Chapter 6
Advances in Lentil Genomics

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Abstract Lentil is a diploid (2n = 2X = 14) self-pollinating crop with a genome size of 4 Gbp. The use of genomics tools in lentil breeding programs has been limited, since available genomic resources are not adequate. Recent advances in high-throughput genotyping and sequencing technologies have brought in new impetus in the development of genetic and genomic resources and high resolution marker-trait association in lentil. Their integration in marker-assisted breeding is expected to improve the precision and efficiency in breeding programs with accelerated and directed genetic gains in crops like lentil. Molecular markers are expected to facilitate indirect selection for difficult traits, introgression of novel genes into adapted varieties, pyramiding genes from different sources, and combining multiple stress resistance. The present review highlights recent advances in lentil genomics and future outlook in the light of rapid advancement in the genomics tools.

Keywords Lentil • *Lens culinaris* • Molecular marker • Genomic resources • Marker assisted breeding

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Introduction

Lentil (*Lens culinaris* ssp. *culinaris* Medicus) is an important grain legume species cultivated throughout the West Asia, North Africa, the Indian subcontinent, North America, and Australia, providing a vital source of dietary protein in human diets and protein-rich straw for animal feed (Erskine et al. 2011). Lentil shares the ability to fix atmospheric nitrogen with other legumes, making it a useful option for soil fertility management in cereal based cropping systems. Lentil also provides rotational benefits to cereal crops in management of weeds, diseases and insect pests, and in many cases offers a profitable, high value crop option for farmers (Rahman et al. 2009). Genetic enhancement programs have been undertaken using conventional breeding approaches to improve yield and adaptability of the crop at national and global levels. Recently, deployment of genomic resources has become an integral part of breeding programs in many crops worldwide. However in comparison to cereal and major food legume crops, there are limited efforts in the development and deployment of molecular tools in lentil. Molecular tools have recently been used by lentil breeders and biotechnologists to understand the genetic basis of a few traits related to biotic (ascochyta blight, anthracnose, rust, fusarium wilt, stemphylium blight) and abiotic (drought, frost, cold, boron, salinity) stresses (Muehlbauer et al. 2006). However, in the genomic era, there is a need to keep pace with the development of new molecular tools and techniques such as transcriptomics and whole genome sequencing. Whole genome sequencing projects have been undertaken for model legumes like *Medicago* and *Lotus*, providing an opportunity to identify putative orthologous gene sequence resources in other legume species, especially those located within the Galegoid clade of the Fabaceae sub-family Papilionoideae. In addition, a draft genome sequence has recently been completed for the warm-season food legumes, soybean (Schmutz et al. 2010) and pigeon pea (Varshney et al. 2011), which belong to the Phaseoloid clade providing further insights into comparative genomics within the Fabaceae family.

In the present chapter, we reviewed lentil genomics, focusing on the present status of genomic resources, molecular markers, genetic engineering and the future outlook in the light of rapid advancement in the genomics tools.

Genome Size

Lentil is a diploid (2n = 2X = 14) self-pollinating crop with a genome size of 4 Gbp (Arumuganathan and Earle 1991). Variable chromosome numbers have been reported in inter-specific hybrids e.g., seven bivalents in the intra-specific hybrids within the members of *L. culinaris* to five bivalents and one quadrivalent in the F_1 hybrids derived from a cross between *L. culinaris* and *L. orientalis*. Several workers have studied karyotypes in *L. culinaris*, and reported similar karyotypes. The length of chromosomes ranged from 3.0 to 9.2 μm. Gupta and Singh (1981) reported two

pairs each of metacentric and sub-metacentric and three pairs of acrocentric chromosomes. The large size of nuclear genome in lentil poses a great challenge to sequence it with the limited resources.

Genomic Resources

Genomic resources are very important for a crop improvement program. Molecular approaches have made limited progress in improving the understanding of the lentil genome. Lentil breeding programs do not yet use marker-assisted selection (MAS) because the genetic maps developed in lentil with restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR) markers are not tightly linked to the genes of interest. Therefore, their use in identification of close trait-marker association remains a major limitation in lentil. In the recent past, however, efforts have been augmented towards the development of genomic resources in lentil.

Microsatellite or SSR Markers

Recently, major efforts have been directed towards the development of microsatellite and gene-specific markers in lentil. Microsatellite or SSR markers are generally co-dominant, unilocus, multi-allelic and species specific. Produced from primers designed to the flanking sequence of mainly di- and trinucleotide repeats, microsatellites have emerged the markers of choice in many plant species to gain understanding of genetic relationships, evolutionary insights and gene mapping. Development of microsatellite markers requires a considerable amount of laboratory effort. Successful isolation of microsatellite markers involves construction and screening of small insert genomic library with SSR motifs, sequencing of the positive clones, designing the primers that can amplify SSR loci, and determining polymorphic SSR primers. The first *Sau*3AI genomic library was constructed from the cultivated accession, ILL5588 and screened with (GT)10, (GA)10, (GC)10, (GAA)8, (TA)10 and (TAA) probes (Hamwieh et al. 2005). Dinucleotide repeats were observed more frequently than trinucleotide repeats or other motifs (Table 6.1). The microsatellite motifs were classified as perfect, imperfect, compound perfect or compound imperfect repeats according to the modified classification of Weber (1990). The simple/perfect repeats were predominant (56.8 %) followed by compound/perfect (16.1 %) whereas compound/imperfect (12.7 %) occurred least often. Among the perfect repeats, (CA/GT)n motifs were the most abundant, comprising 24.2 % of the isolated clones, followed by (AT/TA)n repeats (8.9 %). This led to a set of 30 highly polymorphic SSR markers in lentil. Hamwieh et al. (2009) further developed an additional set of 14 microsatellite markers and used them for genetic diversity analysis of the lentil

