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Regular paper

Application of the High Resolution Melting analysis for genetic mapping of Sequence Tagged Site markers in narrow-leafed lupin (*Lupinus angustifolius* L.)

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Sequence tagged site (STS) markers are valuable tools for genetic and physical mapping that can be successfully used in comparative analyses among related species. Current challenges for molecular markers genotyping in plants include the lack of fast, sensitive and inexpensive methods suitable for sequence variant detection. In contrast, high resolution melting (HRM) is a simple and high-throughput assay, which has been widely applied in sequence polymorphism identification as well as in the studies of genetic variability and genotyping. The present study is the first attempt to use the HRM analysis to genotype STS markers in narrow-leafed lupin (*Lupinus angustifolius* L.). The sensitivity and utility of this method was confirmed by the sequence polymorphism detection based on melting curve profiles in the parental genotypes and progeny of the narrow-leafed lupin mapping population. Application of different approaches, including amplicon size and a simulated heterozygote analysis, has allowed for successful genetic mapping of 16 new STS markers in the narrow-leafed lupin genome.

Key words: HRM, molecular markers, genotyping, narrow-leafed lupin

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INTRODUCTION

Sequence tagged site markers represent single copy sequences in the genome and are commonly used in genetic and physical mapping. Moreover, these markers can be used as skeleton markers that allow comparison between genetic maps among one species or synteny analysis between different species (Croxford *et al.*, 2008; Kroc *et al.*, 2014). In the case of species with unsequenced genomes such an approach is still desirable in comparative studies. The gene-based STS legume anchor markers (Leg markers) used in this study were designed to facilitate the comparative genomics of less known legumes (Fredslund *et al.*, 2006; Hougaard *et al.*, 2008). To date these markers have been successfully applied in the synteny analyses of *Phaseolus vulgaris, Lotus japonicas, Medicago truncatula* and Arachis (Hougaard *et al.*, 2008; Bertioli *et al.*, 2009). Leg markers were also included within a larger set of markers used in the recent synteny analysis of *Lupinus angustfolius* and *Medicago truncatula* (Kroc *et al.*, 2014).

High resolution melting (HRM) is a powerful technique used for genotyping and mutation scanning. This method takes advantage of special saturation dyes properties that fluoresce only in the presence of double stranded DNA. After PCR, amplicons bound to the fluorescent dye are denaturated and the fluorescence fades away. Since various genetic sequences melt at different temperatures, changes in the fluorescence registered during the analysis can be applied to single nucleotide polymorphism (SNP), simple sequence repeat (SSR), small insertion and/or deletion (InDel), as well as length polymorphism detection (Montgomery *et al.*, 2007; Distefano *et al.*, 2012). The HRM assay has been successfully applied in studies of genetic variability and SNP/SSR marker genotyping of various plants, e.g. legumes, including alfalfa (Han *et al.*, 2012), pea (Knopkiewicz *et al.*, 2014), soybean (Monteros *et al.*, 2010) and white lupin (Croxford *et al.*, 2008).

Various molecular markers, such as amplified fragment length polymorphism (AFLP), microsatellite anchored fragment length polymorphism (MFLP), restriction fragments length polymorphism (RFLP), diversity arrays technology (DArT), InDel and STS have already been applied in various studies focused on the narrow-leafed lupin genetic mapping (Nelson *et al.*, 2006; Nelson *et al.*, 2010; Yang *et al.*, 2013; Kroc *et al.*, 2014; Kamphuis *et al.*, 2014). In the case of STS markers, DNA polymorphism has been detected with the aid of restriction enzymes such as either CAPS (cleaved amplified polymorphic sequence) or dCAPS (derived-CAPS) markers or the SNaPshot assay (Life Technologies Inc.) (Nelson *et al.*, 2010; Kroc *et al.*, 2014). However, these techniques have some limitations. Their protocols are multi-stage, time-consuming and labor-intensive. Moreover, in CAPS and dCAPS systems, SNP must create a restriction site, while polymorphism detection requires gel electrophoresis. On the other hand, the SnaPshot assay needs capillary electrophoresis to detect polymorphisms. In contrast to both these methods, HRM is a simple and fast analysis. Furthermore, it can be performed directly after PCR without additional sample processing (Wu *et al.*, 2008).

The main aim of this study was to evaluate the effectiveness of the HRM method in genotyping STS markers in narrow-leafed lupin (*Lupinus angustifolius* L.) as well as HRM method optimization.

