Detecting DNA polymorphism and genetic diversity in Lentil (*Lens culinaris* Medik.) germplasm: comparison of ISSR and DAMD marker

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Abstract Genetic diversity and interrelationships among 31 lentil genotypes were evaluated using 10 Inter-Simple Sequence Repeat (ISSR) and 10 directed amplification of minisatellite DNA region (DAMD) primers. A total of 43 and 48 polymorphic bands were amplified by ISSR and DAMD markers, respectively. Average polymorphism information content (PIC) for ISSR and DAMD markers were 0.37 and 0.41, respectively. All 31 lentil genotypes could be distinguished by ISSR markers into three groups and by DAMD markers into two groups. Various molecular markers show a different efficiency for evaluating DNA polymorphism in lentil and indicate that the patterns of variation are clearly influenced by the genetic marker used. Comparatively, the genetic diversity of examined lentil genotypes by two different marker techniques (ISSR and DAMD) was high and indicated that ISSR and DAMD are effective and promising marker systems for fingerprinting in lentil and give useful information on its genetic relationships.

Keywords Lentil · ISSR · DAMD · Genetic diversity

Abbreviations

DAMD Directly amplified minisatellite DNA
ISSR Inter-simple sequence repeat
PCR Polymerase chain reaction
PIC Polymorphism information content

Introduction

Lentil (*Lens culinaris* Medik. subsp. *culinaris*) is an annual self-pollinated diploid ($2x=2n=14$ chromosomes) species and is an important legume crop cultivated worldwide as human food. Lentil seed is an important source for human consumption, and its plant parts, excluding seeds, are used as animal feed and it also helps in the management of soil fertility (Hamwiah et al. 2009; Toklu et al. 2009).

International endeavors to improve lentil have been promising in some cases (Erskine et al. 1989), but it is not the case everywhere. In South Asia, the yield of lentil remains low and average seed yield on a country basis is below 1.0 t ha^{-1} (SAIC 2009). Lentil has been identified as a narrow adapted crop and the principal constraint of lentil production is its low yield potential because of undesirable plant type (Hanlan et al. 2006). The understanding of the extent of genetic variability within individuals is fundamental and important for crop breeding and conservation of genetic resources and is particularly useful for the efficient use of genetic resources in a breeding program (Talebi et al. 2008). Molecular marker studies have made great contributions to our knowledge of genetic diversity and relatedness in various crops (Abe et al. 2003; Hamwiah et al. 2009). Most of the interspecific and interspecific relationship studies in *Lens* have been carried out using plant morphology (Erskine and Choudhary 1986; Erskine et al. 1989), isozymes (Erskine and Muehlbauer 1991, seed storage proteins (Sultana et al. 2006), random amplified polymorphic DNA (RAPD) markers (Ahmad et al. 1996; Yuzbasioglu et al. 2006), AFLP markers (Toklu et al. 2009), intersimple sequence repeat (ISSR) markers (Fikiru et al. 2007), and SSR markers (Jin et al. 2008; Kushwaha et al. 2013). Although different molecular marker types have been used to study the lentil diversity to date, new high-polymorphism molecular markers are demanded yet. Recently, different studies have demonstrated that specific