Table 6.1 Microsatellite motifs observed in the lentil genomic library

Type		Microsatellite motif	Number	% Occurrence
Simple	Perfect	CA/GT	57	24.2
		CG/GC	2	0.8
		CT/GA	7	3
		CTT/GAA	3	1.3
		AT/TA	21	8.9
		ATT/TAA	7	3
		Others types	37	15.7
		Total	134	56.8
	Imperfect	CA/GT	21	8.9
		CG/GC	0	0
		CT/GA	1	0.4
		CTT/GAA	0	0
		AT/TA	3	1.3
		ATT/TAA	1	0.4
		Others types	8	3.4
		Total	34	14.4
Compound	Perfect		38	16.1
	Imperfect		30	12.7
	Total		68	28.8
Total			236	100

Based on data from Hamwieh A, Udupa SM, Sarker A, Jung C and Baum M. Development of new microsatellite markers and their application in the analysis of genetic diversity in lentils. Breed Sci 2009; 59:77–86

core set. Recently, 126 SSR markers were generated using a magnetic bead capture method at the Washington State University (Weidong Chen and P.N. Rajesh, Personal communication). More than 500 SSRs have been generated from enriched genomic library in lentil (Sabhyata Bhatia, Personal Communication). Still, the lentil-specific SSR markers are limited and not sufficient for the genome-wide coverage to establish genetic relatedness among the closely related germplasm accessions.

Expressed Sequence Tags (ESTs)

ESTs are short DNA sequences of 150–400 bp from a cDNA clone that corresponds to a particular mRNA. These are developed and publicly made available (Rudd 2003). Development of high-throughput functional genomics approaches like Serial Analysis of Gene Expression (SAGE) has led to the generation of more ESTs. The cDNA clones corresponding to the ESTs of interest can be used as RFLP or CAPS based markers (Varshney et al. 2005). The EST sequence data also serve the purpose of identifying SSRs and/or SNPs. Before the ESTs, development of SSR and

SNP markers was expensive and required high resource laboratories, but presently any user can download them from the database and use some special bioinformatic programs like MISA for SSR detection (Thiel et al. 2003; Varshney et al. 2005) and SNipPer for SNP discovery (Kota et al. 2003; Varshney et al. 2005). As on October 2012 there are about 10,163 ESTs available for lentil. Most of the available ESTs (9,513) were published in November 2010 and another remaining 647 ESTs in September 2012 by the University of Saskatchewan. The first EST library was made from a mixture of eight cultivars with varying seed phenotypes. The second cDNA library was prepared from the leaflets of a Canadian cultivar 'Eston' inoculated with *Colletotrichum truncatum*. Kaur et al. (2011) carried out transcriptome sequencing of lentil based on the second-generation technology which permits large-scale unigene assembly and SSR marker discovery. They used tissue-specific cDNA samples from six genotypes (Northfield, ILL2024, Indianhead, Digger, ILL6788, and ILL7537) using Roche 454 GS-FLX Titanium technology, and generated c. 1.38×106 ESTs. *De novo* assembly generated 15,354 contigs and 68,715 singletons. Out of huge ESTs produced, 3,470 SNP and EST-SSRs have been identified. Development of genomic resources has become possible and cost effective with the advent of next generation sequencing of ESTs. Validation of a subset of 192 EST-SSR markers across a panel of 12 cultivated genotypes showed 47.5 % polymorphism from a set of 2,393 EST-SSR markers developed in lentil (Kaur et al. 2011).

Single Nucleotide Polymorphism (SNP)

SNP markers are considered ideal for genetic mapping and diversity assessment in crop plants due to their abundance and relatively even distribution across the genome (Chagne et al. 2007). In addition various technologies exist for evaluation of SNP loci and many of these are amenable to automation for allele calling and data collection. In fact, the availability of extensive sequence database made a new beginning to exploit them as a high-throughput marker system for genome mapping studies. The availability of abundant high-throughput sequence-based markers is essential for detailed genome-wide trait analysis. A significant amount of efforts has been invested in re-sequencing alleles to discover SNPs. There are techniques to detect SNPs such as allele-specific PCR, single base extension and array hybridization methods. Since SNP discovery and genotyping require expensive and sophisticated platforms, the development and exploitation of SNP markers are still restricted to major crop species such as rice (Nasu et al. 2002), wheat (Somers et al. 2003), barley (Kota et al. 2001; Kanazin et al. 2002), maize (Tenaillon et al. 2001), and soybean (Zhu et al. 2003). Recent advances in sequencing techniques have helped in developing SNP assay in lentil. The Department of Primary Industries (Victoria, Australia) and University of Saskatchewan are involved in developing lentil genomic resource by developing SNP and COS marker assay.