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Abbreviations: AFLP, amplified fragment length polymorphism; CAPS, cleaved amplified polymorphic sequence; cM, centimorgan; C_p, crossing point; DArT, diversity arrays technology; del, deletion; dCAPS, derived-CAPS; HRM, high resolution melting; InDel, insertion and/or deletion; Leg markers, legume anchor markers; LOD, log of odds; MFLP, microsatellite anchored fragment length polymorphism; NLL, narrow-leafed lupin; PCR, polymerase chain reaction; RFLP, restriction fragments length polymorphism; RILs, recombinant inbred lines; SNP, single nucleotide polymorphism; SSR, simple sequence repeat; STS, sequence tagged site; T_m, melting temperature

MATERIALS AND METHODS

Plant material. The mapping population of 96 F8 generation recombinant inbred lines (RILs) used in this study was established by the single seed descent method from a cross between a domesticated breeding line 83A:476 and a wild type P27255 (Boersma *et al.*, 2005) and was provided within the framework of cooperation with Dr. Hua'an Yang (Western Australia Department of Agriculture and Food, Perth, Australia).

Young leaf samples were colleted from plants grown in the field and processed for DNA extraction using a DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol with minor changes. These included: (1) an additional incubation step (30 min at room temperature) after the addition of Buffer AP1 and 3 µl of RNase A stock solution, (2) increased duration of the cell lysis step (30 min at 65°C) and (3) increased duration of the precipitation step (15 min on ice).

Polymorphic markers detection. Leg markers were designed with the aid of a bioinformatics pipeline on the basis of the coding sequences of model legumes (*Lotus japonicus, Medicago truncatula*) and legume crop species (*Glycine max, Phaseolus vulgaris*), with *Arabidopsis thaliana* genome sequence data used as a reference genome (Fredslund *et al.*, 2005; 2006). A list of all Leg primer

Table 1. A list of markers selected for HRM analyses and incorporated into the narrow-leafed lupin genetic map.

Marker name	Primer sequences		Amplicon size (bp)	SNP variations in 83A:476 x P27255 mapping population
Leg33MGm_HRM	Forward	TGGGTATTCATTCTGACCCACT	156	43 T>C; 78 G>A; 81 G>T
	Reverse	GAACATAATGTCCAACTACTCCAGAA		
Leg050_HRM	Forward	TTCAAGCCAAATCCAAATGA	82	62G>C
	Reverse	ATGAGATTATGGCCCCATGC		
Leg055_HRM	Forward	CCTGACACAGCAAAGTTATGAGA	89	28A>G
	Reverse	CCAGAGAGAGAGAATTATTTCCAA		
Leg074_HRM	Forward	ACTCAAACTTTGCTGTTCAGGT	144	99_101delTTG; 108 T>C
	Reverse	TCTCTATGATGCATGTTTGGGC		
Leg156_HRM	Forward	TTTTATAATTGCAGTCATATGTGAAA	105	70_71delTG
	Reverse	CCATTTTATGTAGTACTGTGTTTGGTT		
Leg245_HRM	Forward	CAACAACCCACCATAATTTACATAAC	102	60C>A
	Reverse	ACCAGCCTTCCTCAGTTGAA		
Leg256_HRM	Forward	CTTGCATGATGATGAGATATTGAA	151	84C>T
	Reverse	AAGCACGAGCTAAATCATTACAAA		
Leg318_HRM	Forward	ACCTTTGGCTGTCTCTAGGT	150	32G>A
	Reverse	ACAAATGGAGAGGTTGTTTCCA		
Leg325_HRM	Forward	GTTCGTTGCTTAGGTATATTTTCTTTC	100	42A>G
	Reverse	TGCTGCACATACGGTTGAA		
Leg425_HRM	Forward	AAAGACACAATAGTTAGAATGGCTGTT	100	39G>A
	Reverse	CTTTCCTTCCCAGGGTCATC		
Leg435	Forward	TCACTCTCWTCTGAGAAGGCAAG	278	24 C>T; 37 G>A; 72 G>A; 109 G>A; 223 A>C
	Reverse	TCTTGCCAGARTGAATTCTGGCTTTTAGCATAGGC		
Leg445_HRM	Forward	GGAGGACAGATAAGCTTTGATGTAA	118	59T>G
	Reverse	TTGAGGGAAAACCTGTGGAG		
Leg713_HRM	Forward	GTTGGAGCCGATGTTTGATA	86	55A>C
	Reverse	CAGATGTCGGAGATATTTGGTC		
Leg735_HRM	Forward	GCTCCATCATGTTTGCGATA	60	30T>C
	Reverse	ACCGTGGCCATATCAATTTC		
Leg736_HRM	Forward	TTTCATGGCTCTGAAGTGTTT	116	53C>G
	Reverse	TCCATCACTGTATTGAAGGACA		
Leg871_HRM	Forward	ATAACTGATGCAGTTTATTTTCAA	107	51T>A
	Reverse	CATCTCTGCATCATGAAGATG		