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co-dominant SSR markers can detect moderate and high polymorphisms in lentil (Jin et al. 2008; Hamwieh et al. 2009). However, utilization of SSR markers for genome diversity analysis requires sequencing SSR loci and designing the primers, which are not affordable for many laboratories in developing countries (Xiong et al. 2011). Thus, development and applying new molecular marker techniques that do not require any genome sequence information are necessary for many crops and laboratories. Intersimple sequence repeat (ISSR) marker, in addition to its suitability to genetic diversity study, is highly polymorphic, reproducible, cost-effective, and requires no prior information of the sequence (Borner et al. 2002; Zietkiewicz et al. 1994). These facts suggest that ISSR could be an unbiased tool to evaluate the changes of diversity in agronomically important crops (Brantestam et al. 2004). Recently, the utilization of minisatellite core sequences as primers to PCR amplification of minisatellite-rich DNA regions is practicable and the first time reported by Heath et al. (1993) and termed as directed amplification of minisatellite-region DNA (DAMD). To date, DAMD technique has been successfully used to establish DNA fingerprints in wheat (Bebeli et al. 1997), rice (Zhou et al. 1997), *Capsicum* spp. (Ince et al. 2009a), *Origanum*, *Thymus*, *Sideritis* and *Salvia* (Ince et al. 2009b), *Cicer* spp (Amirmoradi et al. 2012), chickpea (Pakseresht et al. 2013), and grapevine (Seyedimoradi et al. 2012), but no published information is available on application of DAMD marker in lentil. This study was designed to investigate the use of DAMD marker-based technique for studying genetic diversity for the first time in lentil genotypes. Objectives of the present study was to determine the potential of DAMD technique to generate polymorphic markers in lentil and to investigate its effectivity in determining genetic relationships among lentil genotypes compared to ISSR markers data.

Materials and methods

Plant materials and DNA extraction

Thirty-one lentil (*L. culinaris* Medik.) genotypes were obtained from most important lentil producing countries of the world to form the germplasm collection (Table 1). Deoxyribonucleic acid (DNA) was extracted from 2 g of young leaves collected from 15-day-old seedling plants of each genotype using the CTAB method (Torres et al. 1993). The final concentration of DNA was determined by agarose-gel electrophoresis using a known concentration of uncut λ DNA as a standard.

ISSR and DAMD markers analysis

A set of 10 ISSR primers was procured from the Biotechnology Laboratory, University of British Columbia,

Table 1 Details of 31 lentil genotypes tested, including pedigree and origin/source

Number	Genotype	Pedigree	Origin
1	FLIP2007-137L	SEL(ILL4605)	ICARDA
2	AKM302	Not traced	Turkey
3	FLIP 2007-100L	ILL7617 X ILLAKM282	ICARDA
4	FLIP 2005-53L	ILL 7716 X ILL590	ICARDA
5	Bilsen-78	Not traced	Yemen
6	Flip 2005-58L	Not traced	ICARDA
7	AKM 46	Not traced	Turkey
8	FLIP2007-128L	ILL7989 X AKM 279	ICARDA
9	FLIP 2006-91L	Not traced	ICARDA
10	FLIP 2006-95L	ILL4400 X ILL 590	ICARDA
11	FLIP 2006-97L	ILL 5883 X ILL590	ICARDA
12	FLIP 2006-99L	ILL 590 X ILL7723	ICARDA
13	PLIP2002-3L	ILL7553 X ILL7117	ICARDA
14	Flip 2005-38L	ILL 4400 X ILL7670	ICARDA
15	FILP 2007-98L	ILL 5722 X ILL6246	ICARDA
16	FLIP 2007-102L	ILL7716 X ILL7715	ICARDA
17	FLIP 2007-103L	ILL590 X ILL7715	ICARDA
18	FLIP 2007-106L	ILL5883 X ILL590	ICARDA
19	FLIP 2007-137L	Not traced	ICARDA
20	FLIP 2008-14L	ILL 7980 XILL 2684	ICARDA
21	FLIP 2006-93L	ILL 5722 XILL 6246	ICARDA
22	FLIP 2007-95L	ILL 5722 X ILL 6999	ICARDA
23	FLIP 2006-94L	ILL 4400 X ILL7670	ICARDA
24	FLIP 2005-32L	ILL7716 X ILL 8008	ICARDA
25	FLIP 2008-21L	ILL590 X ILL7683	ICARDA
26	FLIP2007-129L	ILL590 X ILL 7683	ICARDA
27	PI 299366	Not traced	Chile
28	Bilsen-365	Not traced	Yemen
29	FLIP2003-30L	ILL5871 X ILL6208	ICARDA
30	FLIP 2008-16L	ILL 7978 X ILL2684	ICARDA
31	Local check	Not traced	IRAN

