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Short communication

Development and characterization of InDel markers *for Lupinus luteus* L. (Fabaceae) and cross-species amplification in other Lupin species

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ABSTRACT

Background: Strong artificial selection and/or natural bottle necks may limit genetic variation in domesticated species. *Lupinus luteus*, an orphan temperate crop, has suffered diversity reductions during its bitter/sweet alkaloid domestication history, limiting breeding efforts and making molecular marker development a difficult task. The main goal of this research was to generate new polymorphic insertion–deletion (InDel) markers to aid yellow lupin genetics and breeding. By combining genomic reduction libraries and next generation sequencing, several polymorphic InDel markers were developed for *L. luteus* L.
Results: A total of 118 InDel in silico polymorphic markers were identified. Eighteen InDel primer sets were evaluated in a diverse *L. luteus* core collection, where amplified between 2–3 alleles per locus. Observed heterozygosity (HO; 0.0648 to 0.5564) and polymorphic information content (PIC; 0.06 to 0.48) estimations revealed a moderate level of genetic variation across *L. luteus* accessions. In addition, ten and nine InDel loci amplified successfully *Lupinus hispanicus* Boiss & Reut, and *Lupinus mutabilis* Sweet, respectively, two *L. luteus* close relatives. PCA analysis identified two *L. luteus* clusters, most likely explained by the domestication species history.
Conclusion: The development of InDel markers will facilitate the study of genetic diversity across *L. luteus* populations, as well as among closely related species.

1. Introduction

The genus *Lupinus* comprises more than 200 annual and perennial herbaceous species growing in a wide range of climatic and soil conditions [1]. Lupins have been described as functional food, given the association between their consumption and reduced risk of hypercholesterolaemia, diabetes, and hypertension [2]. *Lupinus luteus*, an old world cultivated lupin, shows higher protein seed content [3] and twice the amount of seed cysteine and methionine than most lupin species [4]. In addition, evaluations of its functional and physicochemical properties have suggested yellow lupin proteins could improve texture and nutritional quality when incorporated in food products [5].

Although some molecular tools have been developed to aid yellow lupin's genetics [6,7,8], an apparent low level of microsatellite polymorphisms [6,7] have suggested the need of diversifying and increasing marker availability for this species. During lupin domestication, a reduced number of naturally occurring mutants were used as progenitors to develop low alkaloid/no pod shattering varieties. This strategy, although successful, reduced the amount of genetic variation contained within lupin breeding populations [9,10]. Reduction of diversity not only limits the generation of better adapted varieties, but also the presence of polymorphic sites in modern breeding lines [11]. Insertions and deletions (InDels) are the second most common type of polymorphisms across species [12], and are distributed throughout the entire genome [12,13]. InDels may result from mechanisms such as transposable elements, slippage in simple sequence replication, and unequal crossover [14]. Due to their high-density occurrence, cost-effectiveness, and ease genotyping, InDels have been increasingly recognized as an important source of molecular markers [12]. InDel markers have been a valuable complement to SNPs and SSRs in *Phaseolus vulgaris* L. and *Glycine max* (L) Merr. [15,16], and haplotype differences in presence/absence variation may explain heterosis and the extraordinary phenotypic diversity in maize [17]. In this study, we present a novel set of 18 validated polymorphic *L. luteus* InDel markers generated by combining

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genomic reduction libraries and next generation sequencing. We also evaluated their ability to cross amplify *Lupinus hispanicus* and *Lupinus mutabilis*, two close relative lupine species.