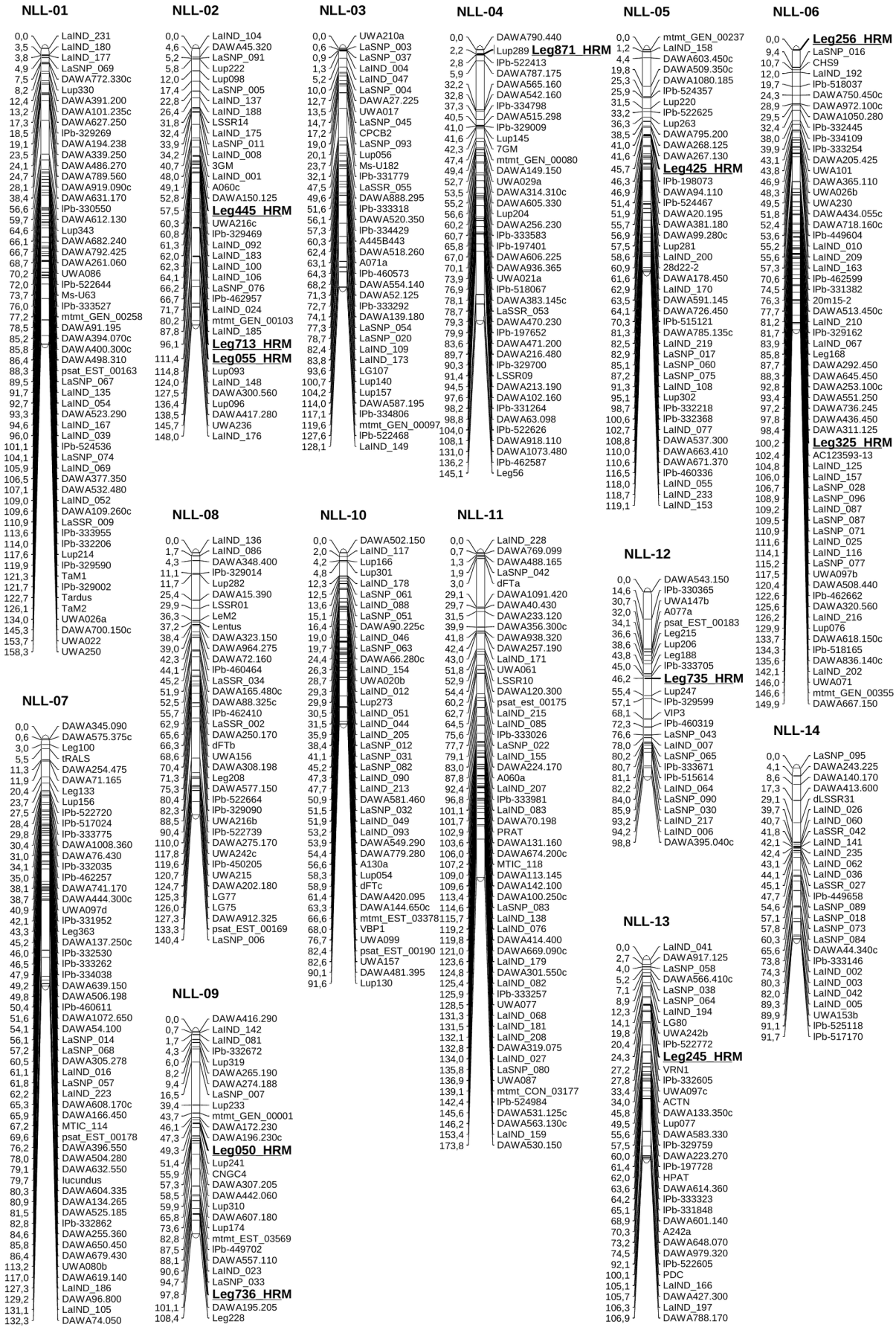

Figure 1. Linkage map of the narrow-leafed lupin genome comprising newly mapped HRM markers.

