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Genome-wide development and utilization of novel intron-length polymorphic (ILP) markers in *Medicago sativa*

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Abstract Alfalfa (*Medicago sativa* L.) is the most widely cultivated forage legume around the world. Though development and application of microsatellite markers in large-scale was reported in this species, a systematic investigation and large-scale exploitation of intron-length polymorphic (ILP) markers has not been conducted. In the present study, the RNA-Seq sequences of alfalfa were aligned with the genomic sequences of *Arabidopsis* to predict the position of introns and develop ILP markers in alfalfa. A total of 693 putative ILPs were identified, and 502 ILP markers were successfully developed. Furthermore, 100 ILP markers exhibited relatively high levels of transferability to leguminous (40.0%–83.0%) and non-leguminous (21.0%–22.0%) species. Polymorphisms in 40 randomly selected MsILP markers were evaluated in 21 alfalfa accessions and collectively yielded 169 alleles with an average of 4.7 alleles per locus. The polymorphism information content (PIC) ranged from 0.15 to 0.87 with an average of 0.60, which indicated a high level of polymorphism in the MsILP markers. For the first time, we developed large-scale ILP markers in alfalfa and demonstrated their utility in transferability, which will be valuable for genetic relationship assessments, comparative genomic studies and marker-assisted breeding of leguminous and non-leguminous species.

Keywords Intron length polymorphism · *Medicago sativa* · Molecular markers · Transferability

Introduction

Alfalfa (*Medicago sativa* L.) is the most widely cultivated forage legume around the world with a global production area of ~30 million ha (Mouttet et al. 2014). Alfalfa not only has huge value as a livestock feed but also plays important roles in reducing erosion and nutrient loss, enhancing soil carbon sequestration and increasing nitrogen fertility. Therefore, alfalfa has been an important component of sustainable agricultural systems for many years (Dien et al. 2011; Gonzalez-Garcia et al. 2010). Besides its applications in agriculture and animal husbandry, alfalfa also has the potential for monoclonal antibody and vaccine production in molecular farming (Zivkovic et al. 2012). In addition, alfalfa's high-fiber content biomass can be used for paper and biofuel production, which makes alfalfa a potentially unique bioenergy crop (Zhou and Runge 2015; Zhou et al. 2015). Traditionally, most of alfalfa cultivars were developed by phenotypic selection,

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which is time consuming and labor-intensive. The molecular breeding approaches based on the efficient and robust molecular markers could enhance efficiency of cultivar development in terms of gain unit cost and time.

Genetic diversity is the basis for breeding programs. Understanding the germplasm diversity and relationships among elite breeding materials is a pre-requisite for the selection of superior parental combinations and increase of heterosis in crop breeding (Chen et al. 2015). Molecular markers are vital tools in both basic and applied genetic research (e.g., the construction of genetic maps and the mapping of genes or quantitative trait loci) and breeding (e.g., marker-assisted selection and genomic selection) and have been developed based mainly on the variations or polymorphisms in DNA sequences (Arruda et al. 2016). Since the 1980s, a number of different molecular markers have been developed, including restriction fragment-length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPD), amplified fragment-length polymorphisms (AFLPs), simple sequence repeat polymorphisms (SSRs) and single-nucleotide polymorphisms (SNPs) (Grover and Sharma, 2016). Introns are non-coding sequences that are widespread and abundant in eukaryotic genomes. Compared to coding sequences (CDS), introns not only actively participate in the control of gene expression but also have more polymorphisms that can be exploited as genetic markers. Intron-length polymorphic (ILP) markers have a number of advantages over the other previously reported DNA markers, such as being gene-specific, co-dominant, hypervariable, neutral, convenient, and reliable and exhibiting high transferability rates between plant species (Yang et al. 2007). Additionally, although the intron itself has no direct impact on gene functions and makes ILP as neutral markers (no phenotypic effect), the expression profiling of functional genes could be regulated by introns, which makes ILP markers reflect the allelic diversity within the genes directly and useful for molecular mapping of the expression QTLs and differentially expressed functional genes associated with various agronomic traits as well as delineating the trait-associated molecular tags rapidly for marker-assisted selection (Badoni et al. 2016; Sharma et al. 2015). All of these characteristics make ILP markers a good choice for the construction of molecular marker maps and investigations of the phylogenetic relationships of related plant species, particularly those that lack or with limited available genomic data, such as alfalfa.