Marker Transferability

Comparative genome mapping has demonstrated different levels of genome conservation among crop species during the course of evolution (Choi et al. 2004; Zhu et al. 2005). The lentil genome has shown different degrees of synteny with other legume crops (Weeden et al. 1992; Simon and Muehlbauer 1997; Phan et al. 2007; Choudhary et al. 2008). Development of PCR-based markers has improved transferability of genetic information among species through comparative genomics, and has facilitated the establishment of phylogenetic relationship in plants species. Since the availability of microsatellite markers in lentil is limited, other legumes offer great scope of marker transferability for genome-wide coverage. Pandian et al. (2000) observed 5 % transferability of chickpea-specific STMS primers in lentil while Reddy et al. (2009) observed successful amplification of 62 % *Trifolium* markers followed by *Medicago* (36 %) and *Pisum* (25 %). Datta et al. (2011) reported transferability of 19 STMS markers from common bean, chickpea, pigeon pea, and soybean. The lack of lentil-specific microsatellite sequences and gene-based markers propelled the mining and transfer of expressed sequence tag-simple sequence repeat (EST-SSR) sequences from the model genome *Medicago truncatula* to enrich an existing intraspecific lentil genetic map (Gupta et al. 2012a). They published 21 clear and reproducible markers showing polymorphism between parents, Northfield and Digger. EST-based intron-targeted amplified polymorphic (ITAP) markers have recently been developed from related crops and applied to lentil. ESTs were compared for phylogenetic distant from *M. truncatula*, *Lupinus albus*, and *G. max* to produce 500 ITAP markers that could be applied to lentil (Phan et al. 2007). Also, 126 *M. truncatula* cross-species markers were used to generate comparative genetic maps of lentil and white lupin and macrosyntenic relationships between lentil and field pea was observed.

Functional Genomics

Differential gene transcript profiles were assessed among resistant (ILL7537) and susceptible (ILL6002) lentil genotypes at 6, 24, 48, 72 and 96 h after inoculation (hai) with *Ascochyta lentis* (AL4 isolate) (Ford et al. 2007). The non-redundant differentially expressed genes for each accession and time point were hierarchically clustered using Euclidean metrics. In total, 25 differentially expressed sequences were up-regulated and 56 down-regulated in ILL7537 whereas 26 were up-regulated and 44 down-regulated in ILL6002. Several candidate defense genes were characterized from lentil including a *b*-1,3-glucanase, a pathogenesis-related protein from the Bet v I family, a pea disease resistance response protein 230 (DRR230-a), a disease resistance response protein (DRRG49-C), a PR4 type gene and a gene encoding an antimicrobial SNAKIN2 protein, all of which have been fully sequenced. Several transcription factors were also recovered at 6 hai and future

aims will be to further biologically characterize these and earlier responses to gain a comprehensive understanding of the key pathogen recognition and defense pathways to *A. lentis* in lentil. Also, the full-length gene sequences will be used in transgenic studies to further characterize function.

Mapping Populations

Mapping populations for important traits are essential genetic resources to establish trait-marker association. Therefore, efforts have been made at ICARDA and national programs to develop mapping populations for key traits in lentil (Table 6.2). RIL (recombinant inbred lines) populations were developed from the crosses made between contrasting parents for the traits of interest through single seed descent method. Indian Institute of Pulses Research (IIPR) has recently developed RIL population from a cross between ILL6002 and ILL7663 in order to identify and map early growth vigor genes in lentil. Identification of markers linked to the genes/QTLs governing these traits will help in development of genotype having high biomass at early stage. For tagging and mapping of genes of earliness, another mapping population was developed from a cross between Precoz (Medium early) and L4603 (early). CSK Himachal Pradesh Agricultural University, Palampur, India has developed recombinant inbred populations involving both intra and intersubspecific crosses that differ for rust reaction, drought tolerance, flowering time, plant vigour, shattering tolerance, seed size and seed weight.

Molecular Maps

The earlier *Lens* genetic linkage maps were constructed by using morphological and isozyme markers (Zamir and Ladizinsky 1984; Tadmor et al. 1987; Vaillancourt and Slinkard 1993). Although genetic mapping (linkage analysis) began in lentil in 1984, the first map comprising DNA based markers was produced by Havey and

Table 6.2 Mapping populations developed for various traits in lentil at ICARDA

Trait	Cross	Population size
Drought	ILL7946 × ILL7979	174
Cold	ILL4605 × ILL10657	153
Earliness	ILL7115 × ILL8009	150
Rust	ILL5888 × ILL6002	152
Fusarium wilt	ILL213 × ILL5883, Precoz × Idleb 2	150
Zn content	ILL5722 × ILL9888	177
	ILL9888 × ILL5480	149
Fe content	ILL9932 × ILL9951	193