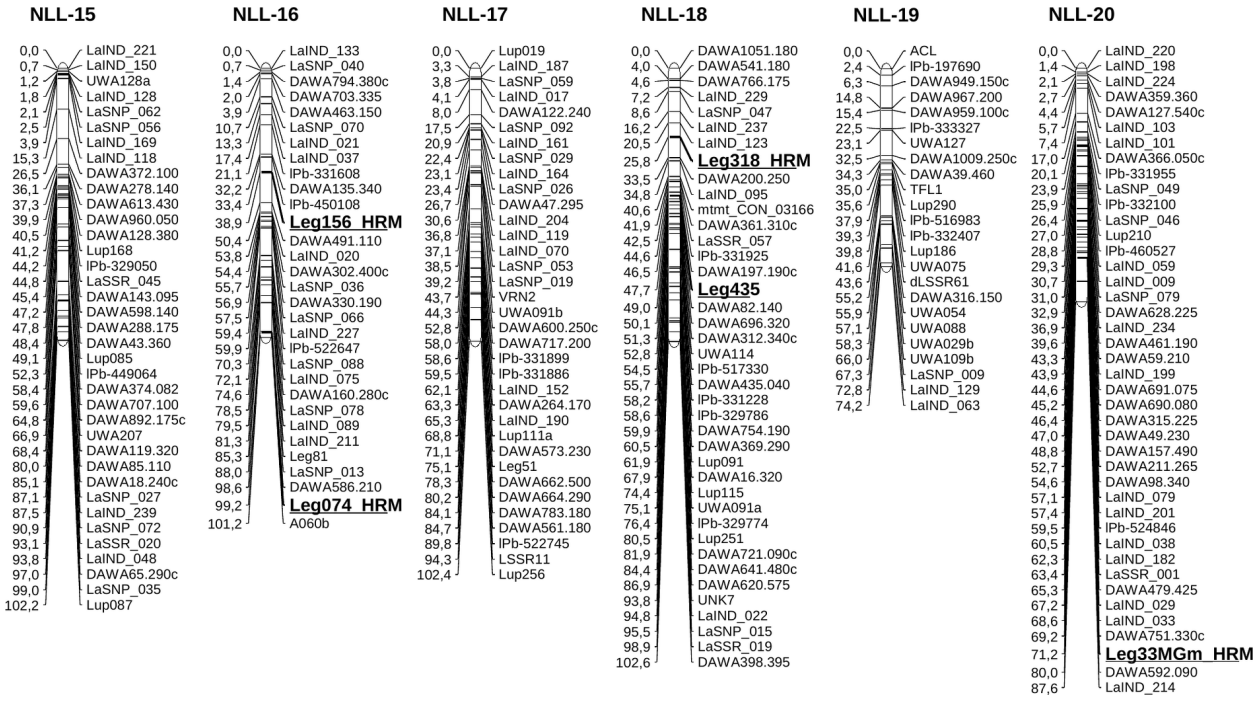

Figure 1. Continued.

pairs is available at http://cgi-www.daimi.au.dk/cgi-chili/GeneticMarkers/table and in Bertioli *et al.* (2009).

Temperature gradient PCR was used for the mapping population parental lines survey to set up the best annealing temperature for each Leg marker using Promega GoTaq® Flexi Polymerase and the protocol provided by the manufacturer. Single PCR products of both parental lines were sequenced using a BigDye Terminator™ v.3.1 Cycle Sequencing Kit (Applied Biosystems) to confirm amplicon identity and further sequence polymorphism detection.

HRM primer pair design and HRM assay. Polymorphic markers of a length over 250 bp were re-designed into smaller amplicons not exceeding 160 bp covering SNP or InDel sites. The only exception was marker Leg435 kept in its original size of 278 bp as a test of the ability of HRM method to differentiate longer fragments. The HRM primers were designed to have a predicted annealing temperature of around 56–62°C using Primer3 (Koressaar & Remm, 2007; Untergrasser *et al.*, 2012), taking care to exclude any possibility of secondary structure formation (Oligo Analyzer 3.1, https://eu.idtdna.com/analyzer/Applications/OligoAnalyzer, Integrated DNA Technologies, Inc).