However, studies of the development and use of ILP markers are currently restricted to only a few species or genes, including the soybean (Shu et al. 2010), rice (Wang et al. 2005), tomato (Wang et al. 2010), foxtail millet (Gupta et al. 2011), cowpea (Gupta et al. 2012) and *Curcuma* (Kita et al. 2016). The related research is lagging behind in alfalfa because less genomic data are available. Recently, advances in high-throughput sequencing methods and bioinformatics analyses have provided the opportunity to collect large numbers of nucleotide sequencing reads at a low cost and have thus made development of ILP markers easier and more cost-effective (Liu et al. 2016; O'Rourke et al. 2015). Hence, in the work described here, we performed a large-scale search and exploitation of candidate ILP markers based on the available RNA-Seq sequences for alfalfa, and the potential for cross-species transferability and genetic diversity assessments of these markers in alfalfa accessions were analyzed.

Materials and methods

Plant material and DNA extraction

The leguminous species barrel medic (*Medicago truncatula* A17), alfalfa (*M. sativa* cultivar ARC), chickpea (common vetch cultivar Lanjian 3), soybean (*Glycine max* cultivar Dongdou 641), crowtoe (*Lotus corniculatus* cv. Mirabal), yellow sweet clover (wild material), and *Sophora alopecuroides* (wild material) and the non-leguminous species *Arabidopsis thaliana* 'Columbia', rice (*Oryza sativa* cv. Kitaake), and tobacco (*Nicotiana tabacum* cv. Samsun NN) were used to examine the transferability of the ILP markers developed in this study. Genomic DNA was extracted from the leaf materials of field plants using a CTAB protocol as described previously (Liu et al. 2015). A total of 21 alfalfa accessions (Suppl. Table 1) were collected from the United States Department of Agriculture National Plant Germplasm System (NPGS) and the National Animal Husbandry & Veterinary Service of the Ministry of Agriculture (MOA), State Grass Germplasm Resources for genetic diversity analyses. The young leaves of 40 individual plants from each accession were bulked as single samples and used for genomic DNA isolation as described above. The quality of DNA was checked on 2% agarose gel, and the quantity was determined at 260 nm using a NanoDrop

ND1000 (Thermo Fisher Scientific, Wilmington, DE). The DNA was normalized to 50 ng/μl for further use.

Sequence retrieval and primer design

A total of 112,626 alfalfa unigene sequences were retrieved from the *M. sativa* gene index 1.2 http://plantgrn.noble.org/AGED/ (AGED) (O'Rourke et al. 2015) and used for the development of the specific intron-based markers. The genome sequences of *Arabidopsis* were downloaded from https://www.arabidopsis.org/index.jsp (TAIR 10) and used as references. The intron positions were determined by identifying the gaps in the alfalfa unigene sequences according to the method described by Yang et al. (2007) with Perl scripts, and the primers flanking both sides of the intron regions were then designed using DNAMAN software.

Primer screening and ILP marker analysis

The polymerase chain reaction PCR reactions were performed in a 10 μl volume containing 5 μl 2 × Power *Pfu* PCR Master Mix (Bioteke Corporation, Beijing, China), 50 ng template DNA, and 10 ng each of the forward and reverse primers. The touchdown PCR procedure was performed as follows: five cycles of denaturation 95 °C for 30 s; the annealing temperature was then decreased by 1 °C for each cycle from 62 to 58 °C to 57–53 °C; 72 °C for 30 s; 30 cycles of 95 °C for 30 s, 58–55 °C for 30 s, and 72 °C for 30 s; and a final extension at 72 °C for 7 min. The PCR products were separated by 6% denaturing PAGE (400 V, 2 h) and visualized by silver staining.

Statistical analysis

The ILP marker profiles were scored in a binary format as present (1) or absent (0) and used for the determinations of the genetic relations. The PIC of each *M. sativa* ILP marker was calculated by applying the formula of Anderson et al. (Anderson et al. 1993), i.e., PIC $=1-\sum(P_{ij})^2$, where P_{ij} is the frequency of the jth allele for the ith locus. The genetic similarity analyses were performed using NTSYS-pc version 2.10e (Rohlf and NTSYS-pc NT, 2000). The data were entered into a binary matrix as discrete variables, and the pair-wise similarities were obtained using Jaccard's coefficients. The matrices of the similarity coefficients were subjected to the unweighted pair group method with arithmetic averaging (UPGMA) to estimate the genetic relatedness among the genotypes and generate the dendrogram.