Canada (Table 2). These primers were chosen based on own lab experiences with other crops. Initially, five genotypes were used for PCR amplification using all the 10 primers. Eight primers gave clear and polymorphic patterns and were used for further analysis of 31 genotypes. Directly amplified minisatellite DNA-polymerase chain reaction (DAMD-PCR) markers (originally derived from the repeat elements of weed rice (Kang et al. 2002) were used in this study. Out of the ten primers screened (Table 2), the seven having a GC content of 50–60 % were selected to generate the DNA fingerprint profiles of all the accessions at 48 °C of annealing temperature (Table 2). Polymerase chain reaction (PCR) amplification was performed in 20 μ l reaction containing 1 $\times$ PCR buffer, 30 ng sample DNA, 2.5 μ M primers, 200 μ M of each dNTP, 1.5–2.5 mM MgCl₂, and 1.5 unit of Taq DNA polymerase

Table 2 Primers used in ISSR and DAMD analyses

	Primer	Sequence 3' to 5'	GC (%)	Annealing temperature
ISSR				
	UBC807	AGAG AG AG AG AG AG AG T	47	48 °C
	UBC828	TGTG TG TG TG TG TG TG A	47	50 °C
	UBC878	GGAT GG AT GG AT GG AT	50	48 °C
	UBC899	CATG GT GT TG GT CA TT GT TC CA	45	50 °C
	UBC822	TCTC TC TC TC TC TC TC A	47	48 °C
	UBC874	CCCT CC CT CC CT CC CT	75	50 °C
	UBC816	CACA CA CA CA CA CA CA T	47	48 °C
	UBC880	GGA GAG GAG AGG AGA	60	52 °C
	UBC815	CTC TCT CTC TCT CTC TG	52	44 °C
	UBC812	GAG AGA GAG AGA GAG AA	47	45 °C
DAMD				
	URP1F	ATCC AA GG TC CG AG AC AA CC	55	48 °C
	URP2F	GTGT GC GA TC AG TT GC TG GG	60	48 °C
	URP6R	GGCA AG CT GG TG GG AG GT AC	65	48 °C
	URP4R	AGGA CT CG AT AA CA GG CT CC	55	48 °C
	URP9F	ATGT GT GC GA TC AG TT GC TG	50	48 °C
	URP13R	TACA TC GC AA GT GA CA CA GG	50	48 °C
	URP17R	AATG TG GG CA AG CT GG TG GT	55	48 °C
	URP25F	GATG TG TT CT TG GA GC CT GT	50	50 °C
	URP30F	GGAC AA GA AG AG GA TG TG GA	50	50 °C
	URP38F	AAGA GG CA TT CT AC CA CC AC	50	50 °C

(Cinnagene, Iran). All amplification were carried out in Eppendorf thermocyclers as follows: 94 °C for 3 min, followed by 35 cycles of denaturation at 93 °C for 45 s, annealing at optimum *T_a* for 45 s, and extension at 72 °C for 90 s. A final extension cycle at 72 °C for 10 min followed. Polymerase chain reaction (PCR) products were separated on 1.2 % agarose gels, stained with ethidium bromide, and scored for the presence or absence of bands.

Data analysis

For each genotype, a binary matrix reflecting specific ISSR/DAMD band presence (1) or absence (0) was generated. Nei's genetic distance (Nei 1973) was determined among the genotypes and used for grouping of the genotypes by unweighted neighbor joining (UNJ) cluster method (Perrier et al. 2003). Tree construction following unrooted NJ tree using similarity matrix was carried through DARwin 5.0.128 (Perrier et al. 2003; Perrier and Jacquemoud-Collet 2006) analysis. Bootstrap analysis using 1,000 bootstrap values was performed for the node construction. Mantel statistic was used to compare the dissimilarity matrices as well as the dendrograms produced by the ISSR and DAMD techniques through NTSY S software. Polymorphic information content (PIC) values were calculated for each ISSR and DAMD primers

according to the formula: $PIC = 1 - \sum (P_{ij})^2$, where P_{ij} is the frequency of the *i*th pattern revealed by the *j*th primer summed across all patterns revealed by the primers (Botstein et al. 1980).

