Research Article

Development of two sequence-specific PCR markers linked to the *le* gene that reduces pod shattering in narrow-leafed Lupin (*Lupinus angustifolius L.*)

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Abstract

Wild types of narrow-leaf lupin (*Lupinus angustifolius* L.) have seed pods that shatter upon maturity, leading to the loss of their seeds before or during the harvest process. Two recessive genes have been incorporated into domesticated cultivars of this species to maximize harvest-ability of the produce. One of these genes is called *lentus* (*le*). Two microsatellite – anchored fragment length polymorphism (MFLP) candidate markers were identified as closely linked to the *le* gene in a recombinant inbred line (RIL) population derived from a domesticated x wild type cross. The candidate MFLP markers were isolated from the gel, re-amplified by PCR, cloned and sequenced. The MFLP polymorphisms were converted into sequence-specific PCR-based markers. Linkage analysis by MapManager indicated that one of the markers, LeM1, was 2.6 centiMorgans (cM) and the other, LeM2, was 1.3 cM from the gene, with both being on the same side. The correlation between the marker genotype and the plant phenotype for the *le* gene is 95% for the Australian cultivars, and approximately 36% on wild types tested. These markers may be useful in marker assisted selection for the *le* gene when introgressing wild material into lupin breeding programs.

Key words: pod shattering, Lupin, MFLP, molecular marker, marker assisted selection.

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Introduction

An essential characteristic of a modern grain crop is the ability to retain its seeds long enough to allow mechanical harvesting at full maturity. In the genus *Lupinus*, this characteristic has long been present in two species - *Lupinus albus* L., an old crop species of the Mediterranean region, and *L. mutabilis* (sweet) which has been cultivated in the Peruvian Andes for over 2000 years (Gladstones, 1967).

Narrow-leaf lupin (*Lupinus angustifolius* L.) is the most widely cultivated grain legume crop in Australia. Originally introduced into Western Australia (W.A.) towards the end of the 19th century (Gladstones, 1994) as a green manure crop, these plants were essentially wild. Wild types of *L. angustifolius* have seed pods that shatter upon maturity, making harvesting very difficult. Research aimed at finding plants with non-shattering pods was reported to have begun in Germany in 1929 (Hanelt, 1960). They were successful in selecting a strain that had a reduced pod shatter (von Sengbusch and Zimmermann, 1937) apparently related to a large reduction in the thick-

ness of the pod wall (Atebekova, 1958). Domestication of this species in W.A. began in 1960 with the discovery of two natural mutant (recessive) genes, *lentus* and *tardus* for reduced pod-shattering (Gladstone, 1967) and, in 1961 a gene for nil-vernalisation requirement and early flowering (Gladstones, 1969).

One of the genes for reduced pod-shatter, known as *tardus* (*ta*), affects the sclerenchyma strips of the dorsal and ventral pod seams, fusing the two halves to such an extent that separation of the two halves is greatly impeded. It was considered analogous to a gene previously found in *L. luteus*. The second gene *lentus* (*le*) modified the orientation of the sclerified endocarp of the pod, resulting in a reduction of torsional forces upon drying, and hence reduced pod shatter. This gene was also associated with a reduction in the thickness of the pod wall, but not to the extent of the previously found strain of von Sengbusch and Zimmerman (1937), leading to the conclusion that the controlling genes were not the same (Gladstones, 1967). This modification was associated with a change in internal pod pigmentation that gave the immature pods a purplish tinge and the inside surface of mature pods a bright yellowish-brown color (Gladstones, 1967). In addition, this trait is also associated with development of a reddish pigmentation within the stem of plants older than two months. The first Australian

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cultivar to carry both these genes was Uniharvest, released in 1971. Apart from Fest (released 1973) all Australian and many European cultivars released since then carry both of these genes.