Table 6.3 Molecular maps developed in lentil

Population used for mapping	No. of loci	Type of markers	Genetic map length (cM)	References
RILs (ILL5588×L692-16-1)	177	RAPD, AFLP, RFLP	1,073	Eujayl et al. (1998a)
F2 (ILL5588×ILL7537)	114	RAPD, ISSR	784	Rubeena et al. (2003)
Lens culinaris ssp. *Culinaris*×*L. c.* ssp. *orientalis*	161	RAPD, ISSR, AFLP, SSR	2,172	Duran et al. (2004)
RILs (ILL5588×L692-16-1)	283	SSR, AFLP	751	Hamwieh et al. (2005)
F2 (L830×ILWL77)	199	SSR, ISSR and RAPD	3843.4	Gupta et al. (2012a, b)
RIL (ILL5588×ILL5722)	196	RAPD, ISSR, EST-SSR, and SSR	1156.4	Gupta et al. (2012a, b)

Muehlbauer (1989). Subsequent maps were published by several workers (Table 6.3). With the development of PCR based markers, the number of mapped markers across the *Lens* genome increased dramatically (Kumar et al. 2011). The first extensive map comprised 177 RAPD, AFLP, RFLP and morphological markers was constructed using a RIL population from a cross between a cultivated *L. culinaris* ssp. *culinaris* cultivar and a *L. culinaris* ssp. *orientalis* accession (Eujayl et al. 1998a). Rubeena et al. (2003) published the first intraspecific lentil map comprising 114 RAPD, inter simple sequence repeat (ISSR) and resistance gene analogue (RGA) markers. Rubeena et al. (2006) reported F_2 map comprising 72 markers (38 RAPD, 30 AFLP, 3 ISSR and one morphological) spanning 412.5 cM. The first *Lens* map to include SSR markers was that of Durán et al. (2004). Hamwieh et al. (2005) added 39 SSR and 50 AFLP markers to the map constructed by Eujayl et al. (1998a) to produce a comprehensive *Lens* map comprising 283 genetic markers covering 715 cM. Subsequently, the first lentil map that contained 18 SSR and 79 cross genera ITAP gene-based markers was constructed using a F_5 RIL population developed from a cross between ILL5722 and ILL5588 (Phan et al. 2007). The map comprised seven linkage groups that varied from 80.2 to 274.6 cM in length and spanned a total of 928.4 cM. Gupta et al. (2012a) used 196 markers including new 15 *M. truncatula* EST-SSR/SSR using a population of 94 RIL produced from a cross between ILL5588 and ILL5722 and clustered into 11 linkage groups (LG) covering 1156.4 cM. An intersubspecific F_2 *Lens* linkage map consisting of 199 PCR-based markers (28 SSRs, 9 ISSRs and 162 RAPDs) mapped on to 11 linkage groups covering a distance of 3,847 cM has been constructed (Gupta et al. 2012b).

Quantitative Trait Loci (QTL) Mapping

Molecular markers linked to desirable genes/QTL have been reported for marker-assisted selection in lentil (Table 6.4). Morphological markers viz., cotyledon (*Yc*), anthocyanin in stem (*Gs*), pod indehiscence (*Pi*), seed coat pattern (*Scp*), flower

Table 6.4 Molecular markers linked to desirable genes/QTL for marker-assisted selection in lentil

Traits	QTL/genes	Type of markers	References
Ascochyta blight resistance	*QTL*	RAPD	Ford et al. (1999)
	Ra/2	RAPD, SCAR	Chowdhury et al. (2001), Taran et al. (2003)
	QTLs	AFLP	Rubeena et al. (2006)
Anthracnose resistance	*LCt-2*	AFLP, RAPD	Tullu et al. (2003), Taran et al. (2003)
Fusarium wilt resistance	*Fw*	RAPD, SSR	Eujayl et al. (1998b), Hamwieh et al. (2005)
Cold winter hardiness	*Frt*	RAPD, SSR	Eujayl et al. (1999)
		RAPD, SSR, AFLP	Kahraman et al. (2004)s
Earliness and plant height	QTL	RAPD, SSR, AFLP	Tullu et al. (2008)
Plant structure, growth habit and yield	QTL	RAPDs, ISSRs, AFLPs, SSRs	Fratini et al. (2007)
Stemphylium blight resistance	QTLs	SSRs, SRAPs, RAPDs	Saha et al. (2010a)
Rust resistance	*R*	STS, SSRs, RFPLs, RAPDs, CAPS, dCAPS	Saha et al. (2010b)
Ascochyta lentis resistance	*QTLs*	RAPD, ISSR, EST-SSR, SSR	Gupta et al. (2012a, b)