Results

ISSR markers analysis

Intersimple sequence repeat (ISSR) analysis revealed clear and scorable bands per primer and in total, 58 bands both polymorphic and monomorphic were obtained using eight primers in 31 lentil genotypes (Table 3). Forty-three out of 58 bands (73 %) were polymorphic, with an average of 5.4 polymorphic bands per primer. The average polymorphic information content (PIC) was 0.37, ranging from 0.29 to 0.45. The highest PIC value was obtained by UBC899. The genetic relationships among the 31 lentil genotypes based on ISSR markers were estimated by NJ cluster method of the genetic distance matrix (Fig. 1). Among all genotypes, 29 could be divided in to three distinct clusters. Group I included eight genotypes that originated from ICARDA, except for Bilsen-78 from Yemen. Group II was composed of six

Table 3 Polymorphism and PIC values in lentil genotypes as revealed by ISSR and DAMD markers

Primer	No. of amplified bands	No. of polymorphic bands	Polymorphism (%)	PIC value
ISSR				
UBC807	9	7	0.78	0.42
UBC816	6	4	0.67	0.35
UBC878	5	4	0.80	0.37
UBC860	6	4	0.67	0.29
UBC822	8	6	0.75	0.35
UBC812	7	5	0.71	0.32
UBC899	9	7	0.78	0.45
UBC815	8	6	0.75	0.41
DAMD				
URP 1F	8	5	0.63	0.39
URP 2F	9	7	0.78	0.41
URP 6F	8	7	0.88	0.45
URP 4R	7	6	0.85	0.39
URP9F	10	8	0.80	0.44
URP 13R	7	6	0.86	0.34
URP 17R	11	9	0.82	0.46

genotypes. In this cluster, AKM302 from Turkey were grouped with other genotypes from ICARDA. Group III was composed of 15 genotypes. PI299366 from Chile, Bilsen-365 from Yemen, and local check genotypes from Iran closely clustered into this cluster (Fig. 1). FLIP2005-58L and FLIP2006-95L did not group into any cluster.

Fig. 1 NJ phylogenetic tree using ISSR molecular data in 31 lentil genotypes. Bootstrap values are indicated at the node of each cluster

DAMD markers analysis

Direct amplification of minisatellite region DNA (DAMD) with 10 microsatellite core sequences as primers was used on genomic DNA templates of the 31 lentil genotypes. Seven primers produced clear and scorable band (Table 3). The number of amplified bands per primer varied from 7 to 11. Total number of bands was 60 of which 48 were polymorphic with an average of 6.8 polymorphic bands per primer. Polymorphic information content (PIC) values ranged from 0.34 to 0.46, with an average value of 0.41 per locus (Table 3). Cluster analysis grouped lentil genotypes in two distinct clusters (Fig. 2). Cluster I contained 13 genotypes. Most of the genotypes in this cluster were same with genotypes in cluster III obtained by ISSR markers. Cluster II included 16 genotypes that all of them originated from ICARDA, except AKM302 from Turkey. The remaining two genotypes (Bilsen-78 from Yemen and FLIP2..8-14L from ICARDA) did not group in any of the clusters (Fig. 2).

Combined analysis

The general dendrogram (Fig. 3) that was constructed using the combined data of the ISSR and DAMD marker divided genotypes in four clusters was relatively similar to those obtained separately with each marker techniques. Similarity between genotypes clustering in ISSR and DAMD analyses

Fig. 2 NJ phylogenetic tree using DAMD molecular data in 31 lentil genotypes. Bootstrap values are indicated at the node of each cluster

Fig. 3 NJ phylogenetic tree using combined ISSR+DAMD molecular data in 31 lentil genotypes. Bootstrap values are indicated at the node of each cluster

based on mantel coefficient test was not significant ($r=0.12$). Cluster analysis by combined data showed significant positive correlation with ISSR ($r=0.66^{**}$) and DAMD ($r=0.81^{**}$) markers.