All PCR-HRM analyses were performed in 96-well plates using a LightCycler 480 (Roche) and either LightCycler 480 High Resolution Melting Master (Roche) or a combination of GoTaq® Flexi Polymerase (Promega) with LightCycler® 480 ResoLight Dye (Roche). All data were recorded and analyzed using LightCycler® 480 Gene Scanning Software. During the optimization step, the quality and the specificity of the PCR products were verified by agarose gel electrophoresis and/or LightCycler® 480 T_m Calling Software.

LightCycler 480 High Resolution Melting Master. The HRM reactions were carried out in a final volume of 10 µl containing 1x LightCycler 480 High Resolution Melting Master (Roche), 0.2 µM of each primer, 1.5–3.5 mM $MgCl_2$ (Roche) and 25 ng of template DNA. After an initial denaturation step of 10 min at 95°C, 45–65 cycles were carried out with a repeated denaturation step at 95°C for 10 sec, an annealing step at 54–62°C for 15 sec and an extension step at 72°C for 10 sec. After the amplification, samples were heated to 95°C for 1 min and then cooled to 40°C for 1 min to encourage duplex formation. HRM curve data were obtained by melting over increasing temperatures from 65 to 95°C at a rate of 25 acquisitions per 1°C. This protocol applies to markers: Leg33MGm, Leg245, Leg256, Leg318, Leg325, Leg425, Leg435, Leg445, Leg713, Leg735, Leg736 and Leg871.

Combination of GoTaq® Flexi Polymerase and LightCycler® 480 ResoLight Dye. Single HRM reactions were conducted in 10 µl containing 1× GoTaq® Flexi Buffer (Promega), 0.2 mM of each dNTP (Thermo Scientific), 1.5–3.5 mM $MgCl_2$ (Roche), 1 unit of GoTaq® Flexi Polymerase (Promega), 0.2 µM of each primer, 0.5 µl LightCycler® 480 ResoLight Dye (Roche) and 25 ng of template DNA. Markers were amplified with initial denaturation at 95°C for 10 min, then 45 cycles of repeated denaturation steps at 95°C for 10 sec, annealing steps at 52–56°C for 15 sec and extension steps at 72°C extension for 30 sec, followed by a melting cycle as described above. This protocol applies to markers: Leg050, Leg055, Leg074 and Leg156.

Linkage analysis. Integration of the Leg markers into the newest reference linkage map of narrow-leafed lupin (Kamphuis *et al.*, 2014) was performed using MapManager software version QTXb20 (Manly *et al.*, 2001). In the mapping analysis only the skeleton markers from Kamphuis *et al.* (2014) were incorporated. The new markers were added to the map with a P-value≤0.001. The Kosambi mapping function was applied for conversion of the recombination rate into genetic map distance (cM). Graphic illustration of linkage groups was performed with the aid of MapChart software (Voorrips *et al.*, 2002).

RESULTS AND DISCUSSION

Sixteen Leg primer pairs were incorporated in the evaluation of HRM method effectiveness in markers genotyping for the purpose of their genetic mapping in the narrow-leafed lupin genome (Table 1). The genetic map constructed on the basis of skeleton markers from Kamphuis *et al.* (2014) was longer than originally as a result of different mapping software application (2290.1 cM *versus* 2263.9 cM and average spacing between skeleton loci of 2.92 cM *versus* 2.9 cM). Newly mapped Leg markers were distributed in ten linkage groups (Fig. 1, Table 2) In two cases map intervals between adjacent markers exceeded 10 cM, whereas all the remaining newly mapped markers were tightly linked (Table 2). The new linkage map length slightly increased and was 2362.6 cM with an average of 2.96 cM between adjacent loci. The linkage groups varied in length from 74.2 cM (NLL-19) to 173.8 cM (NLL-11) (Fig. 1).

One of the main criterion of our HRM assay was to keep the amplicon size under 160 bp. Melting of smaller amplicons results in more significant differences of melting temperature (T_m) among genotypes, which greatly simplifies sample differentiation (Gundry *et al.*, 2003; Liew *et al.*, 2004). Secondary structures were avoided at the primer pairs design level since its formation influence the reaction kinetics, efficiency and specificity. The optimization of the PCR profile mainly involved $MgCl_2$ concentration (1.5 to 3.5 mM in 0.5 mM steps), since an optimal Mg^{2+} concentration is es-

Table 2. HRM markers map position and linkage details.