Since field based selection for the *le* gene requires that the F_2 progeny be maintained for at least 8 weeks from sowing to ensure that this trait is retained, it would be more efficient to use molecular markers that could be used to evaluate progeny within the first weeks of growth. Recently, two molecular genetic maps have been produced in *L. angustifolius* on which *le* has been mapped (Boersma *et al.*, 2005; Nelson *et al.*, 2006). No marker was located within 5 centiMorgans (cM) of the *le* gene on the map of Nelson *et al.* (2006). Conversely, several markers on the map of Boersma *et al.* (2005) were closely linked to the *le* gene. However, the MFLP markers in the map of Boersma *et al.* (2005) are not implementable in practical lupin breeding as the multiple bands produced by their primer combinations may cause confusion, makes it impossible to multiplex several markers for high efficiency and, is too tedious and expensive to be implemented in a lupin breeding program for screening large numbers of plants. The aim of this research is to develop simple, sequence-specific PCR based markers linked to the *le* gene desirable for marker assisted selection (MAS) in lupin breeding.

Material and Methods

Plant materials and phenotyping of *le* gene

A population of F_8 recombinant inbred lines (RILs) of a domestic x wild type (DxW) cross of *L. angustifolius* was previously developed by the Department of Agriculture and Food, Western Australia (DAFWA) using as parents lines P27255 (wild) and 83A:476 (domesticated). Part of the resulting RIL population has previously been used to produce linkage maps for *L. angustifolius* (Boersma *et al.*, 2005; Nelson *et al.*, 2006).

The full population consisting of two parents and 115 RILs, was sown in a screen-house during the winter growing season with 25-30 plants of each RIL grown in 1.5 meter rows. Plants were either rated as *LeLe* (wild, shattering; pigment absent) or *lele* (domesticated, reduced shattering; pigment present) based on the method described by Gladstones (1967).

Marker development

The parents and 89 RILs were previously subject to MFLP tests involving 10 SSR-anchor primers each in combination with 16 *Mse*I-primers (Boersma *et al.*, 2005). Two MFLP markers having the best correlation to the *le* phenotyping data were selected. DNA fragments from candidate MFLP markers were isolated from MFLP gels, re-amplified by PCR, ligated into plasmids (pGEM-T Easy Vector, Promega) and cloned into *E. coli* according to the manufacturer's instructions. Plasmid DNA with MFLP fragment inserts were isolated from *E. coli*, and sequenced using the BigDye Terminator system (Applied Biosystems). To ensure the accuracy of DNA sequencing, at least five clones of *E. coli* were used to prepare the plasmid DNA containing the inserts, and sequenced separately. A sequence-specific primer was designed near the *Mse*I-end internal to the SSR-*Mse*I fragment. (Yang *et al.*, 2002, 2004; You *et al.*, 2005). Primers were designed so that the annealing temperature was approximately 54 °C based on calculations using the nearest-neighbor model (http://www.sigmaaldrich. com).

Testing of converted markers

Testing of the converted markers was achieved by PCR, using the sequence-specific primer in combination with the SSR anchor-primer from which the original MFLP polymorphism was produced (Yang *et al.*, 2001, 2002). Marker DNA fragments were amplified in a10 µL PCR consisting of 1.5 µL of the template DNA (approximately 100 ng), 0.5 unit of Taq polymerase (Fisher Biotec, Perth), 5 pmol of each primers, 67 mM Tris-HCl (pH 8.8), 2 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 0.45% Triton X-100, 4 µg gelatin, and 0.2 mM dNTPs. The sequence-specific primers were labeled with γ-^{33}P based on a previously reported method (Yang *et al.*, 2001, 2002, 2004). PCR was performed on a thermocycler (Hybaid DNA Express) with each cycle comprising 30s at 94 °C, 30s at the annealing temperature (see below), and 1 min at 72 °C. The annealing temperature of the first cycle was 60 °C, and decreased 0.7 °C in each subsequent cycle until the temperature reached 54 °C. The final 25 cycles used an annealing temperature of 54 °C. The selective PCR products were separated on a 5% polyacrylamide denaturing sequencing gel (7 M urea) using a 38 x 50 cm (0.4 mm in thickness) Sequi-Gen GT sequencing cell (Bio-Rad). After electrophoresis at 55W for about 3h, the gel was dried on a gel drier (Model 583, Bio-Rad). Marker bands were detected by autoradiography (Yang *et al.*, 2002; You *et al.*, 2004) with overnight exposure of the X-ray film to the dried gel.