Results and discussion

Intron identification and development of the ILP markers

In the present study, a total of 112,626 alfalfa unigene sequences that represented 19% of the predicted 800 Mbp *M. sativa* genome (O'Rourke et al. 2015) were aligned with the genomic sequences of *Arabidopsis*. The average sequence size of these alfalfa unigenes was 1352 bp, and the largest spanned 15,768 bp. The alfalfa unigene sequences were considered to be homologous to the *Arabidopsis* CDS only when at least 200 bp overlapped with 80% similarity. Two thousand and seventy alfalfa unigene sequences exhibited significant hits with the *Arabidopsis* genome, and 446 unigene sequences had one or more intron insertion sites. The total number of introns present in *Arabidopsis* was 693, with an average of 1.55 introns per unigene sequence. The maximum number of introns present in a single unigene sequence was 14 in the case of *Arabidopsis*. A total of 502 ILP markers (0.4%) were generated from 446 unigene sequences with an average frequency of ~32.95 ILP markers per megabase of genomic sequence (Suppl. Table 2). The remaining unigene sequences either exhibited no BLAST hits, or the sequences were inappropriate for designing primers. In the present study, the ILP marker identification rate (0.4%) in the alfalfa was quite comparable to that reported for cowpea (0.9%) (Gupta et al. 2012) but lower than those reported for foxtail millet (20.6%) and rice (41.4%) (Muthamilarasan et al. 2014; Wang et al. 2005). Previous studies have demonstrated that although larger numbers of intron positions are highly conserved in orthologous genes across species, family and even kingdom boundaries, the numbers and densities of introns may vary dramatically between organisms due to the different rates of lineage-specific intron loss and/or gain (Wang et al. 2014). Furthermore, differences in the query sequence type, search criteria, database size and tools used for ILP marker development may also have contributed to these discrepancies.

Physical mapping in *M. truncatula* and functional annotation of ILP

To locate the relatively physical positions of ILP markers on *M. truncatula* chromosomes, the 446 ILP targeted alfalfa unigene sequences were compared to the *M. truncatula* genome sequence using BLASTN search under stringent conditions with thresholds of 95% identity and 90% coverage, and the physical map was drawn with MapChart 2.0 (Voorrips, 2002). The results showed that 501 of 502 ILP markers physically located on the eight chromosomes of *M. truncatula* with an average marker density of 1.3 markers/Mb (Fig. 1). The average marker density was greatest in chromosome 1 (2.1/Mb), followed by chromosome 2 (1.5/Mb), and a minimum was observed in chromosome 6 (0.6/Mb). An extensive analysis of the chromosome-wide distribution and frequencies of these physically mapped ILP markers revealed a high frequency of markers mapped to chromosome 1 (113 markers, 22.6%) and a minimum frequency mapped to chromosome 6 (20 markers, 4.0%; Suppl. Table 3).

To evaluate the potential functions of the ILP-targeted unigenes, the corresponding gene ontology (GO) term annotations of the 446 unigenes were retrieved from the *M. sativa* gene index 1.2 and functionally classified using WEGO software (Ye et al. 2006). Suppl. Fig. 1 completely summarizes the categorization of these unigenes according to biological process and molecular function. Of the 446 unigenes, 385 were annotated and classified into 27 categories. Many unigenes were found in several categories. In the biological process category, the two most over-represented GO terms were cellular process (359 genes, 93.2%) and metabolic process (336 genes, 87.3%), followed by response to stimulus (246 genes, 63.9%) and biological regulation (166 genes, 43.1%). The categories based on molecular functions classified the unigenes into the following eight groups: binding (296 unigenes, 76.9%), catalytic activity (221 genes, 57.4%), structural molecule activity (79 genes, 20.5%), transporter activity (44 genes, 11.4%), enzyme regulator activity (23 genes, 6.0%), translation regulator activity (13 genes, 3.4%), transcription regulator activity (12 genes, 3.1%), and molecular transducer activity (three genes, 0.8%).

Cross-species transferability of the ILP markers

To assess the cross-species transferability of the newly developed ILP markers, 100 of the MsILP markers were selected and tested in seven leguminous (barrel medic, alfalfa, chickpea, soybean, crowtoe, yellow sweet clover and *Sophora alopecuroides*) and three non-leguminous species (*Arabidopsis*, rice, and tobacco) (Suppl. Table 4). The markers that produced consistent amplification profiles in other species were scored as cross-transferable, and the majority of the ILP markers produced significant length variations in the ten species (Suppl. Fig. 2). As presented in Table 1 and Suppl.