2. Materials and methods

DNA from two *L. luteus* accessions, Core 18 and Core 227 (**Table S1**), was extracted from young leaves using CTAB buffer [18]. DNAs were further purified and quantified using DNeasy mini spin columns (Qiagen) and a Qubit® 2.0 Fluorometer (Life Technologies), respectively. Genomic reduction was accomplished using a previously described protocol [19]. Briefly, 450 ng of total genomic DNA, of each DNA sample, was separately double-digested using 3 U of the restriction enzymes *Eco*RI and BfaI (New England Biolabs, Beverly, MA). DNA fragments were then ligated with 5′-TEG biotinylated/3′-phosphorylated *Eco*RI adapters and 3′-phosphorylated BfaI adapters [20]. Small DNA fragments were excluded from the samples using Chroma Spin-400 columns (ClonTech, Mountain View, CA). DNA fragments containing the biotin labeled *Eco*RI adapters were isolated using M-280 streptavidin beads (Invitrogen, Carlsbad, CA) and resuspended in 100 μL of TE [20]. A sample specific PCR amplifications was conducted using 1 μL of streptavidin-cleaned DNA fragments and primers containing complementary EcoRI and BfaI adaptor + restriction DNA and unique 5′ barcode sequences [20]. Amplifications were carried out in 50 μL PCR reactions using 1X Advantage HF 2 PCR Master Mix (ClonTech, Mountain View, CA) and 0.2 μM of each primer. Thermocycling profiles and amplified DNA visualization were conducted following standard conditions [19,20]. DNA concentrations for each PCR reaction were measured fluorometrically using a Quant-iT picogreen dye (Invitrogen, Carlsbad, CA) and pooled in equimolar amounts. DNA from a pooled PCR samples was separated electrophoretically in a 1.5% Metaphor agarose gel (Cambrex BioScience, East Rutherford, NJ), and visualized using ethidium bromide staining. A single 500–650 bp gel slice was removed and DNA fragments extracted using a Qiaquick column (Qiagen, Germantown, MD). A single micro-bead sequencing run was conducted using a Roche-454 GS FLX and Titanium reagents (Branford, CT) at the Brigham Young University DNASC (Provo, UT). DNA reads were trimmed and separated into MID barcode pools representing the two *L. luteus* genotypes using the process-tagged sequences function in CLCBio Workbench v. 4.0 (Katrinebjerg). InDels were identified by combining both *L. luteus* sequencing pools into a single de novo assemblage. Contigs were built using the Roche Newbler assembler v. 2.3, with a minimum overlap length and identity of 50 bp and 95%, respectively. The minimum contig length was ≥200 bp. Custom perl scripts were used to identify putative InDels within contigs when the coverage depth at the InDel was ≥10 and the minor allele frequencies were at least 20% of the reads. InDels explaining 1-bp difference and those located within homopolymer repeats were discarded. Flanking primer pairs were designed for InDel containing contigs using Primer3 implemented in Geneious® 6.1.8 [21] with expected amplicon lengths between 150–500 bp and an optimal annealing temperature (Ta) of 60°C. Oligonucleotides were synthesized by IDT (Integrated DNA Technologies, Inc.).

Eighteen primer pairs flanking InDels of at least 3 bp (Table 1) were randomly selected to genotype 164 *L. luteus* accessions (**Table S1**) belonging to a seed core collection previously reported [6,7]. The *L. luteus* accessions were from several origins (Poland, Russia,

Table 1
Characteristics and genetic properties of 18 newly developed InDel markers for *Lupinus luteus* L.

