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Rubeena · R. Ford · P. W. J. Taylor

Construction of an intraspecific linkage map of lentil (*Lens culinaris* ssp. *culinaris*)

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Abstract The first intraspecific linkage map of the lentil genome was constructed with 114 molecular markers (100 RAPD, 11 ISSR and three RGA) using an F_2 population developed from a cross between lentil cultivars ILL5588 and ILL7537 which differed in resistance for ascochyta blight. Linkage analysis at a LOD score of 4.0 and a maximum recombination fraction of 0.25 revealed nine linkage groups comprising between 6 and 18 markers each. The intraspecific map spanned a total length of 784.1 cM. The markers were distributed throughout the genome, however markers were clustered in the middle or near the middle of the linkage groups, suggesting the location of centromeres. Of 114 mapped markers, 16 (14.0%) were distorted, usually at the end or middle of the linkage groups. The utility of ISSR and RGA markers for mapping in lentil was explored, and the primer with an (AC) repeat motif was found to be useful.

Keywords *Lens culinaris* ssp. *culinaris* · Intraspecific genetic map · RAPD · ISSR · RGA

Introduction

Lentil (*Lens culinaris* ssp. *culinaris*), a self-pollinating diploid ($2n = 2x = 14$), is an important pulse crop grown widely throughout the Indian subcontinent, Middle East, northern Africa, southern Europe, North and South America and Australia. Lentil is predominantly grown for human consumption as a rich source of protein. The crop is generally grown in rotation with cereals to break cereal disease cycles and to fix atmospheric nitrogen, thus reducing the demand for nitrogen fertilizers.

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Rubeena (✉) · R. Ford · P. W. J. Taylor
BioMarka, Joint Centre for Crop Innovation,
School of Agriculture and Food Systems,
The University of Melbourne, Parkville, Victoria, Australia 3010
e-mail: rubeena7@hotmail.com
Fax: +61-3-83445037

The development of a detailed genetic map, on which markers associated with desirable traits are identified, is a valuable tool to improve breeding efficiency. Genetic maps have been developed in almost all the agricultural crops (O'Brien 1993). In order to maximize the polymorphism for map construction, interspecific mapping populations have previously been used for *Lens* (Havey and Muehlbauer 1989; Muehlbauer et al. 1989; Weeden et al. 1992; Tahir et al. 1993; Vaillancourt and Slinkard 1993; Tahir and Muehlbauer 1994; Eujayl et al. 1998). However, maps based on interspecific populations may not represent the true recombination distances of the cultivated species (Causse et al. 1994; Lefebvre et al. 1995). Also, reduced recombination or chromosomal rearrangements between species within an interspecific cross may lead to segregation distortion (Tadmor et al. 1987). Maps developed from crosses between cultivars are the most useful for breeding applications as they identify polymorphic markers within the cultivated gene pool and are therefore more likely to be present in crosses involving other cultivated genotypes (Menéndez et al. 1997). Crossing within the cultivated species may also negate the problem of linkage drag often encountered in crosses derived from wild species (Saliba-Colombani et al. 2000). Genetic maps based on intraspecific crosses have also been recommended for the mapping of quantitative trait loci due to less segregation distortion (Havey and Muehlbauer 1989). To date, no comprehensive lentil map based on an intraspecific mapping population has been reported.

Earlier studies to develop a rudimentary map of the *Lens* genome with isozymes and other morphological and cytological markers were conducted using interspecific populations (Zamir and Ladizinsky 1984; Tadmor et al. 1987). The first *Lens* map incorporating restriction fragment length polymorphisms (RFLPs) was developed by Havey and Muehlbauer (1989) from an interspecific cross (*L. culinaris* × *L. orientalis*). Weeden et al. (1992) constructed an expanded *Lens* map using an interspecific cross of *L. ervoides* × *L. culinaris*, with isozymes, morphological and DNA markers, giving ten linkage

groups. Subsequently, all of the earlier maps were reviewed and a preliminary linkage map of *Lens* was developed with ten linkage groups, which included seven morphological, 38 RFLP, 25 isozymes and six other loci (Tahir et al. 1993).