Marker name	Adjacent markers name [a]	LOD[b]	Distance b/t adjacent markers (cM)[a]	Linkage group
Leg33MGm_HRM	DAWA751.330c	18.0	2.0	NLL-20
	DAWA592.090	9.6	8.8	
Leg050_HRM	DAWA196.230c	18.5	2.0	NLL-09
	Lup241	16.8	2.1	
Leg055_HRM	Leg713_HRM	5.6	15.3	NLL-02
	Lup093	15.7	3.4	
Leg074_HRM	DAWA586.210	22.3	0.6	NLL-16
	A060b	18.5	2.0	
Leg156_HRM	IPb-450108	14.9	5.5	NLL-16
	DAWA491.110	7.5	11.5	
Leg245_HRM	IPb-522772	15.6	3.9	NLL-13
	VRN1	15.0	2.9	
Leg256_HRM	LaSNP_016	9.3	9.4	NLL-06
Leg318_HRM	LaIND_123	14.1	5.3	NLL-18
	DAWA200.250	9.8	7.7	
Leg325_HRM	DAWA311.125	19.7	1.8	NLL-06
	AC123593-13	16.3	2.2	
Leg425_HRM	DAWA267.130	14.8	4.1	NLL-05
	IPb-198073	22.3	0.6	
Leg435	DAWA197.190c	20.6	1.2	NLL-18
	DAWA82.140	20.9	1.3	
Leg445_HRM	DAWA150.125	14.3	4.7	NLL-02
	UWA216c	16.1	2.8	
Leg713_HRM	LaIND_185	10.6	8.3	NLL-02
	Leg055_HRM	5.6	15.3	
Leg735_HRM	IPb-333705	21.5	1.2	NLL-12
	Lup247	8.9	9.2	
Leg736_HRM	LaSNP_033	17.3	3.1	NLL-09
	DAWA195.205	16.2	3.3	
Leg871_HRM	Lup289	21.4	0.0	NLL-04
	IPb-522413	23.2	0.6	

[a]HRM marker and preceding and succeeding marker in the linkage group. [b]linkage with preceding and succeeding marker