Discussion

Knowledge of genetic variation and genetic relationship between lentil genotypes is important for efficient utilization of germplasm resources and is more important to know which marker techniques and how many of the markers represent variation in the entire genome and should be used in order to derive reliable diversity pattern (Saini et al. 2004). Various molecular markers show a different efficiency for evaluating DNA polymorphism in lentil (Toklu et al. 2009). Directly amplified minisatellite DNAs (DAMDs) are tandem repeats of 10–60 bp long DNA sequence motif and are widely distributed throughout the eukaryotic genomes. Variation in the tandem repeat copy number of minisatellite is proved to be the source of the polymorphism in several organisms (Hu et al. 2011). Various species may share common motifs known as core sequences (Nakamura et al. 1988). Intersimple sequence repeat (ISSR) amplification (Zietkiewicz et al. 1994) is a relatively recent technique which can differentiate closely

related genotypes. It is based on the amplification of a single primer containing a microsatellite ‘core’ sequence anchored at the 3’ or 5’ end by 2–4 selective, often degenerate, nucleotides. The DNA fragments amplified are flanked by inversely oriented, adjacent microsatellites. Our results indicate that the patterns of variation are clearly influenced by the genetic marker used. The differences in the chromosomal location of the two types of markers can influence the diversity assessment (Kojima et al. 1998). It is possible to hypothesize that in some species the differences may be higher than in others, and this is the case of lentil (Sonnante and Pignone 2001). In this study, both the ISSR and DAMD primers yielded high numbers of polymorphic bands per primer, 5.4 and 6.8, respectively. The DAMD markers produced higher number of polymorphic bands per primer in lentil. This result was not unexpected, since the DAMD technique amplifies potentially polymorphic minisatellite regions (Ince et al. 2009a). A high level of polymorphism were detected using the same ISSR and DAMD markers in chickpea in *Cicer* spp. by Amirmoradi et al. (2012) and Pakseresht et al. (2013). These results suggested the presence of a considerable polymorphism at studied molecular markers and revealed a high level of genetic diversity in the existing lentil germplasm. In this study, all the genotypes possess the largest genetic diversity (except for origin-unknown resources). These results may be explained by this fact that lentil is originating from the Near East region (Sonnante and Pignone 2001), and these regions are a part of the Fertile Crescent, where lentil was first domesticated (Hamwieh et al. 2009).

In this study, the average genetic distance obtained by ISSR and DAMD (0.46, 0.49) was the same and this relatively high genetic dissimilarity among studied genotypes indicating the presence of a considerable genetic diversity in these genotypes which can be considered for future parental selection in breeding program. Based on these results, we anticipate that the source of detected diversity is different, as each technique targets different regions of the genome. Intersimple sequence repeat (ISSR) and DAMD markers showed high level of polymorphism and PIC value. These techniques were more informative than the previously used biochemical and molecular methods to study variation and genetic relationships in lentil, such as isozymes, storage proteins (Erskine and Muehlbauer 1991; Sultana et al. 2006), AFLP markers (Toklu et al. 2009), and RAPD markers (Yuzbasioglu et al. 2006). It was evident from the results that the dendrogram based on ISSR molecular markers was not completely in accordance with the dendrogram based on DAMD markers. There are several possible explanations for such results: some of them connected with nature and structure of different molecular markers that designed from various regions of genome (Pakseresht et al. 2013). Another problem was the possibility of overestimating genetic similarity because fragments with the same size could have different origins

(Amirmoradi et al. 2012). In conclusion, in this study, we provide additional data for identification of lentil genotypes in addition to previous analyses of RAPD, AFLP, and SSR studies. We have demonstrated that DAMD-PCR markers can be used as reliable techniques for detecting levels of DNA polymorphism and genetic relationship in lentil.

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