Confirmation of linkage

The two converted sequence – specific markers were tested on all 115 F_8 RILs of the population derived from the DxW cross. The marker score data and *le* phenotype data of the 115 F_8 RILs were merged and analyzed using MapManager (Manly *et al.*, 2001) to determine the genetic linkage between the markers and the *le* gene.

Further testing of the markers was carried out on all 23 Australian historical and current cultivars and on 36 landrace accessions from the Australian Lupin Collection, to examine correlation of their phenotype with the *le* markers' genotypes. Landrace accessions were selected on the basis of their geographic origins.

Results

Phenotyping of *le* gene

Phenotyping on the individual plants from each of the 115 RILs revealed that 55 RIL lines were homozygous for *le* (reduced shatter), and 60 lines were homozygous for *Le* (shattering). The segregation of RILs with the *lele* genotype to plants with the *LeLe* genotype fits the expected 1:1 ratio ($\chi^2 = 0.217$, $P = 0.641$) for a single gene.

DNA sequencing of candidate MFLP markers

Among the markers originated from 153 sets of MFLPs during a mapping study (Boersma *et al.*, 2005), two dominant markers designated as DAWA323.150 and DAWA468.290 were identified as candidate markers for development as sequence-specific markers tagging the *le* gene.

Marker DAWA323.150 mapped as1.2 cM from the *le* gene in the map based on the 89 RILs (Boersma *et al.*, 2005), was present in RILs with *lele* genotype, but was absent in RILs with *LeLe* genotype (Figure 1). DNA sequencing found that MFLP marker DAWA323.150 is a 157 bp fragment including the sequence of SSR-anchor primer MF51 (5-GGGAACAACAACAAC-3') and the primer *Mse*I-CGA (5-GATGAGTCCTGAGTAACGA-3') (Table 1).

Marker DAWA468.290 was also present in plants with *lele* genotype but absent in plants with *LeLe* genotype (Figure 2). This marker had not been shown in the map (Boersma *et al.*, 2005) as it was also calculated to be 1.2 cM from the *le* gene, and therefore co-segregated with marker DAWA323.150. DNA sequencing revealed that marker DAWA468.290 is a 284 bp fragment including the anchor primer sequence MF42 (3- GTCTAACAACAACAACA AC-5') and the sequence of primer *Mse*I-CCA (5'-GATGA GTCCTGAGTAACCA-3') (Table 2).