Primer	Sequence	Allele size (bp)	Ta(C)	A	PIC	Ho	GenBank
GR1_INDEL02120	F: TCTGGAGGAAATAAAAAACTGTAGG R: GCAACGATAATATCCATAACCGTC	159–162-163	60	3	0.22	0.2343	KX778774 KX778775 KX778776
GR1_INDEL15589	F: GTGTACTAAACTCAAGCCAATWTATGC R: GATAACAAAATTTGTATGGCATTGAC	140–144	58	2	0.27	0.3287	KX778777 KX778778
GR1_INDEL21885	F: GATTGTCGTGGATCAGAAGC R: ATAAACCAATGAATAAATGTTGAAC	181–192	57	2	0.09	0.0933	KX778779 KX778780
GR2_INDEL06804	F: TCCAGACAGAATTTTTGTAACTTCAAAGCA R: CCACGAAGGAAGCCACTTGAATCC	320–323	60	2	0.06	0.0648	KX778785 KX778786
GR2_INDEL08379	F: TGGCATACCTGAAATTATTATCAAGCTTTT R: TGGCCTGACCGAGGCTTGGC	524–537	60	2	0.08	0.0873	KX778789 KX778790
GR2_INDEL10199	F: TGGCTGGTTTGAAAGTCTATTTAAAGGCAA R CACCTTGAGACTTCCTTGTTCCTTACTTAC	428–437	60	2	0.48	0.5564	KX778793 KX778794
GR2_INDEL10592	F: TGGGAGCACATTTACGTTTCCA R ACTGTTTTATTCATAGTTGCTTAGAAAGAC	414–421	60	2	0.05	0.0534	KX778795 KX778796
GR2_INDEL11357	F: GGACAGAGTTATTTGGGTGGATGGGGA R: TGGCATCAAATGGAAGACCATATAGCCCC	250–262	60	2	0.18	0.2002	KX778797 KX778798
GR2_INDEL13347	F:CATGTCCGAGCCGGGAACATCCA R:CGTAAAGGACAAGAGGAAGTTTCCTACTGA	206–212-222	60	3	0.20	0.2215	KX778801 KX778802 KX778803
GR2_INDEL14402	F: TCTCATTCTTTGACCAATAAACCAAGACAC R:TGGAGTTATCAACAACAAGAATAGACACTC	317–333	60	2	0.35	0.4458	KX778804 KX778805
GR2_INDEL14515	F:CGTCGAGCCATAAAGCAAACAAGTGA R: TGTCTCATCGGAATTGGACAAGGTATTAAA	277–283	60	2	0.26	0.3141	KX778806 KX778807
GR2_INDEL15354	F:GCTTCACTTTGACGTCGCCAGGG R:CCTTGAAGTCGTGGTAAACATTCAAGGAGA	206–217	60	2	0.35	0.4589	KX778810 KX778811
GR2_INDEL03758	F:GCCCCACTGGATCCGAGAAAGACC R:TCCAAGTTGGCTAAAGCCATTGTATCCTTC	297–306	60	2	0.37	0.4854	KX778783 KX778784
GR2_INDEL07358	F:CCCAACTGCTTTTAACTGATCTTGGCGGG R: TCGGCTCTCCACATTGCAGCCA	596–612	60	2	0.37	0.4969	KX778787 KX778788
GR2_INDEL01779	F: CCACCCAAGACAGATCCATCATACA R:TGCATCACATGTGCAGCTTGGCT	286–309	60	2	0.26	0.3104	KX778781 KX778782
GR2_INDEL09538	F: GGCAGGCCACACAAACAGGAGG R:AAGAGGATAGAAGTGTCATTACAAGTTGTC	261–276	60	2	0.18	0.2002	KX778791 KX778792
GR2_INDEL12780	F: GTCAGACATACTCCAATGAGTTCAGGT R:TCTTCTCATTTAATCACATACACCATTTTG	301–289	60	2	0.34	0.4308	KX778799 KX778800
GR2_INDEL15167	F: TCACATCGCTTACCTCATTGTTCCGGG R:CCGTACTGGACGGTCTGAGCAGTCT	292–288	60	2	0.32	0.3866	KX778808 KX778809

Note: *Ta* = annealing temperature; A = number of alleles sampled; Ho = observed heterozygosity; PIC = polymorphic information content.

Germany, Denmark, Ukraine, Hungary, Netherlands, Belarus, Morocco, Portugal, Israel, Argelia, ex Federal Republic of Yugoslavia, Australia, Spain, and Chile), and included wild accessions, old local varieties, and breeding lines.

