amplifying products are listed in Table S2. In the validation experiment, 312 primers were used: 48 primers from the e-validated list and 264 primers from Table S1. E-validated primers are coded with prefix "PUT" and other primers with "UN" (Table 3).

3.2. Structural and functional annotation of ESTs and EST–SSRs

Contig length ranged between 199 and 2599 bp (Fig. S1). The most prevalent contig length was 600–799 bp, followed by 400–599 and 800–999. Functional GO-SLIM analysis showed that the distribution of unigenes among GO, Domain, and Enzyme categories was 55.57%, 39.91%, and 4.52%, respectively (Fig. S2). This distribution was consistent with the categorization of the EST–SSRs into functional GO, Domain, and Enzyme categories, which accounted for 54.25%, 42.38%, and 3.70%, respectively (Fig. S3). In the GO category Biological Process, the first four processes were oxidation–reduction process, ribosome biogenesis, translation and regulation of transcription, and DNA dependent (Fig. S4). A similar trend was observed using GO Biological Process analysis of the EST–SSRs, in which the ranking of processes was as follows: oxidation–reduction process, regulation of transcription, DNA dependent, ribosome biogenesis, and translation (Fig. S5). The first four functions of the unigenes in the GO category Molecular Function were DNA binding, nutrient reservoir activity, structural constituent of ribosome, and zinc ion binding (Fig. S6). However, in the GO category Molecular Function, for the EST–SSRs, the first four functions were ATP binding, DNA binding, structural constituent of ribosome, and zinc ion binding (Fig. S7). In the GO category Cellular Process, the first four functions of the unigenes were nucleus, cytosol, plasma membrane and chloroplast (Fig. S8) and for the EST–SSRs the first four functions were cytosol, plasma membrane, nucleus, and integral to membrane (Fig. S9).

3.3. Frequency and distribution of EST–SSRs

The frequency of SSRs was 6.89 per kb of sequence analyzed (374 SSRs/2575 kb of sequence). There were 21 SSR repeat patterns (Fig. S10). The most prevalent were trinucleotide, followed by mono-, tetra-, and pentanucleotide repeat patterns. The most frequently observed repeat was AG/CT, followed by AAG/CTT, AAC/GTT, ATC/ATG, and AT/AT (Fig. S10).

3.4. Validation of EST–SSRs

Among the synthesized 312 primers, 219 successfully amplified *Lens* DNA. A diverse panel of 22 *Lens* genotypes, consisting of *L. culinaris* advanced breeding lines, parents of mapping populations, wild taxa, and genotypes of *L. nigricans*, *L. culinaris* ssp. *orientalis*, and *L. lamottei* was tested to identify polymorphic markers. A total of 57 polymorphic primers were found. The number of alleles amplified ranged from 2 to 17 for each primer and the polymorphic information content (PIC) ranged between 0.10 and 0.91. The average number of alleles produced per primer was seven.

4. Discussion

MIRA assembly is very flexible. Short reads, such as ESTs, can easily be assembled into contigs and specific trimming further improves the quality of the sequences (http://mira-assembler.sourceforge.net/docs/DefinitiveGuideToMIRA.html) [23]. The

number of SSR-containing sequences detected was very high compared to those in other studies; however, they yielded fewer polymorphic markers. These polymorphic markers successfully discriminated the test genotypes and grouped genetically more closely related individuals. Simple sequence-based markers are the most robust and easy to use in marker systems. Moreover, sequencing-based gel separation technologies can now detect differences of a very few bases among alleles. It can be seen from the results of the present experiment that each polymorphic marker generated multiple alleles and that for several, up to 17 alleles were observed. Similar results were obtained with other crops for which EST databases were used to develop robust genic SSR markers [10–14,29]. Recently, Kumar et al. [25] reviewed the recent development of genic SSR markers in lentil [6,7,26,27] and Andeden et al. [28] developed 78 polymorphic SSR markers in lentil. However, the number of polymorphic genic SSR markers is still limited. Kaur et al. [6] validated a subset of de novo discovered 192 EST–SSR markers across a panel of 12 cultivated lentil genotypes, observing 47.5% polymorphism using a set of 2393 EST–SSR markers. Kaur et al. [26] found 40 polymorphic markers after testing 516 EST–SSRs. Andeden et al. [28] developed (CA)n, (GA)n, (AAC)n, and (ATG)n repeat-enriched libraries and by sequencing these libraries found 78 polymorphic SSR markers using a set of 15 Turkish lentil genotypes. They observed 21.6% polymorphism (of the 360 primers validated, 78 were polymorphic). In the present study, a test of 219 markers on 22 cultivated and wild lentil genotypes yielded 26% polymorphism. Verma et al. [7] reported 42.59% polymorphism in 54 markers tested on 22 lentil, *Medicago*, *Glycine*, and *Vigna* genotypes. The inclusion of additional genera contributed to the high percentage of polymorphism that they reported. The use of SSR markers for diversity analysis or grouping of genotypes based on genetic relatedness in lentil or other closely related food legumes has been reported by several workers [29–32]. Kaur et al. [6] and Verma et al. [7] also found comparable grouping ability of the test polymorphic markers. Verma et al. [7] and Andeden et al. [28] reported a lower number of alleles amplified per locus (2.3 and 5.1, respectively) than found in our study (7 alleles). Wong et al. [33] classified four gene pools in lentil using genotyping by sequencing (GBS) of 60 genotypes. These were primary, secondary, tertiary, and quaternary gene pools, formed by *L. culinaris/Lens orientalis/Lens tomentosus*, *L. lamottei/Lens odemensis*, *Lens ervoides*, and *L. nigricans*, respectively.

The distributions of functional annotation categories were dissimilar between the total ESTs and EST–SSRs. It is noteworthy that reducing the detail of the GO categories improved the authenticity of the annotation data. It was observed that most functional annotations remain the same between the unigenes and EST–SSRs. Functional annotations of the EST–SSR flanking regions indicated the involvement of the translated portion of the genome. This finding is important for the development of functional markers in lentil. The lentil genome sequencing project is under way. The most recent draft (version 0.7) has approximately 150× coverage, producing scaffolds covering about half of the genome. The initial assembly resulted in useful SNPs suitable for marker-assisted selection [34].

5. Conclusions

A polymorphic set of 57 markers was developed in lentil. Of these, 14 amplified the same SSRs reported by Kaur et al. [6]. The markers were further validated among diverse lentil genotypes. They could be used by the lentil research community for molecular breeding. Development of dense genetic maps is a prerequisite for adoption of genomic tools in lentil [35] and recently EST–SSR and SNPs were mapped in lentil [26,36]. Lists of unigene sequences for the polymorphic markers are available in the Cool Season Food Legume database (https://www.coolseasonfoodlegume.org/).

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cj.2016.06.012.

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