colour (*W*), radiation frost tolerance locus (*Rf*), early flowering (*Sn*) and ground colour of the seed (*Gc*) were mapped as qualitative markers because they exhibited monogenic dominant mode of inheritance (Eujayl et al. 1998a; Duran et al. 2004; Hamwieh et al. 2005; Tullu et al. 2008). Further analysis for the association between DNA markers and Fusarium wilt resistance (Fw) gene was confirmed (Eujayl et al. 1998b; Hamwieh et al. 2005). However, only SSR59-2B was closely linked with Fw at 19.7 cM (Hamwieh et al. 2005). Anthracnose disease resistance (Lct-2) was mapped by Tullu et al. (2003). To date, quantitatively inherited traits have been mapped by Duran et al. (2004) who detected five QTLs each for the height of the first ramification and flowering time, three for plant height, seven for pod dehiscence, and one each for shoot number and seed diameter. Five and four QTLs were identified for winter survival and winter injury, using a RIL population of 106 lines derived from WA8649090 × Precoz (Kahraman et al. 2004). In this study, the experiments were conducted at multiple locations and only one of five QTLs was expressed in all environments. Mapping of Ascochyta blight resistance using an F_2 population derived from ILL7537 × ILL6002 identified three QTLs accounting for 47 % (*QTL*-1 and *QTL*-2) and 10 % (*QTL*-3) of disease variation. Recently, QTLs conferring resistance to Stemphylium blight and rust diseases using RIL populations were identified in lentil (Saha et al. 2010a, b). Though the use of F_2 populations in identification of QTLs has been done widely in lentil, their use in marker-trait analysis has led to identification of only major QTLs. Thus, several minor QTLs were overlooked in such populations and identification of environmental responsive QTLs was difficult. Because quantitative traits are influenced by both genetic and environmental effects, RILs or near isogenic lines (NILs) are more suitable populations to accurately dissect their components. For ascochyta blight, three QTLs each were

detected for resistance at seedling and pod/maturity stages (Gupta et al. 2012a). Together these accounted for 34 and 61 % of the total estimated phenotypic variation and demonstrated that resistance at different growth stages is potentially conditioned by different genomic regions. The flanking markers identified may be useful for MAS and pyramiding of potentially different resistance genes into elite backgrounds that are resistant throughout the cropping season.

Application of Genomic Resources

Genomic resources can be deployed in lentil improvement programs following either molecular marker or transgenic approaches.

Determination of Molecular Diversity

Genetic diversity analysis has been studied among a set of cultivated and wild lentils using various molecular marker system and genetic materials. Earlier studies have used RFLP, AFLP and RAPD markers to assess genetic diversity and phylogenetic analyses within and among *Lens* species (Havey and Muehlbauer 1989; Abo-el-Wafa et al. 1995; Ahmad and McNeil 1996; Sharma et al. 1995, 1996; Ford et al. 1997) and gene mapping (Eujayl et al. 1998b; Tullu et al. 2003; Duran et al. 2004; Kahraman et al. 2004; Hamwieh et al. 2005). As a part of the CGIAR's Generation Challenge Program (GCP), International Center for Agricultural Research in the Dry Areas (ICARDA) has identified a composite collection of lentil germplasm and characterized them by using microsatellite markers. ICARDA holds the largest global collection of lentil with >11,000 accessions. From this collection, a global composite collection of 960 accessions (Table 6.5) representing landraces, wild relatives, elite breeding lines and cultivars was established (Furman 2006). The results indicated two major clusters separating south Asia (Nepal, India, Pakistan and Afghanistan) from the Middle East and western countries (Fig. 6.1, Hamweigh et al. in progress). The major output of this study was a reference set which represents around 15 % (135 accessions) of the global composite collection representing all the geographical regions. This set has been phenotyped for different biotic and abiotic stresses, and emerged as a useful genetic resource to start with. Recently, a set of microsatellite markers was used to study the genetic diversity of lentil mini core set. The mini core collection comprised 109 accessions from 15 countries representing 57 cultigens (including 18 breeding lines) from 8 countries and 52 wild accessions (*L. culinaris* subsp. *orientalis*, *L. culinaris* subsp. *tomentosus* and *L. culinaris* subsp. *odemensis*) from 11 countries. The total alleles detected across the microsatellite loci were 182, with a mean of 13 alleles per locus. Wild accessions were rich in allelic variation (151 alleles) compared to cultigens (114 alleles). The genetic diversity index for the microsatellite loci in the wild accessions ranged from 0.16 (SSR28 in *L. culinaris*

Table 6.5 Composition of core germplasm representing 10 % of the global lentil collection by ICARDA

Country	No. of acc.	Country	No. of acc.	Country	No. of acc.
Afghanistan	30	Germany	10	Romania	2
Albania	1	Greece	17	Russian	13
Algeria	11	Guatemala	1	Saudi Arabia	1
Argentina	6	Hungary	3	Scg	4
Armenia	3	India	192	Slovakia	1
Azerbaijan	4	Iran	103	Spain	17
Bangladesh	6	Iraq	11	Sudan	2
Belgium	1	Italy	6	Syria	70
Brazil	2	Jordan	46	Tajikistan	5
Breeding	35	Lebanon	9	Tunisia	8
Bulgaria	6	Libyan	1	Turkey	69
Canada	3	Macedonia	3	Turkmenistan	1
Chile	27	Mexico	8	Ukraine	5
China	1	Morocco	14	United States	10
Colombia	3	Nepal	28	Unknown	7
Croatia	1	Netherlands	1	Uruguay	1
Cyprus	9	Norway	1	Uzbekistan	2
Czech Republic	6	Pakistan	27	Yemen	12
Egypt	25	Pal	4	Yugoslavia	2
Ethiopia	49	Poland	4	Sum	960
France	5	Portugal	5		