The first map of *Lens* involving random amplified polymorphic DNA (RAPD) markers was developed by Eujayl et al. (1997), and spanned 206 cM of an interspecific genome created from a cross in which one of the parents was 50% wild (*L. culinaris* ssp. *orientalis*). The most recent and extensive linkage map of *Lens* comprised 177 markers [89 RAPD, 79 amplified fragment length polymorphism (AFLP), six RFLP and three morphological markers] and covered 1,073 cM of the *Lens* genome (Eujayl et al. 1998). This was constructed using a recombinant inbred line (RIL) population created from the same cross previously used for mapping (Eujayl et al. 1997). Recently, linkage relationships between morphological markers and RAPD markers have been reported (Emami et al. 1999; Hoque et al. 2002).

An advantage of DNA markers over morphological and isozyme markers is the high amount of polymorphism (Paterson et al. 1991) which can be generated to saturate a linkage map. The polymerase chain reaction (PCR) technique using RAPD markers (Welsh and McClelland 1990; Williams et al. 1990) has been used to construct genetic maps of several grain legume species (Kaga et al. 1996; Menéndez et al. 1997; Eujayl et al. 1998; Freyre et al. 1998; Laucou et al. 1998; Kaló et al. 2000; Lambrides et al. 2000; Santra et al. 2000). Inter-simple-sequence repeats (ISSR) have also been successfully used to map the genomes of wheat (Kojima et al. 1998) and chickpea (Santra et al. 2000; Winter et al. 2000). Recently, resistance gene analogue (RGA) markers have been mapped on linkage maps (Kanazin et al. 1996; Timmerman-Vaughan et al. 2000; Flandez-Galvez et al. 2003; Huettel et al. 2002). Although, not all of the amplified products may correspond to disease resistance genes, they should all contain conserved sequences of leucine-rich repeats (LRR), kinase and/or nucleotide-binding sites (NBS), which are likely to be involved in signal transduction pathways (Chen et al. 1998).

The main objective of the investigation reported here was to develop an intraspecific genetic map of the lentil genome based on RAPD, ISSR and RGA markers that can be used to elucidate the loci governing desirable traits that segregate in the population.

Materials and methods

Plant material and DNA extraction

One hundred and fifty F_2 plants, developed from an intraspecific cross between ILL5588 (ascochyta blight-susceptible) and ILL7537 (ascochyta blight-resistant), were used as the mapping population (Nguyen et al. 2001). Plants were grown in a growth chamber maintained at 20 ± 2 °C under a 16/8-h (light/dark) photoperiod. Genomic DNA was extracted from young leaves following the modified CTAB micropreparation procedure of Taylor et al. (1995). The DNA was RNase-treated and subsequently quantified on agarose gel by comparison with standard lambda DNA markers.

RAPD analysis

A total of 307 decamer primers were screened on the parents to determine their potential to produce a reproducible and clear polymorphism. The reaction conditions used by Ford et al. (1999) were used for RAPD analysis except that the PCR volume was reduced to 12.5 μl. PCR was performed using a PTC-100 or PTC-200 thermocycler (MJ Research, Waltham, Mass.). The amplification products were resolved on 1.4% agarose gels in Tris-borate EDTA (TBE) buffer and stained with ethidium bromide. The parents were screened for the presence/absence of bands and polymorphic, repeatable and clear markers were screened on the whole population.

ISSR and RGA analysis

Eighty-five ISSR primers from the University of British Columbia (UBC primer set no. 9, Vancouver, B.C.) were screened on the parents for polymorphism. The PCR reaction consisted of PCR buffer (Invitrogen, Carlsbad, Calif.), 3 m*M* $MgCl_2$, 0.24 m*M* each dNTPs, 0.24 μM primer, 1 U of *Taq* polymerase and 40 ng of template DNA. The reaction volume was 12.5 μl. The PCR conditions were similar to those used by Ratnaparkhe et al. (1998), and the products were resolved on 2% agarose gel in TBE buffer and stained with ethidium bromide.

One RGA primer (Pto kinase) was also screened. The method for RGA analysis was that developed by Chen et al. (1998), except that the PCR volume was adjusted to 12.5 μl. The PCR products were resolved on 5% polyacrylamide gel (PAGE) in TBE buffer (Sambrook et al. 1989) and detected by silver staining (Promega, Madison, Wis.).