Marker LeM1: Based on the DNA sequence of MFLP marker DAWA323.150, a sequence-specific primer LeMS1 (3'-TTAACGAACCTACCATTTG-5') was designed near the *Mse*I end of the original MFLP marker (Table 1). The dominant MFLP marker was successfully converted into a dominant simple PCR based marker by
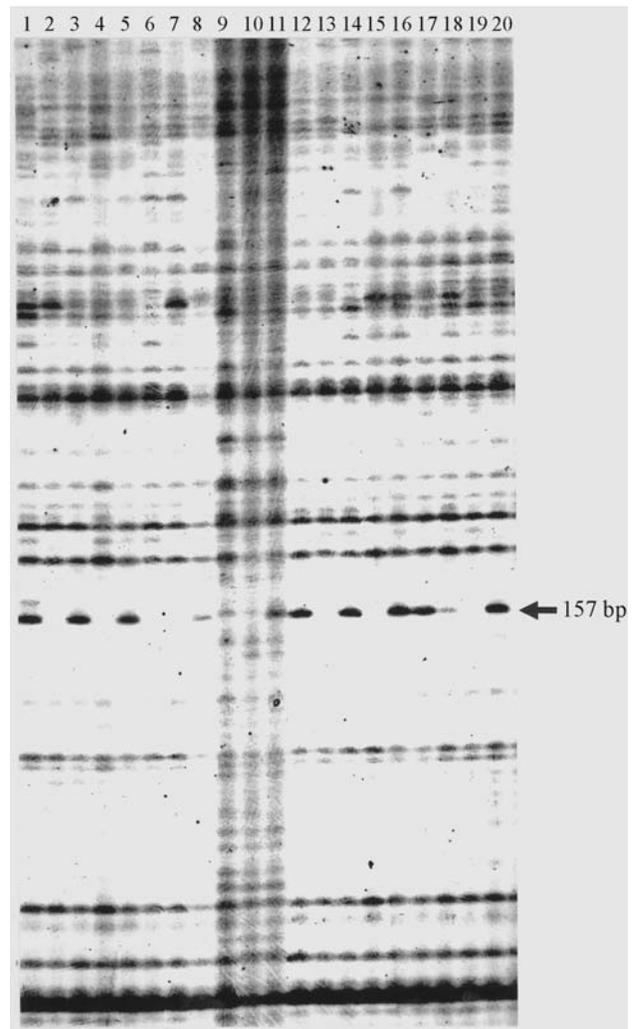

Figure 1 - MFLP fingerprinting on 20 RILs derived from a domesticated x wild cross in *Lupinus angustifolius* generated by SSR-anchor primer MF51 in combination with primer *Mse*I-CGA. Eight RILs having the *LeLe* genotype are (DxW) 58 (Lane 2), 60 (Lane 4), 65 (Lane 6), 66 (Lane 7), 70 (Lane 9), 73 (Lane 10), 77 (Lane 13), and, 85 (Lane 19). Eleven RILs: (DxW) 57 (Lane 1), 59 (Lane 3), 64 (Lane 5), 69 (Lane 8), 74 (Lane 11), 76 (Lane 12), 78 (Lane 14), 82 (Lane 16), 83 (Lane 17), 84 (Lane 18), and 86 (Lane 20) have the genotype *lele*. Note that all of the 20 plants except one (RIL 80, Lane 15) showed correct correlation between the *le* phenotype and the marker genotype. Arrow indicates the candidate MFLP marker linked to the *le* allele at 157 bp.

Table 1 - DNA sequence of the MFLP marker DAWA323.150 showing primers giving rise to sequence-specific marker LeM1.

Position	Sequence
1	*GATGAGTCCT GAGTAACGA*[1]A CCTACCATTT G[2]CCTAAACAA TATATTGTTT
51	ACTGGTTGTT GTTGTTGTTC TTCTTCTTCT TCCTCTTCTT CCTCTTCTTC
101	TTCTTCTTCT TACCATTTGC CTAAACAATA TATTGTTTAC TGGTTGTTGT
151	TGTTCCC[3]

[1]Primer *Mse*I-CGA (5'-GATGAGTCCTGAGTAACGA-3') is in italic.
[2]Sequence-specific primer LeBMS1 (5'-TTAACGAACCTACCATTTG-3').
Note: The first 4 bp in primer LeBMS1 corresponds with the recognition site of *Mse*I (T/TAA), while the first nucleotide (T) was replaced by G when the *Mse*I-adaptor was ligated onto the restriction fragments in MFLP (Yang *et al.* 2001).
[3]Annealing site of SSR-anchor primer MF51 (3'-GGGAACAACAACAAC-5').