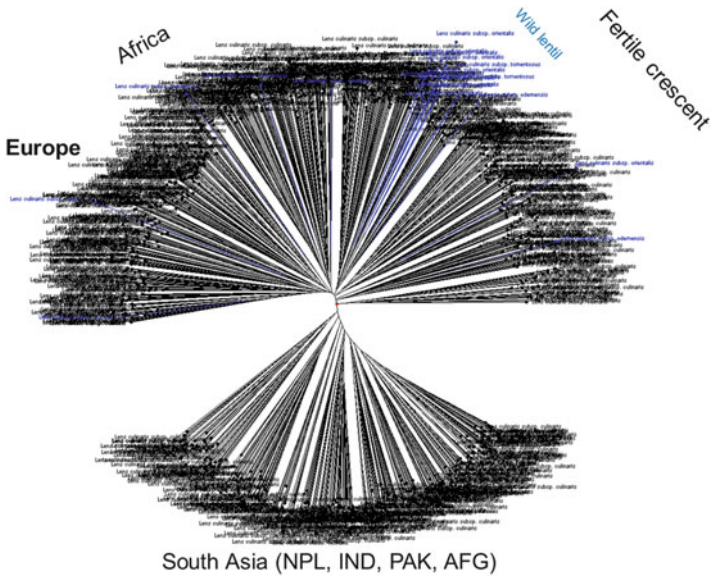

Fig. 6.1 Cluster analysis of wild and cultivated lentil accessions using 22 microsatellite markers. The results indicated two major clusters separating south Asia (Nepal, India, Pakistan and Afghanistan) from the Middle East and western countries

subsp. *odemensis*) to 0.93 (SSR66 in *L. culinaris* subsp. *orientalis*) with a mean of 0.66, while in the cultigens, genetic diversity varied between 0.03 (SSR28) and 0.87 (SSR207) with a mean of 0.65. Cluster analysis indicated two major clusters (Fig. 6.2), mainly one with the cultigens and the other with the wild accessions (Hamwieh et al. 2009).

Recently, comparative genomics approach has provided significant opportunities for analysis of genetic diversity in lentil. The conserved primers (CPs) based on *M. truncatula* EST sequences flanking one or more introns were used to sequence amplicons in 175 wild and 133 domesticated accessions. This analysis of the sequences confirmed that *L. nigricans* and *L. ervoides* are well-defined species at the DNA sequence level. *Lens culinaris* subsp. *orientalis* is the progenitor of domesticated lentil, *L. culinaris* subsp. *culinaris*, but a more specific area of origin can be suggested in southern Turkey. The study detected the divergence, following domestication, of the domesticated gene pool into overlapping large seeded (megasperma) and small-seeded (microsperma) groups and observed that lentil domestication led to a loss of genetic diversity of approximately 40 % (Alo et al. 2011).

Testing the Hybridity of F_1s

Making crosses between diverse parents is difficult in practice in lentil because of very small flowers leading to increase the chances of selfing. In addition to this, differentiating F_1 plants from selfed ones also becomes difficult due to low phenotypic diversity between the parents. Hence molecular markers have been found very useful to detect the hybridity of F_1 plants in lentil. Solanki et al. (2010) used molecular markers in lentil and detected only 21 % plants as true hybrids. These results suggest that molecular markers can reduce the time and money required to grow a population from selfed or admixed plants and increase the efficiency of plant breeders in selection of recombinant plants.

Marker Assisted Selection

Ideally, the genes controlling a trait of interest are the perfect marker for MAS. However, this is often made difficult because cloning of a gene is labor intensive and time consuming. Alternatively, marker(s) that are tightly linked to and flanking a gene locus that conditions a sizable genetic variation for the trait may be selected for with the premise that the associated chromosomal region contains the functional gene(s). Often, genetically linked markers to traits of interest are identified by coarse mapping and these have limited use in MAS because of the distance and hence chance of recombination between marker and actual gene locus. Therefore, genomic regions where the trait is mapped should be fine mapped at high resolution and be validated across genetic backgrounds in order to determine their utility in