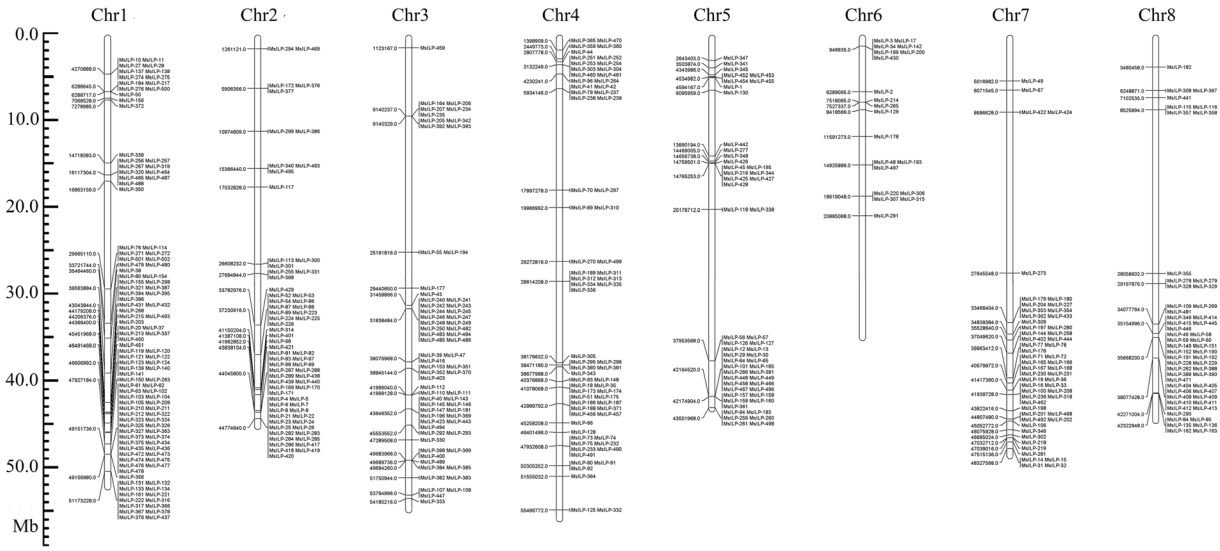

Fig. 1 Distribution of the 501 ILP markers on the *Medicago truncatula* chromosomes

Table 1 Percent transferability of the 100 ILP markers to the different leguminous and non-leguminous species

No.	Investigated crop	Transferability
1	*Medicago truncatula*	82.0%
2	*Medicago sativa*	83.0%
3	*Vicia sativa*	65.0%
4	*Glycine max*	52.0%
5	*Lotus corniculatus*	51.0%
6	*Melilotus officinalis*	70.0%
7	*Sophora alopecuroides*	40.0%
8	*Arabidopsis*	21.0%
9	*Oryza sativa*	21.0%
10	*Nicotiana tabacum*	22.0%
Average		50.7%

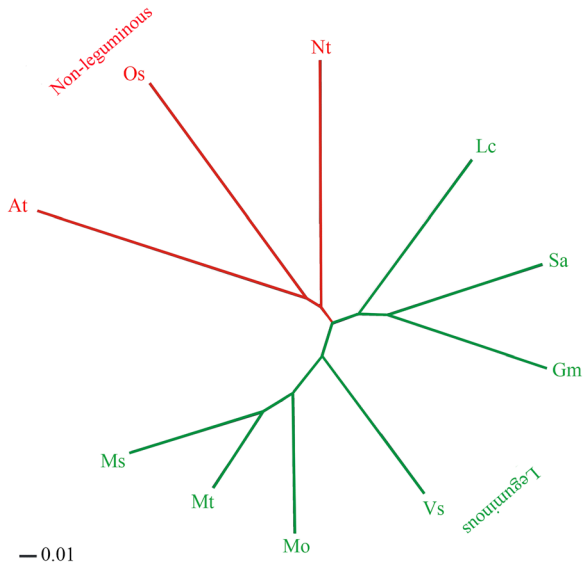

Fig. 2 Genetic relationships between the leguminous and non-leguminous species based on 87 *Medicago sativa* ILP markers using NJoin clustering