Marker cross amplification was determined by including six accessions each of *L. hispanicus* and *L. mutabilis* (**Table S1**). DNA extraction and quantification was conducted as mentioned above. Amplification of InDel containing regions was carried out in 20 μL PCR reactions containing 100 ng of genomic DNA, 0.2 mM dNTPs, 2 mM MgCl2, 1X PCR buffer, 2.5% DMSO, 1 U of GoTaq® DNA Polymerase (Promega) and 5 pmol of each reverse and forward SSR primer. Thermal cycling consisted of 95°C for 4 min and 35 cycles of 1 min at 95°C, 1 min at 57–60°C, 1 min at 72°C, and a final step of 72°C for 5 min. PCR products were separated on 6% denaturing polyacrylamide gels, run in TBE buffer at 60 W for 3–4 h and visualized using silver stain procedures [6]. PCR fragments with different sizes were scored as different alleles and following a codominant fashion. Standard population genetics metrics were calculated using PopGene version 1.32 and Molkin 3.0v [22]. A principal component analysis (PCA) was conducted on the correlation matrix using the REML estimation method implemented on JMP® Genomics 6.1 (SAS Institute Inc., Cary, NC) and computed from the InDel data matrix.

3. Results and discussion

Advances in crop genomics have allowed the generation of high density markers, not only facilitating genome marker saturation, but also significantly increasing map-based cloning and marker-assisted selection efficiencies [12]. This is particularly critical for minor crops like yellow lupin, where its orphan condition has limited the availability of genomic tools to aid genetic and breeding research efforts [7]. Although SNP markers have been used in most genetic studies, research on genome structural variation have pointed out that SNPs may not fully capture the genomic variation responsible of phenotypic differences across varieties of the same species [17]. By combining genomic reduction libraries and next generation sequencing, we identified a total of 118 in silico polymorphic InDel markers between two *L. luteus* accessions (**Table S2**). Eighteen of these InDels were used to genotype a diverse *L. luteus* Core Collection (Table 1).

All 18 InDel loci were polymorphic, with allele numbers ranging from 2 to 3 per locus (Table 1). Similar results were observed in maize, where whole genome searches found an average of 2.76 alleles per locus, but with a wider range of alleles, ranging from 2 to 107 for each InDel locus [12]. All *L. luteus* InDel loci showed allelic frequencies significantly deviated from expected Hardy–Weinberg proportions ($P < 0.001$). Observed heterozygosities (Ho) and polymorphic information contents (PIC) ranged from 0.0648 to 0.5564 (average 0.298), and 0.06 to 0.48 (average 0.247), respectively, indicating a moderate to low level of genetic variation. These values, although higher than those obtained for *L. luteus* microsatellite data [6], still suggest a trend of limited genetic variation for yellow lupin. In contrast, PIC values for InDel loci in maize, estimated using 345 genomes, ranged from 0.50 to 0.80, with an average PIC of 0.55 [12]. This apparent low level of genetic variation has also been observed in other lupin species, such as *Lupinus angustifolius* [23] and *Lupinus polyphyllus* [24].

Results from PCA analysis showed that first and second components accounted for 23.7%, 11.3% of the total variation detected among individuals, respectively (Fig. 1). One main cluster contained ~80% of *L. luteus* accessions, in accordance with Ho and PIC low to moderate levels of variation. The rest of the accessions were scattered into a more disperse and low denser cluster with no clear country of origin pattern (Fig. 1; Table S1). The lack of a clear pattern following geographical accession origins (country) could be explained by several reasons. For instance, the number of accessions may not have been large enough to allow a clear pattern to arise, and/or the wide geographic distribution across the Mediterranean region, mainly due to human introductions, could have homogenized any natural genetic distinctiveness of this species [25]. Nevertheless, most wild accessions, such as Core 98, 102, 104, and 226 were included in the dispersed cluster, suggesting that the main division observed across *L. luteus* germplasm could be consequence of the lupin domestication history.

The 18 InDel loci were also tested for cross amplification in two other *Lupinus* species, *L. hispanicus* and *L. mutabilis*. Both lupins showed a similar amplification success of ~50% (Table 2), although the sister *L. hispanicus*/*L. luteus* phylogenetic relationship [1] would have predicted higher numbers for *L. hispanicus*.