using the sequence-specific primer LeBMS1 in combination with SSR-anchor primer MF51. F_8 RILs with the genotype *lele* developed the 126 bp marker band, while plants with genotype *LeLe* did not develop this band (Figure 3). We designated this sequence-specific dominant marker linked to the *le* gene as LeM1.
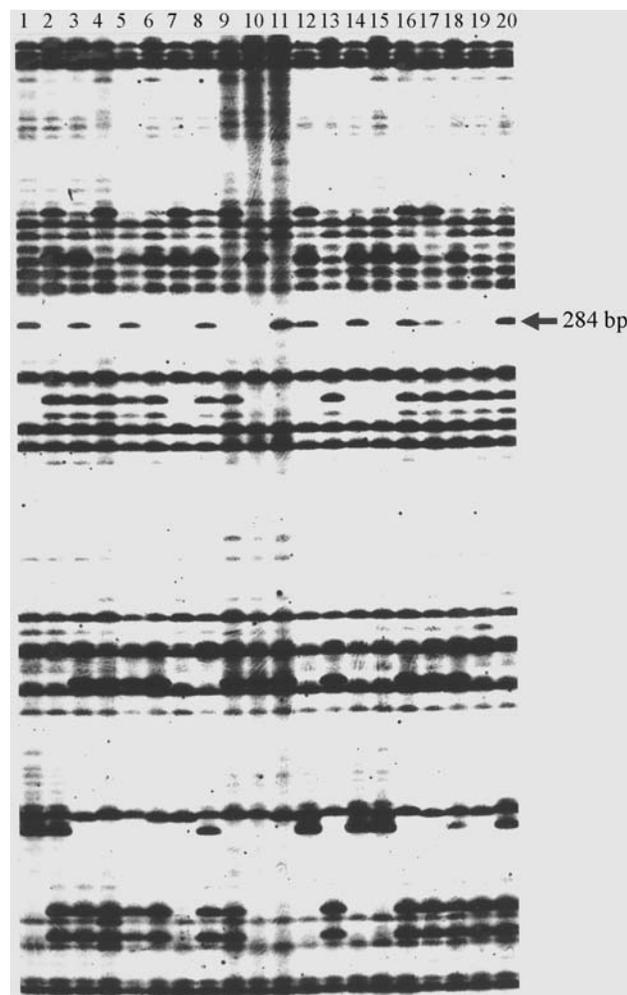

Figure 2 - MFLP fingerprinting on 20 RILs in *Lupinus angustifolius* generated by SSR-anchor primer MF42 in combination with primer *Mse*I-CCA. RILs portrayed in this figure are the same as in Figure 1. Arrow indicates the candidate MFLP marker linked to the *le* allele at 284 bp.

Marker LeM2: A sequence-specific primer LeMS2 (3'-AGAAAAAGATGAATGCACG-5') was designed near the *Mse*I end based on the sequence of MFLP marker DAWA468.290 (Table 2). The marker was successfully converted into a simple PCR based marker by using primer LeMS2 in combination with SSR-anchor primer MF42. Plants with genotype *lele* produced a 204 bp marker band, but the marker fragment is absent in plants with *LeLe* genotype (Figure 4). We designated this marker as LeM2.

Confirmation of linkage

Markers LeM1 and LeM2 were tested on the parents and 115 RILs of the DxW population. Marker results and RIL genotypes showed imperfect correlation, with LeM1 having six differences and, LeM2 having three differences between the marker result and RIL genotypes. The three RILs which showed inconsistence between phenotype and marker genotype of LeM2 also showed the inconsistency with LeM1. Map distances were calculated by MapManager (Manly *et al.*, 2001) to be 1.3 cM (LeM2) and 2.6 cM (LeM1) from the gene (Figure 5).

Among the 23 Australian cultivars, only the first released cultivar Uniwhite, carries the shattering alleles *LeLe*, and all the other 22 cultivars have the reduced-shattering genotype *lele*. The 22 cultivars having the *lele* genotype were correctly scored by the markers. However, the marker genotype and *Le* phenotype of Uniwhite did not match (Table 3).

The Australian lupin collection holds many accessions of wild and landrace *L. angustifolius* collected or received from a range of locations. Most of these accessions have been rated as either 'shedding' (shattering; no *le* or *ta*), 'reduced shedding' (carrying at least one gene reducing pod shatter) or 'non-shedding' (presumably carrying at least 2 genes for the reduction of pod shatter).