Table 5, a total of 260 alleles were collectively yielded based on 87 transferable MsILP markers in ten species. The highest amplification percentage (83%) was observed in *M. sativa*, the lowest (21%) was observed in *Arabidopsis* and rice, and the average was 51%. Of the 100 MsILP markers, 31 (31%) produced amplifications in all of the legume species, 86 (86%) were amplified in at least one legume species, and only three markers (3%), i.e., MsILP-10, MsILP-23 and MsILP-68, produced amplification in all ten species. All 87 of the transferable MsILP markers exhibited the discriminatory power in terms of clearly separating the leguminous and non-leguminous species into two distinct groups (Fig. 2). As expected, the average transferability of these MsILP markers in leguminous species (63.29%) is strikingly higher than that in non-leguminous species (21.33%), and especially high in two *Medicago* spp. (82.50%), indicated that these markers will be more useful in assessing genomic relationships in leguminous species, especially in *Medicago* genus.

Previous studies have demonstrated that the success rate of intron amplification depends on the product size; the success rate decreases as the product size increases (Gupta et al. 2012; Wang et al. 2010). With targeted introns larger than 1 kb, 60% of ILP markers have been found to fail in the cowpea (Gupta et al. 2012). Wang et al. (2010) reported that the success rate of intron amplification was greater than 78% when the EST sequences were less than 300 bp in the tomato, and with intron lengths limited to 500 bp, 88.2% of ILP markers can produce successfully amplified stable products in the soybean (Shu et al. 2010). In the present study, ILP primers were only designed for the query unigenes containing possible introns positions that were expected to be below 400 bp according to the *Arabidopsis* genome sequences, and the amplification rate in alfalfa was 83%, which is consistent with the results revealed in the tomato and soybean studies mentioned above.

To examine whether the PCR products were truly amplified or homologous to the target genes, the electrophoretic bands produced by primer pair MsILP-23, which amplified variant alleles from 162 to 175 bp in the leguminous and non-leguminous species (Suppl. Fig. 3), were isolated and sequenced (by the Shanghai Sangon Biotech Company). As illustrated in Fig. 3, the multiple sequence alignment revealed that the exon regions at the two end positions of the intron were well conserved among the plants, but large differences, including length variations and point mutations, were observed in the intron region in the middle (Fig. 3). Similar observations have also been reported in different plants, such as rice (Wang et al. 2005), *Hypericum perforatum* (Ferreira et al. 2009), foxtail millet (Gupta et al. 2011; Muthamilarasan et al. 2014) and cowpea (Gupta et al. 2012). These findings indicate that the PCR products obtained from all species must result from specific amplifications and reflect the conserved natures of the CDS compared with the non-coding genomic and intron DNA sequences (Guo et al. 2006; Liu et al. 2015; Scoles et al. 2009). Hence, the high levels of cross-

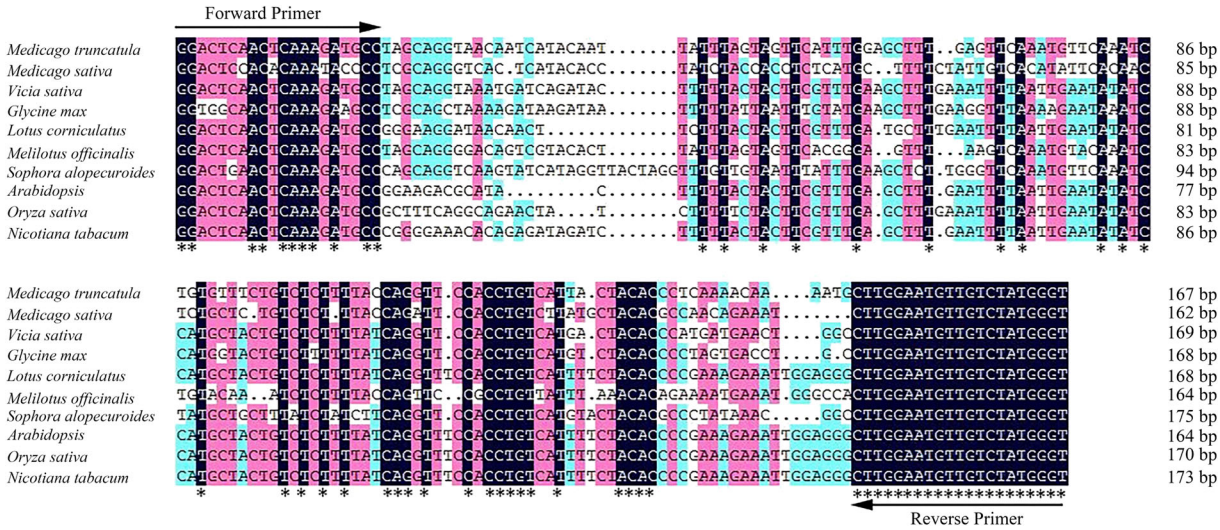

Fig. 3 Multiple alignment of sequences amplified from 10 plant species by primer pair MsILP-23. The asterisks denote similar sequences, and the points represent deletions