Marker nomenclature

Each RAPD marker was designated a two-part name. The first part referred to the primer with which the polymorphism was observed [capital letter followed by a two digit number which corresponded to an Operon Technologies (Alameda, Calif.) primer], while the second part in subscript corresponded to the approximate size (in basepairs) of the marker. One RAPD primer from the University of British Columbia (UBC227) was also used. The marker was named as UBC227 followed by the marker size (subscript).

The ISSR markers were named "UBC" followed by a three digit number and the size of the marker (subscript). The ISSR markers were italicized. The RGA markers were named by the primer name followed by a lower case letter (a, b or c) indicating markers produced by the same primer in descending order of size.

Inheritance and linkage analysis

The markers were analyzed by a chi-square test for goodness-of-fit to the expected Mendelian segregation ratio (3:1) of a dominant locus in an F_2 population ($P < 0.05$). Linkage analysis was performed using MAPMAKER/EXP version 3.0 (Lander et al. 1987). Linkage groups were established at a LOD score of 4.0 and a recombination fraction (θ) of 0.25 by two-point analysis using the "group" command. The best marker order of the linkage group having eight or fewer markers was identified using the "compare" command, whereas the order of the groups with more than eight markers was identified using the "order" and "try" commands. The marker order of each linkage group was verified using the "ripple" command. The kosambi mapping function (Kosambi 1944) was used to convert the recombination fractions into additive genetic distance (centiMorgans).

Results

Polymorphic markers for mapping

The screening of parents (ILL5588 and ILL7537) with RAPD primers revealed 59 polymorphic primers (19.2%) which generated 102 reproducible and segregating markers (1.7 markers/primer). Of the ISSR primers screened, only seven were polymorphic, which collectively produced 13 clear and reproducible markers for mapping. Three RGA markers produced with the Pto kinase primer pair (Chen et al. 1998) were also used for mapping. Thus, a total of 118 segregating markers were used to construct the lentil linkage map (Table 1). The size of the markers varied from approximately 250 bp to 1,400 bp.

Linkage mapping

Of the 118 markers screened in the F_2 population, 114 (96.6%) were mapped and four (two RAPD and two ISSR) remained unlinked. The analysis revealed nine

Table 1 Molecular markers used for construction of the genetic linkage map of lentil

Marker type	Number of primers/primer pairs screened	Number of polymorphic primers/primer pairs	Number of polymorphic markers	Number of mapped markers
RAPD	307	59 (19.2%)	102 (1.7 markers/primer)	100 (98.0%)
ISSR	85	7 (8.2%)	13 (1.9 markers/ primer)	11 (84.6%)
RGA	1	1 (100%)	3 (3 markers/primer pair)	3 (100%)
Total	393	67	118	114 (96.6%)