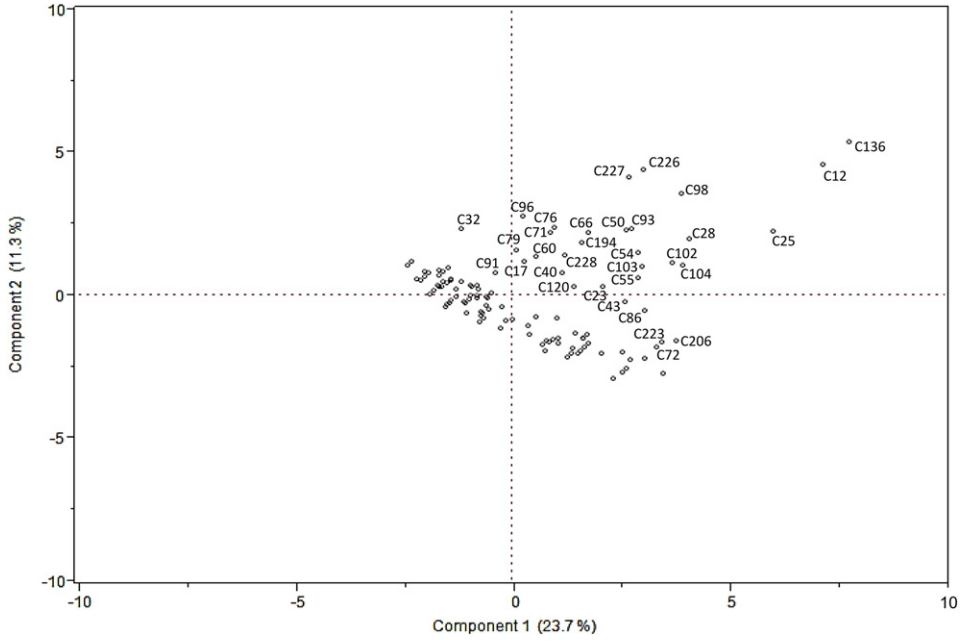

Fig. 1. Genetic relationships among 164 *L. luteus* individuals, of several origins, based on the first two principal components (PC1, PC2) derived from a multivariate analysis (PCA) of 18 InDel marker data. Accession identification was provided for *L. luteus* genotypes not belonging to the main observed cluster. C = Core (**Table S1**).

Table 2
Cross-species amplification of 18 InDel loci in two closely related species of *L. luteus* L.

Primer	Cross-amplification
GR1_INDEL02120	–
GR1_INDEL15589	–
GR1_INDEL21885	–
GR2_INDEL06804	*L. hispanicus; L. mutabilis*
GR2_INDEL08379	*L. mutabilis*
GR2_INDEL10199	–
GR2_INDEL10592	–
GR2_INDEL11357	–
GR2_INDEL13347	*L. hispanicus; L. mutabilis*
GR2_INDEL14402	*L. hispanicus; L. mutabilis*
GR2_INDEL14515	*L. hispanicus; L. mutabilis*
GR2_INDEL15354	*L. hispanicus; L. mutabilis*
GR2_INDEL03758	*L. hispanicus*
GR2_INDEL07358	*L. hispanicus; L. mutabilis*
GR2_INDEL01779	*L. hispanicus*
GR2_INDEL09538	–
GR2_INDEL12780	*L. hispanicus; L. mutabilis*
GR2_INDEL15167	*L. hispanicus; L. mutabilis*

By combining the use of genomic reduction libraries and next-generation sequencing, we were able to develop a set of polymorphic InDel markers, which could prove useful in population genetic and breeding studies. In addition, their success to cross-amplify old and new world lupins, would most likely increase their usefulness as molecular tools across the *Lupinus* genus.

Conflict of interest

The authors declare that they have no competing interests.

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Supplementary material

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