Of the 36 landraces, marker LeM1 designated only six accessions as *Le* ('shedding' (shattering)) and LeM2 designated seven as *Le*, despite all but one of them having previously been rated as *Le* ('shedding', Australian Lupin Collection database, 2005). Only one of the accessions was designated as *Le* by both markers. Similarly, both markers

Table 2 - DNA sequence of the MFLP marker DAWA468.290 showing primers giving rise to sequence-specific markers LeM2.

Position	Sequence
1	GATGAGTCCT GAGTAACCA[1] C TCATAGTTTA TAAACTCTCC AATTGTTTGT
51	TATTCTCATG TATTATATCC TATCTCACAA AGAAAAAGAT GAATGCACG[2]A
101	GTTTTTTAGT ACAAATTTCA TCCTACACTG AATCTGTTGA AATTGAAATG
151	AATATCACAA AACATTTTGC TGCAGTGTCG GTCTTGCTTT ACCATTCGTG
201	GTGGCAACAC TTACACGTCA AGCAAAGTCA TTGATGGATG CCCCACCTAC
251	TGTGATAAAA GCTTGGTTGT TGTTGTTGTT AGAC[3]

[1]Primer *Mse*I-CCA (5'-GATGAGTCCTGAGTAACCA-3').
[2]Sequence-specific primer LeMS2 (5'-AGAAAAAGATGAATGCACG-3').
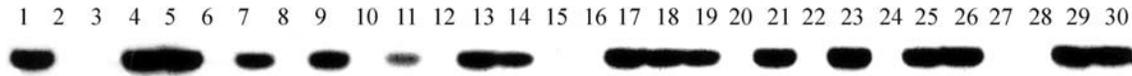[3]Annealing site of primer MF42 (3'-GTCTAACAACAACAACAAC-5').

Figure 3 - Screening molecular marker LeM1 on the parents and 28 F_8 derived RILs of *Lupinus angustifolius* using PCR with sequence-specific primer LeMS1 (5'-TTAACGAACCTACCATTTG-3') and SSR anchor primer MF51. Lines with the *lele* genotype which developed a 126 bp band include the domesticated parent plant 83A:476 (Lane 1) and 21 RILs: (DxW) 67 (Lane 4), 69 (Lane 5), 71 (Lane 7), 74 (Lane 9), 76 (Lane 11), 78 (Lane 13), 79 (Lane 14), 82 (Lane 17), 83 (Lane 18), 84 (Lane 19), 86 (Lane 21), 90 (Lane 23), 93 (Lane 25), 94 (Lane 26), 97 (Lane 29) 98 (Lane 30). Plants with the *LeLe* genotype which did not produce the marker band are the wild type parent P27253 (Lane 2) and 11 RILs: 66 (Lane 3), 70 (Lane 6), 73 (Lane 8), 75 (Lane 10), 77 (Lane 12), 81 (Lane 16), 85 (Lane 20), 89 (Lane 22), 92 (Lane 24), 95 (Lane 27), 96 (Lane 28). RIL DxW 80 (Lane 15) also produced no band despite carrying the *lele* allele.
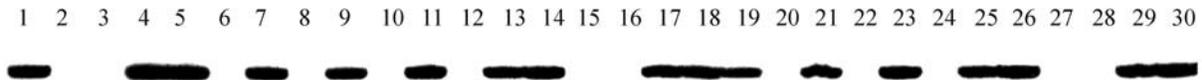

Figure 4 - Screening molecular marker LeM2 on the parents and 28 F_8 derived RILs of *Lupinus angustifolius* using PCR with sequence-specific primer LeMS2 (3'-GAAAAAGATGAATGCACG-5') and SSR anchor primer MF42. The length of the band fragment produced here is 204 bp. RILs portrayed in this figure are the same as in Figure 3.
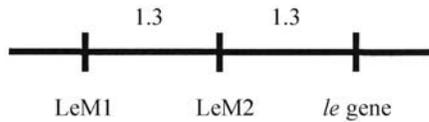