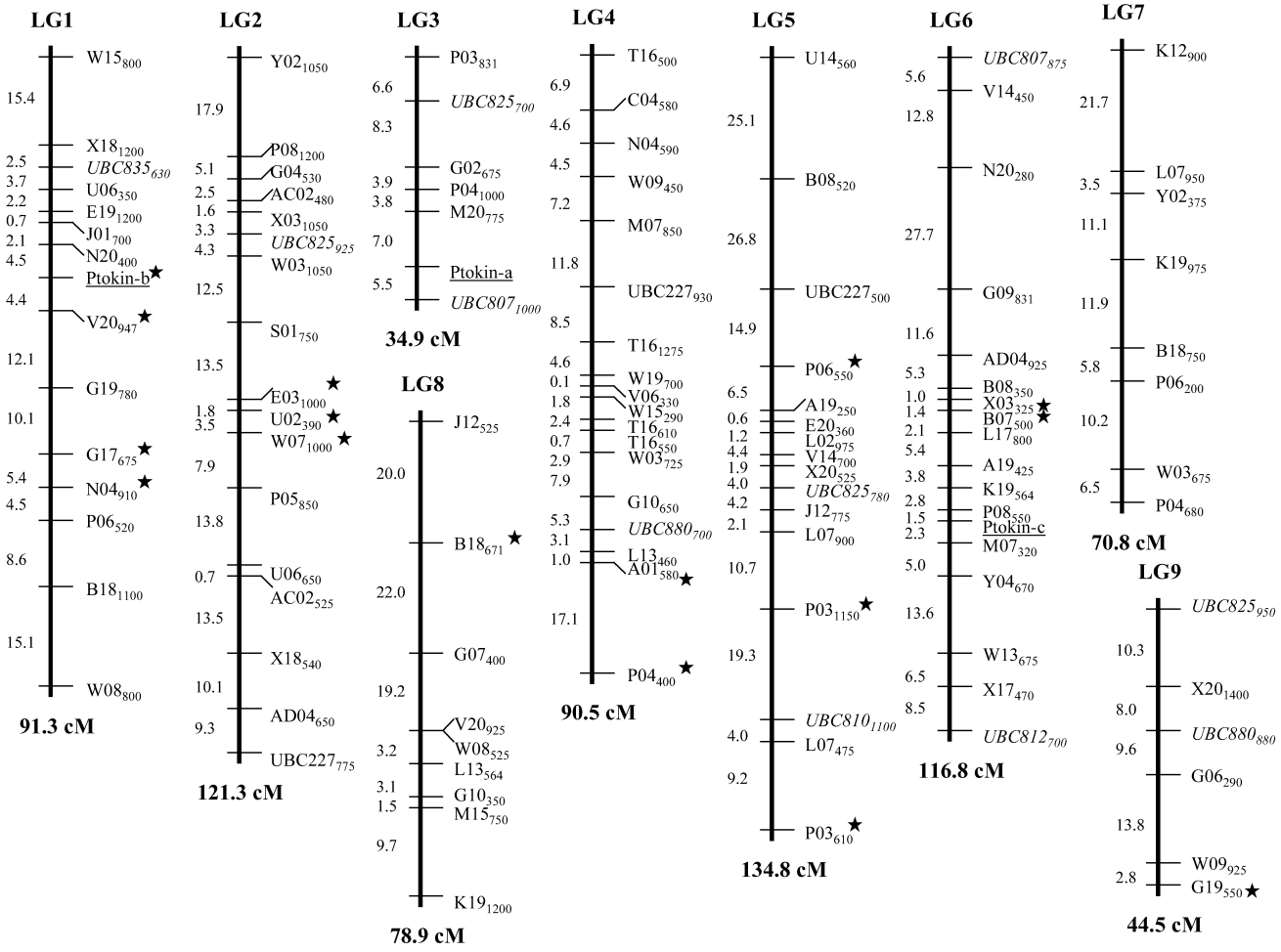

Fig. 1 Intraspecific map of lentil at a LOD score of 4.0. The linkage groups are named (*LG1–LG9*). Loci names are indicated on the *right side* of the *vertical bars* and genetic distances, in centiMorgans (kosambi function), are on the *left side* of the *vertical bars*. Length of the linkage groups is written at the *end* of the linkage groups. ISSR markers are *italicized* and RGA markers are *underlined*, whereas RAPD markers are *normal type*. The distorted markers are indicated by *stars*

Table 2 Characteristics of the intraspecific genetic linkage map of lentil

Linkage group	Length (cM)	Number of markers	Average distance between markers (cM)
LG1	91.3	15	6.1
LG2	121.3	17	7.1
LG3	34.9	7	5.0
LG4	90.5	18	5.0
LG5	134.8	16	8.4
LG6	116.8	18	6.5
LG7	70.8	8	8.9
LG8	78.9	9	8.8
LG9	44.5	6	7.4
Total	784.1	114	6.9

linkage groups varying in length from 34.9 cM to 134.8 cM (Fig. 1). Three linkage groups (LG2, LG5 and LG6) were longer than 100 cM, whereas four linkage groups (LG1, LG4, LG7 and LG8) were less than 100 cM but longer than 70 cM. Only two linkage groups (LG3 and LG9) were less than 50 cM in length. The number of marker loci per linkage group ranged from 6 to 18 (Table 2). The map spanned a total length of 784.1 cM with an average marker density of 6.9 cM between adjacent markers. RAPD and ISSR markers were distributed throughout all linkage groups of the genome. However, ISSR markers were not mapped on LG7 and LG8. RGA markers were observed on LG1, LG3 and LG6. All three of the RGA markers were inherited from the resistant parent ILL7537.