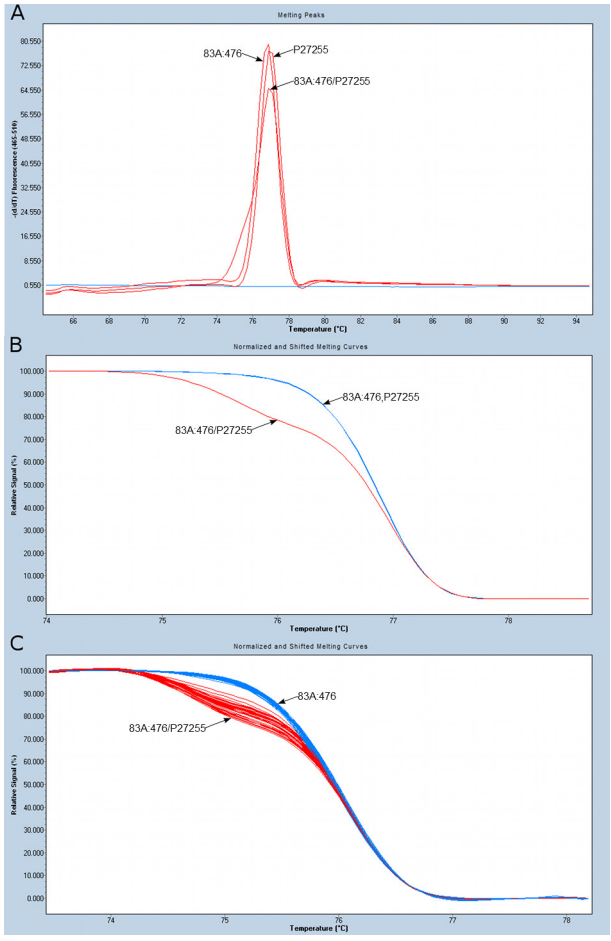

Figure 2. High resolution melting curves of Leg736 (116 bp) containing a SNP substitution (C>G).
(**A**) Verification of the PCR amplicon specificity by T_m Calling analysis. Melting curves profile of the parental genotypes (83A:476 and P 27255) and the simulated heterozygote (83A:476/P 27255). Single peaks for each analyzed genotype indicate a proper specificity of the conducted analysis. (**B**) Normalized and shifted melting curves of the parental genotypes (83A:476 and P 27255) and the simulated heterozygote (83A:476/P 27255) amplicons. Parental genotypes of Leg736 were indistinguishable due to existing C>G mutation type causing only a slight T_m difference between both amplicons. To overcome this problem melting profile generated by a mixed template of both parental DNA (a simulated heterozygote) was analyzed apart from individual parental genotypes. (**C**) Normalized and shifted melting curves of Leg736 amplicons after addition of 83A:476 parental line to each mapping population progeny sample. Figure presents the results for 88 RILs analyzed simultaneously on 96-well plate together with the mapping population parental lines as standards). Arrows represent: mapping population parental line 83A:476 and RILs with same genotype as well as simulated heterozygote 83A:476/P27255 and corresponding RILs. Application of this approach resulted in successful mapping of Leg736 marker in the NLL-09 linkage group.

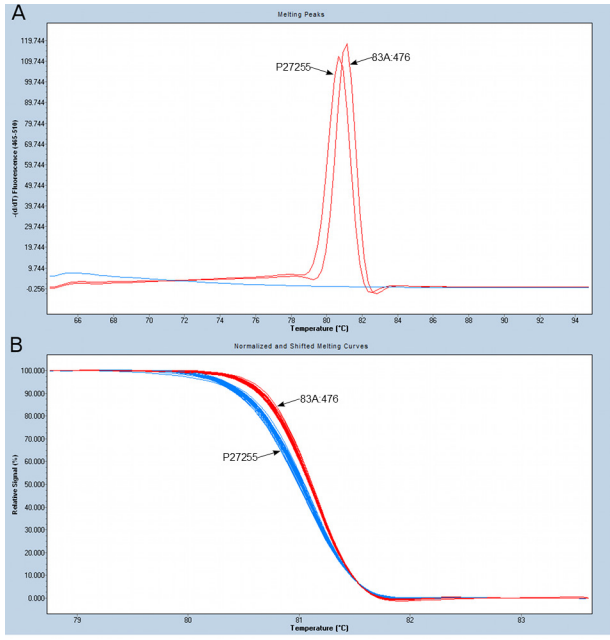

Figure 3. HRM assay of Leg435 in narrow-leafed lupin mapping population.
(**A**) Verification of the PCR amplicon specificity by T_m Calling analysis. Arrows represent the mapping population parental lines 83A:476 and P 27255. Single peaks of the analyzed genotypes indicate a proper specificity of the conducted analysis. (**B**) Normalized and shifted melting curves of the 278-bp Leg435 amplicons. Parental genotypes contain five SNP substitution (C>T; G>A; G>A; G>A, A>C). Figure presents the results for 88 RILs analyzed simultaneously on 96-well plate together with the mapping population parental lines as standards). Arrows represent genotypes of the mapping population parental lines 83A:476, P 27255 and also their corresponding RILs. All of the RILs analyzed have been assigned to one of the parental genotypes and Leg435 marker was successfully mapped in the NLL-18 linkage group.

sential to ensure the best specificity and yield of the PCR products (Montgomery *et al.*, 2007). The annealing temperature for the primer pairs tested mostly oscillated around 56°C.

The analysis of the actual HRM data was always preceded by the examination of amplification fluorescence data. The crossing point (C_p) which corresponds to the cycle number at which the fluorescence signal of the PCR product rises above the background should remain below 30 cycles for each reaction. This indicates an adequate amount of template DNA and a suitable amplification efficiency (LightCycler® 480 Real-Time PCR System – Technical Note No. 1 High Resolution Melting: Optimization Strategies). In the case of four analyzed markers (Leg050, Leg055, Leg074, Leg156) the amplification carried out with the aid of LightCycler® 480 High Resolution Melting Master (Roche) was not effective enough and required more than 30 cycles to achieve C_p and even more than 60 cycles to achieve the plateau phase. Application of a previously optimized GoTaq® Flexi Polymerase (Promega) and LightCycler® 480 ResoLight Dye (Roche) combination instead of the HRM commercial reagent kit allowed the required C_p and amplification efficiency to be reached.