Figure 5 - Genetic linkage of the two molecular markers LeM1 and LeM2 and the reduced-pod-shatter gene *lentus* (*le*) of *Lupinus angustifolius*, as analyzed by MapManager. Distances between adjacent points given in centiMorgans (on the left side) between adjacent points on the linkage map are non-cumulative.

designated as *le* the one accession listed as 'non-shedding', received from Belarus (Table 4). Accessions correctly designated as *Le* or *le* were collected (or received) from eight countries including Belarus, France, Greece, Italy, Morocco, Portugal, Spain, Syria. Accessions from the other eight source countries including Cyprus, Chile, Germany, India, Israel, Turkey, USA and the USSR were incorrectly genotyped by the two markers. In total, 13 out of 36 (36%) land-race accessions showed a positive correlation between marker score and pod-shatter genotypes for one or both markers.

Discussion

We have successfully developed two sequence-specific PCR markers linked to the *Le* gene in *L. angustifolius* in this study. Both markers are simple PCR based, are cost-effective, and could be used for MAS in lupin breeding. Previously Boersma *et al.*, (2005) had mapped marker DAWA323.150 from which LeM1 was derived as being only 1.2 cM from the gene based on 89 RILs. This distance has now been re-calculated as being 2.6 cM from the gene on the basis of a population of 115 RILs, including the 89 RILs previously used by them. LeM2 which had not previously been placed on the map has been positioned mid-way between the *Le* gene and LeM1 at 1.3 cM. The genetic distance between the marker and the gene determines the ac-

Table 3 - Correlation of marker score and *Le* phenotype of 23 Australian cultivars of *Lupinus angustifolius*.

Cultivars	Year of release	Lentus phenotype	Presence of LeM1 marker band	Presence of LeM2 marker band
Uniwhite	1967	***LeLe***	+	+
Uniharvest	1971	*lele*	+	+
Unicrop	1973	*lele*	+	+
Marri	1976	*lele*	+	+
Illyarrie	1979	*lele*	+	+
Yandee	1980	*lele*	+	+
Chittick	1982	*lele*	+	+
Danja	1986	*lele*	+	+
Geebung	1987	*lele*	+	+
Gungurru	1988	*lele*	+	+
Yorrel	1989	*lele*	+	+
Warrah	1989	*lele*	+	+
Merrit	1991	*lele*	+	+
Myallie	1995	*lele*	+	+
Kalya	1996	*lele*	+	+
Wonga	1996	*lele*	+	+
Belara	1997	*lele*	+	+
Tallerack	1997	*lele*	+	+
Tanjil	1998	*lele*	+	+
Moonah	1998	*lele*	+	+
Quilinock	1999	*lele*	+	+
Jindalee	2000	*lele*	+	+
Mandelup	2004	*lele*	+	+

Note: Both LeM1 and LeM2 are dominant markers, where the marker band is linked to the *le* allele. The one cultivar incorrectly identified by the markers, Uniwhite, is presented in bold.
+ = band present.
- = band absent.

Table 4 - Correlation of marker score and *Le* phenotype of 36 Wild and Landrace accessions of *L. angustifolius* from the Western Australian Lupin Collection.