Four of the linkage groups (LG5, LG6, LG7 and LG8) contained gaps between adjacent markers of more than 20 cM, the maximum distance being 27.7 cM. LG4 was the most dense with 18 markers and an average marker density of 5.0 cM. LG3 also had an average marker density of 5.0 cM but comprised only seven markers. The sparsest linkage group (LG7) comprised eight markers, with an average density of 8.9 cM. Six linkage groups (LG1, LG2, LG4, LG5, LG6 and LG8) showed clustering of mainly RAPD markers in the middle or near the middle of the linkage groups.

Chi-square analysis ($P < 0.05$) revealed 17 markers (15 RAPD, one ISSR and one RGA) which did not segregate according to the expected Mendelian ratio of a dominant marker in a diploid F_2 progeny. However, 16 of the 17 distorted markers were mapped, the exception being the single ISSR marker. The distorted markers were not more specific to either of the parents. In general, the aberrant markers were located on all the linkage groups except LG3 and LG7 and at the end or middle of the linkage groups.

Discussion

This linkage map constructed from the F_2 population from the cross between lentil cultivars ILL5588 and ILL7537 is the first report of a linkage map of lentil in which an intraspecific mapping population within the cultivated gene pool has been used. RAPD primers were 19.2% polymorphic between the parent cultivars, which was sufficient polymorphism to map the genome. In comparison, Eujayl et al. (1998) observed 29.7% RAPD primer polymorphism between the parents ILL5588 and the wild relative L692-16-1(s). The higher polymorphism was most probably due to the presence of the wild genome in the male parent. All previous *Lens* maps were constructed using a wild relative as a parent in the development of the mapping population so as to increase the polymorphism for mapping (Havey and Muehlbauer 1989; Weeden et al. 1992; Tahir et al. 1993; Eujayl et al. 1997, 1998).

Linkage analysis

The lentil map spanned 784.1 cM with 114 markers and a density of 6.9 cM between adjacent markers, which is relatively small compared to the genome size of lentil (approximately 4,063 Mbp/C; Arumuganathan and Earle 1991). However, the map length of field pea is 700–800 cM based on cytological studies (Hall et al. 1997a, b), and since lentil and field pea have approximately the same genome size (Arumuganathan and Earle 1991), the map length detected in the present study could therefore represent the entire lentil genome. Cytological studies have to be conducted to confirm this hypothesis.

The previous *Lens* maps (Havey and Muehlbauer 1989; Weeden et al. 1992; Eujayl et al. 1997) spanned 333 cM, 560 cM and 206 cM, respectively, with relatively few markers. However, the most recent map, made using an interspecific RIL population, spanned 1,073 cM and comprised 177 markers (Eujayl et al. 1998). If an equal number of markers were placed on the intraspecific map developed in the current study, with the present marker density, a projected distance of 1,217 cM would be mapped. This would be approximately 13% longer than the map of Eujayl et al. (1998). The projected increase in map length is in accordance with the results obtained by Causse et al. (1994) in rice where a 25% larger map length was detected using an intraspecific population than that detected using an interspecific population. Lefebvre et al. (1995) also detected this in pepper, where an intraspecific map was 60% longer than an interspecific map. The increased intraspecific map length is due to

higher recombination frequency due to homology between very closely related chromosomes.

In the lentil map, only four unlinked markers were detected, whereas 37 markers were found to be unlinked in the map developed by Eujayl et al. (1998). The lower number of unlinked markers in the lentil map was most probably due to the use of an intraspecific cross as there is more homology among chromosomes of the same species. Another explanation is that recombination frequency between closely linked markers in a RIL population is twofold higher than in F_2 population (Taylor et al. 1978). Increases in recombination frequency pose difficulties in the establishment of linkage between markers that are more than 20 cM apart in a RIL population (Burr et al. 1988; Paran et al. 1995).

Linkage analysis at a LOD score of 4.0 and a maximum recombination fraction of 0.25 produced nine linkage groups which were not equal to the haploid chromosome number of lentil ($n = 7$). Since reducing the LOD score to 3.0 did not affect the number of linkage groups identified (data not shown), more markers may need to be mapped to merge smaller linkage groups to larger ones. Eujayl et al. (1998) observed 15 groups, of which seven were large and eight were small fragments. The clustering of markers in the middle or near the middle of the linkage groups may indicate the location of centromeres, which experience up to tenfold less recombination than other areas of the genome (Tanksley et al. 1992). A similar trend of clustering of markers was previously observed in field pea and chickpea (Laucou et al. 1998; Winter et al. 2000).