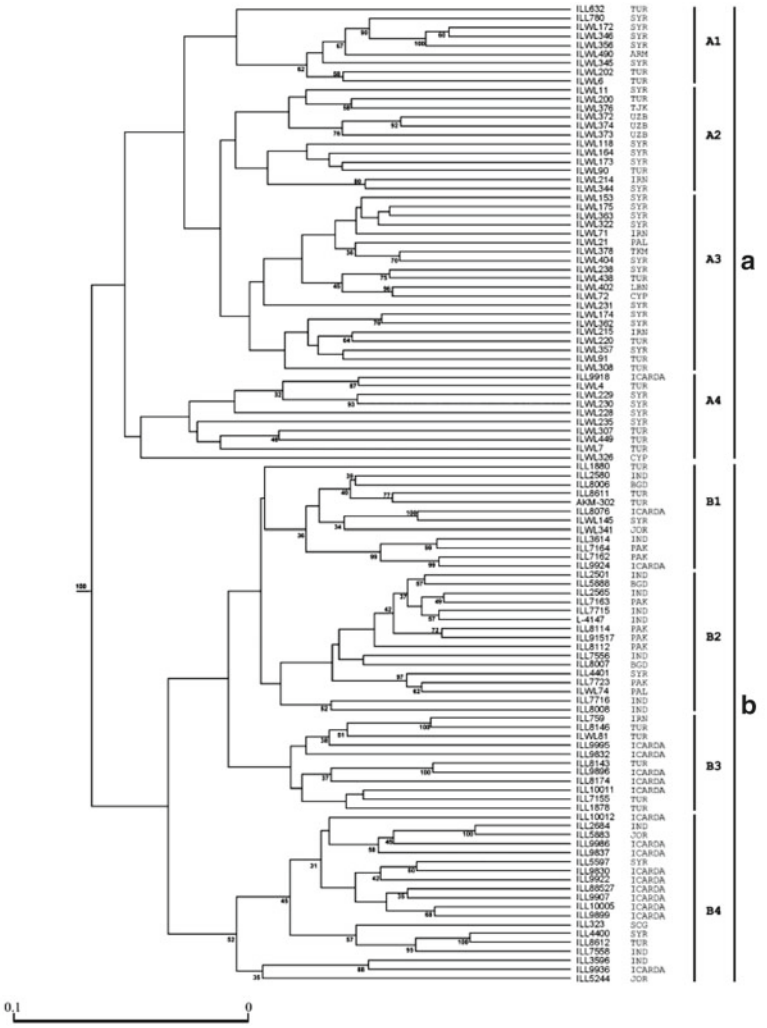

Fig. 6.2 Cluster analysis of wild and cultivated lentil accessions using 14 microsatellite markers. The groups are denoted on the *right side* as A or B, and the subgroups as A1, A2, A3, A4, B1, B2, B3, and B4. The origins of 109 lentil accessions are listed closed to the genotype numbers. Bootstrap values of above 30 % are indicated at the nodes. The abbreviations of the countries: Bangladesh (BGD), India (IND), Iran (IRN), Jordan (JOR), Pakistan (PAK), Syria (SYR), Turkey (TUR), Serbia and Montenegro (SCG), Palestine (PAL), Armenia (ARM), Cyprus (CYP), Uzbekistan (UZB), Tajikistan (TJK), Turkmenistan (TKM), Lebanon (LBN)

MAS. Also, physical characterization of genomic regions of interest will facilitate cloning of the gene to develop direct markers (candidate genes) and/or physically closer markers to the gene, increasing the reliability for MAS. The most useful marker system for MAS should be locus specific, highly reproducible and easy to discern. These include sequence tagged site (STS), sequence characterized amplified region (SCAR) or allele specific amplified primer (ASAP), specific polymorphic locus amplification test (SPLAT) and PCR-based RFLP markers. When locus specific markers are not polymorphic among the parental lines used in the breeding programs, sequence discriminative methods are required. These include SNP, cleaved amplified polymorphic site (CAPS) and derived CAPS (dCAPS) markers. Meanwhile, there are several markers available for different traits that have the potential for use in MAS and gene pyramiding. Two QTLs governing Ascochyta blight resistance were identified on LG I and II in lentil for which dominant and partial dominant gene actions were observed (Nguyen et al. 2001). These QTLs may represent the effects of the two major dominant genes previously reported for resistance in ILL7537. These include SCARW19 and SCARB18 linked to and flanking the *AbR*1 resistance loci (Nguyen et al. 2001; Taran et al. 2003). These enabled successful pyramiding of *AbR*1 and *ral*2 resistance loci together with the *LCt*2 anthracnose resistance loci (Taran et al. 2003).

Genetic Manipulation Through Transformation

Transgenic approach uses functional genes which are not available within the crossable gene pool. Thus cloned genes are important genomic resources for making genetic manipulation through transformation. Commonly, the particle bombardment and the *Agrobacterium tumefaciens* infection methods have been used to introduce genes with novel functions. With the explosion of sequence information available in the databases, transformation systems have also become useful tools to study gene function via RNA interference 'knockout', T-DNA insertion or transforming a genotype lacking a particular gene. Thus a robust, reproducible and efficient transformation system combined with a protocol to regenerate complete fertile plants from transformed cells is essential to fully study plant gene functions.