Landrace (Perth n.)	Origin	*Lentus* phenotype	Presence of LeM1 marker band	Presence of LeM2 marker band
P20650	Portugal	*LeLe*	+	+
P20653	Portugal	*LeLe*	+	+
P20711	Italy	*LeLe*	+	+
P20712	Italy	*LeLe*	+	-
P20714	Italy	*LeLe*	+	+
P20724	Italy	*LeLe*	+	+
P20729	Russia	*LeLe*	+	+
P20730	Russia	*LeLe*	+	+
P20733	Germany	*LeLe*	+	+
P20736	Israel	*LeLe*	+	+
P21517	Israel	*LeLe*	+	+
P21624	Turkey	*LeLe*	+	+
P21625	Syria	*LeLe*	+	+
P22609	Turkey	*LeLe*	+	+
P22665	Spain	*LeLe*	-	+
P22666	Spain	*LeLe*	-	+
P22669	Spain	*LeLe*	+	+
P22820	Portugal	*LeLe*	+	-
P22829	Portugal	*LeLe*	+	-
P23051	France	*LeLe*	+	-
P24025	India	*LeLe*	+	+
P26042	USA	*LeLe*	+	+
P26045	Greece	*LeLe*	+	+
P26235	Spain	*LeLe*	+	-
P26263	Portugal	*LeLe*	+	-
P26307	Spain	*LeLe*	+	+
P26308	Spain	*LeLe*	+	+
P26436	Greece	*LeLe*	+	+
P26465	Greece	*LeLe*	-	+
P26652	Italy	*LeLe*	+	+
P26675	Cyprus	*LeLe*	+	+
P27055	Belarus	*lele*	+	+
P27253	Morocco	*LeLe*	-	-
P27434	Syria	*LeLe*	-	+
P27968	Greece	*LeLe*	-	+
P28003	Chile	*LeLe*	+	+

Note: Both LeM1 and LeM2 are dominant markers, where presence of the marker band is linked to the *le* allele. Accessions for which the *Le* phenotype correlates with either or both of the two markers are presented in bold.
+ = band present.
- = band absent.

curacy of marker assisted selection when the marker is used in the breeding program. Since markers LeM1 and LeM2 are both on the same side of the *le* gene, and LeM2 is closer to the gene than LeM1, it would be appropriate to use marker LeM2 in MAS if both markers are applicable.

For MAS, it would be ideal to develop so-called 'perfect Markers' where the DNA fragments of the markers are actually on the gene of interest, in which case the linkage between the markers and the gene is almost unbreakable (Ellis *et al.*, 2002). However, perfect markers are very difficult to develop. Most of the molecular markers being implemented in practical plant breeding programs are 'imperfect' where certain genetic distances exist between the markers and the gene of interest in the chromosomes (Staub and Serquen, 1996; Gupta *et al.*, 1999). The two markers linked to the *Le* gene in lupin reported in this paper, are like-wise imperfect. Over long periods of time and in the crossing in plant breeding programs, genetic recombination may occur between the marker locus and the gene locus, and consequently plants not having the target gene may carry the marker DNA sequence, and *vice-versa* (Sharp *et al.*, 2001, You *et al.*, 2005). In MAS, a molecular marker can be used to screen the progeny from a cross only if the marker is polymorphic among the parents so that the desirable allele can be distinguished from the undesirable ones (Eagles *et al.*, 2001). Fortunately, all the modern Australian cultivars having the *lele* genotype show the target bands for markers LeM1 and LeM2. However, testing of these markers on land-races that were fully shattering (having the *LeLe,TaTa* genotype) resulted in only 13 of the 36 landrace accessions correlating the marker bands with genotype. Eleven of those 13 accessions were correctly identified by either one or the other of the two markers, indicative of the degree of crossing over that has occurred between the marker and the *le* gene in the wild, even though it only occurs at an estimated 1.3-2.6% of the time in any given cross. The results suggest that these markers may be of use in MAS to screen the progeny of approximately one third of wild accessions when they are crossed with modern Australian cultivars. This study although not exhaustive, also suggests that these markers would be more likely useful when testing progeny of seed material originating from Greece, Morocco (source of DxW parent P27255), Portugal, Spain and Syria, although seed received from other sources including collections (*e.g.* France, Belarus) should not be ruled out.

When markers LeM1 and LeM2 are used to screen the F_2 progenies from DxW crosses, they enable breeders to eliminate the approximately 25% of plants which are homozygous for the *LeLe* allele. However, since both markers are dominant, they cannot differentiate homozygous *lele* F_2 plants from the heterozygous *Lele* plants. Therefore, further selection for the *lele* genotype may be necessary in later generations of the breeding cycle.

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