Due to the unavailability of co-dominant markers for lentil, dominant markers (RAPD, ISSR and RGA) were used for mapping. Dominant markers are unable to distinguish heterozygotes from homozygotes, however, they allow many polymorphic markers to be quickly identified, which is useful for mapping the genome. They have been used to construct linkage maps in various legume crops (Menéndez et al. 1997; Eujayl et al. 1998; Laucou et al. 1998; Santra et al. 2000) and also to extend the existing linkage map of rye (Masojć et al. 2001). Molecular maps based on RAPD markers using F_2 populations have previously been successfully constructed (Simon and Muehlbauer 1997; Lambrides et al. 2000; Thoquet et al. 2002).

In the lentil map, 14.4% markers were distorted, which is comparable to the 9.0% and 13.3% detected in the maps using F_2 populations of *Phaseolus vulgaris* (Nodari et al. 1993) and *Glycine* spp. (Keim et al. 1990), respectively. However, this is in contrast to the 26.5% of marker distortion previously detected in an interspecific F_2 segregating *Lens* population (Havey and Muehlbauer 1992). PCR-derived marker distortion levels as high as 39.6% and 40.6% have been reported in F_2 intraspecific populations of *Medicago truncatula* and *M. tornata*, respectively (Jenczewski et al. 1997). However, Eujayl et al. (1998) observed only 8.4% marker distortion using an interspecific RIL *Lens* mapping population, most probably due to previous selection against segregation distortion at the F_2 stage (Eujayl et al. 1997). However, in another cross, a segregation distortion of 83.3% was reported (Eujayl et al. 1997). In the lentil map, distorted loci were observed at the ends or middle of linkage groups. A similar trend was also observed by Eujayl et al. (1998) in RIL map of *Lens*.

Mapping of ISSR and RGA markers

None of the primers screened that contained $(AT)_8$ or $(TA)_8$ repeats produced amplification products. This was surprising since (AT) repeats were previously reported to be the most abundant repeat type in plant genomes (Wang et al. 1994). Similarly, even though several annealing temperatures were assessed, $(AT)_n$ primers did not produce distinct bands in wheat (Nagaoka and Ogihara 1997). Of the six ISSR primers that did produce markers for mapping, five were di-nucleotide repeats [$(AG)_8$YC, $(AC)_8$T, $(AG)_8$T, $(GA)_8$T, $(GA)_8$A] and one was a penta-nucleotide repeat $(GGAGA)_3$. The repeat $(GA)_n$ was previously found to be abundant in the related chickpea genome (Hüttel et al. 1999). In the lentil map, 4 out of 11 mapped ISSR markers were obtained from (AC) repeat motif. The (AC) repeat motif was also found to be useful for mapping in wheat (Nagaoka and Ogihara 1997; Kojima et al. 1998). Three RGA markers derived from the Pto kinase primer were also mapped in lentil, one each on LG1, LG3 and LG6. Mapping of RGA sequences can be useful in locating markers tightly linked to resistance genes and in identifying candidate resistance gene loci (Kanazin et al. 1996). RGA markers have been reported to be associated with *fusarium* resistance in chickpea (Huettel et al. 2002). Graham et al. (2002) reported the mapping of resistance-like genes in the region of disease resistance genes in soybean.

Comparison between the intraspecific lentil map and the interspecific map developed by Eujayl et al. (1998) is difficult due to the different primers used and the inability to determine whether the same polymorphic marker is segregating in both populations. However, as suggested by Eujayl et al. (1998), the development of co-dominant PCR-based markers such as simple-sequence repeats will be more beneficial in enriching the map and will enable easier comparison between maps. The present study explored the utility of ISSR and RGA markers in mapping of the lentil genome, and this lentil map will be useful in locating the gene loci governing desirable traits such as ascochyta blight resistance that may segregate in the population and for their marker-assisted selection. The markers can also be integrated for developing a more saturated genetic linkage map of lentil.

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