High resolution melting of the PCR products can detect most homozygous mutations, however, some homozygous SNP have melting curves identical to those of the wild-type. This might be caused by overlong amplicons that influence the reaction sensitivity. Moreover, A>T and C>G mutation types result in only a slight T_m difference between amplicons (usually less than 0.4°C), which makes their detection more difficult (Liew *et al.*, 2004). In this case, addition of DNA of a known genotype to each unknown sample before PCR, results in heteroduplex formation which enables differentiation of homozygous and wild-type genotypes (Palais *et al.*, 2005). This approach was successfully utilized by Croxford *et al.* (2008) to detect SNP markers in *Lupinus albus*. In our studies, the parental genotypes of six markers (Leg-33MGm, Leg074, Leg156, Leg713, Leg736 and Leg871) were difficult to distinguish, therefore, the comparison of melting profiles generated by each parent separately and

also by a mixed template containing DNA from both parental genotypes (a simulated heterozygote 1:1 mixed sample) was applied. On the basis of this approach, the distinction of both genotypes for these markers was feasible (Fig. 2).

Amplicon size has a significant impact on HRM method resolution. Han *et al.* (2012) reported successful employment of the HRM assay in SNP genotyping of 51–149 bp amplicons in alfalfa; Wu *et al.* (2008) analyzed 68–198 bp amplicons in almond; while De Koeyer *et al.* (2010) were able to detect SNP with amplicon sizes between 50–230 bp in potato. As mentioned above, the size of the HRM markers designed in our studies was kept under 160 bp, but one marker (Leg435) was used with its original, longer sequence size of 278 bp to test the possibility of SNP detection in longer amplicons (Fig. 3). According to Reed & Wittwer (2004), the melting of amplicons longer than 300 bp induces a depletion of sensitivity and specificity in single SNP detection. On the other hand, the increased number of polymorphic sites within the amplicon results in an improvement of HRM analysis resolution, thus enabling larger PCR product utilization (Hofinger *et al.*, 2009). Most of the re-designed HRM markers in our studies covered one polymorphic site, but in the case of three markers, two (Leg074), three (Leg33MGm) or even five (Leg435) SNP and/or InDel were incorporated in the amplicon sequence. The increased number of polymorphic sites of marker Leg435 is probably the explanation of its successful HRM analysis. Knopkiewicz *et al.* (2014) also used the HRM method to successfully analyze 400 bp and 600 bp amplicons covering both three and seven SNPs, respectively.

The HRM technique offers a very sensitive and rapid method for SNP genotyping that after optimization does not require DNA restriction or electrophoresis to detect sequence polymorphisms. In our studies, melting curve analyses using LightCycler® 480 T_m Calling Software (Roche) proved to be completely sufficient and in some cases even more sensitive than standard agarose electrophoresis. Thus, in comparison with other popular genotyping methods, i. e. CAPS/dCAPS, the HRM technique is less time-consuming and labor-intensive. When the most appropriate chemistry set is selected, the important advantage of the HRM method is its flexibility in new markers examination. New primer set is the only requirement for each newly analyzed marker, while the chemistry remains unchanged in each analysis. No additional reagents are needed to detect any existing polymorphic site. HRM assay is therefore more profitable than other methods, i.e. CAPS/dCAPS requiring specific restriction endonucleases. The main obstacle in the HRM technique application is the requirement of a specialized and expensive equipment to conduct analyses. Fortunately, as qPCR thermocyclers adapted to HRM assay are becoming more and more popular, their cost gradually decreases.

The new Leg markers with its associated DNA sequence could potentially be used in the synteny analyses between the genome of *Lupinus angustifolius* and model legumes. Synteny between *Lupinus angustifolius* and *Medicago truncatula* as well as *Lotus japonicus* were previously undertaken, showing examples of marker colinearity between their genomes (Nelson *et al.*, 2010; Nelson *et al.*, 2006; Kroc *et al.*, 2013). The current reference map of Kamphuis *et al.* (2014) was not involved in any synteny analysis and as it incorporated a significant number of new markers, their positions/order have changed compared to the previous map versions. It is therefore difficult to assess if the newly mapped Leg markers are involved and extend any previously reported synteny blocks.

In the course of our studies the optimized HRM markers were analyzed in the mapping population of narrow-leafed lupin. As a result, all of the 16 analyzed markers were successfully mapped in lupin genome. The incorporation of new Leg markers into the narrow-leafed lupin genetic map opens the possibility of synteny analyses with other legume species. Furthermore, the different approaches we applied in the HRM marker optimization process, might serve as a good starting point in overcoming difficulties when implementing HRM assays in other species. We conclude that the HRM assay proved to be an effective method for genotyping of STS markers in narrow-leafed lupin despite optimization problems encountered. It is therefore a good alternative to other popular genotyping methods.

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