Following the initial report of shoot regeneration (Bajaj and Dhanju 1979) from apical meristems, it has been achieved routinely with different explants such as apical meristems (Bajaj and Dhanju 1979), stem nodes (Polanco et al. 1988; Singh and Raghuvanshi 1989; Ahmad et al. 1997), cotyledonary node (Warkentin and McHughen 1992; Sarker et al. 2003a), epicotyls (Williams and McHughen 1986), decapitated embryo, embryo axis and immature seeds (Polanco and Ruiz 2001) and cotyledonary petioles (Khawar and Özcan 2002). The induction of functional roots on *in vitro*-developed shoots has been the major challenge in lentil micro propagation. The difficulty to induce roots is thought to be associated with the use of cytokinin to obtain multiple shoots from the initial explants (Mohamed et al. 1992; Sarker et al. 2003b). Among the several studies conducted on root induction

from shoots, Fratini and Ruiz (2003) reported 95 % rooting efficiency from nodal segments cultured in an inverted orientation in media with 5 µM indole acetic acid (IAA) and 1 µM kinetin (KN). Sarker et al. (2003b) reported 30 % rooting efficiency on MS medium supplemented with 25 mg/l indole butyric acid (IBA). More recently Newell et al. (2006) obtained 100 % rooting efficiency on nodal micro-cuttings placed inverted in a mixture of sphagnum peat, coarse river sand and perlite at a 0.5:2:2 ratio, and concluded that the improved rooting efficiency was due to greater aeration.

To date, transformation of lentil has been reported through *A. tumefaciens-mediated* gene transfer (Warkentin and McHughen 1992; Lurquin et al. 1998; Sarker et al. 2003a) and biolistic transformation including electroporation (Chowrira et al. 1996) and particle bombardment (Gulati et al. 2002; Mahmoudian et al. 2002). Warkentin and Mc-Hughen (1992) reported the susceptibility of lentil to *A. tumefaciens* and later evaluated a number of explant types including shoot apices, epicotyl, root, cotyledons and cotyledonary nodes. All explants showed transient *b-glucuronidase* (GUS) expression at the wound sites except cotyledonary nodes, which were subsequently transformed by Sarker et al. (2003b). Öktem et al. (1999) reported the first transient and stable chimeric transgene expression on cotyledonary lentil nodes using particle bombardment. Gulati et al. (2002) reported regeneration of the first fertile transgenic lentil plants on MS medium with 4.4 µM benzyladenine (BA), 5.2 µM gibberellic acid (GA3) and chlorsulfuron (5 nM for 28 days and 2.5 nM for the rest of the culture period), followed by micrografting and transplantation in soil. The first successful work was reported by Barton et al. (1997), using pCGP1258 plasmid construct on four lentil genotypes. Khatib et al. (2007) have developed herbicide-resistant lentil through *A. tumefaciens* mediated transformation. This was achieved with the same plasmid construct pCGP1258, harbouring the *bar* gene conferring resistance to the herbicide glufosinate ammonium that was transformed using *A. tumefaciens* strain *AgL0*. Three lentil lines, ILL5582, ILL5883 and ILL5588, were used and a high selection pressure of 20 mg/l of glufosinate was applied to the explants for 18 weeks. Surviving shoots were subsequently grafted onto non-transgenic rootstock and plantlets were transferred to soil and acclimatized. The presence of the transgene was confirmed by PCR and the gene function was confirmed via herbicide application. Recently, Akcay et al. (2009) reported the production of transgenic lentil plants via *Agrobacterium*-mediated transformation and the stable transmission of the *nptII* and *gus*A genes in the subsequent generations. However, these studies were mostly confined to establish transformation techniques rather than the introduction of genes into improved varieties. Khatib et al. (2011) reported for the first time the introduction of the *dreb1a* gene into lentil for enhancing drought and salinity tolerance. The PCR results confirmed the insertion and stable inheritance of the gene of interest and *bar* marker gene in the plant genome. The Southern blot analysis revealed integration of a single copy of the transgene. The *DREB1A* gene driven by rd29A promoter transcribed in the transgenic plants by inducing salt stress in form of sodium chloride solution. The results showed that mRNA was accumulated and thus the *DREB1A* gene was expressed in the transgenic plants.

Conclusion

Pace of development of genomic resources and enabling technologies is still slow in lentil. Limited population size, low heritability, lack of candidate genes, low marker density and the difficulty in identifying beneficial alleles are the main limiting factors in genomics enabled improvement. More concerted efforts are required for developing more number of SSR and SNP markers in lentil because both are breeder friendly, highly polymorphic, evenly distributed throughout the genome and highly reproducible. The next generation sequencing technology has opened new opportunity of fast development of sequence based markers. These sequencing methodology are widely used in major legumes such as chickpea and soybean. Application of markers to practical breeding programs worldwide is still limited, and thus, more molecular maps, and genomics approaches including more gene sequences need to be developed for broadening our understanding of the complex nature of lentil genome. Genome sequencing in lentil is underway and it is expected to leverage vast genetic information to be used by lentil breeders. Lentil breeders can play an important role in development of trait specific mapping populations and precise phenotyping to establish the association of gene sequences/markers with desirable traits. Access to high-throughput genotyping and sequencing technologies is expected to speed up the genetic gain across the target environments in lentil. These developments ultimately will increase the utilization of genomic resources in genetic improvement of lentil and will lead fast track development of improved cultivars.

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