species amplification of the ILP markers developed in our study could be useful for linkage map construction and comparative genomic studies.

Assessment of genetic diversity

Forty MsILP primer pairs were randomly selected from the 83 markers that were successfully amplified in the alfalfa, and their potentials for genetic studies were tested in 21 alfalfa accessions (Suppl. Table 1). Thirty-six MsILP markers (90%) were found to be polymorphic across the 21 accessions and collectively yielded 169 alleles with an average of 4.7 alleles per locus and a range of two to 11 alleles per locus (Suppl. Table 6). The expected heterozygosity (*He*) ranged from 0.16 in the primers MsILP-40, MsILP-53 and MsILP-54 to 0.88 in the primer MsILP-75, with a mean of 0.65 (Suppl. Table 6). The PIC ranged from 0.15 to 0.87, with an average of 0.60, which was higher than those reported for foxtail millet (0.20), cowpea (0.34), rice (0.45) and maize (0.48) (Gupta et al. 2012; Liu et al. 2012; Muthamilarasan et al. 2014; Wang et al. 2005). The primers MsILP-40, MsILP-53 and MsILP-54 had the lowest PIC, while the highest PIC was found in primer MsILP-75. PIC values greater than 0.5 have been suggested to

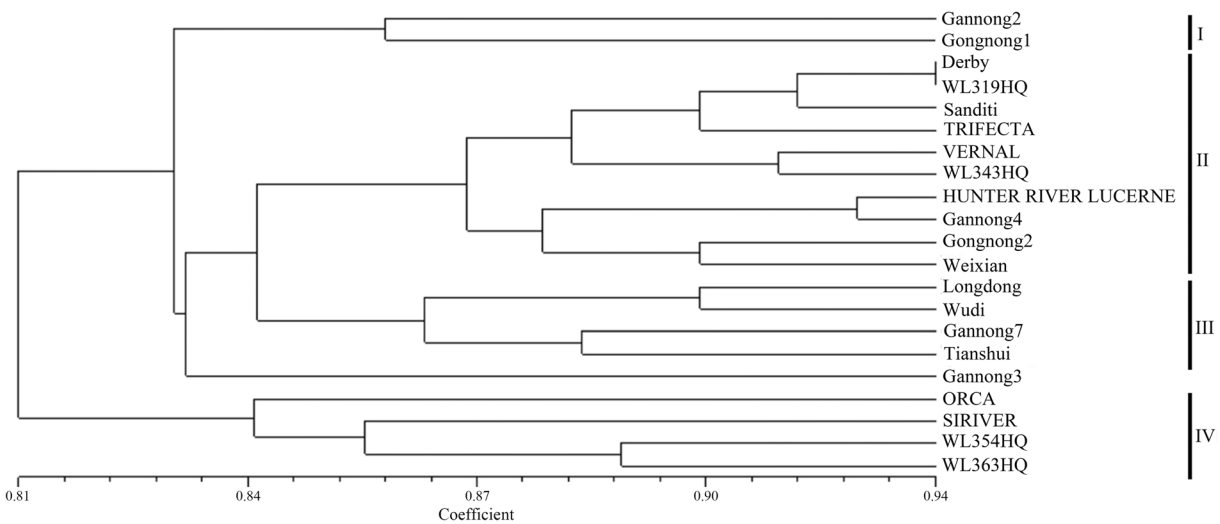

Fig. 4 The dendrogram of 21 alfalfa accessions based on UPGMA analysis using 36 polymorphic MsILP markers

indicate informative markers, and loci with PIC values greater than 0.7 are suitable for genetic mapping (Liu et al. 2015). In the present study, 25 and 15 of the ILP markers and PIC values greater than 0.5 and 0.7, respectively, which indicates high levels of polymorphisms in these markers and their potential for genetic diversity and genetic mapping analyses.

Compared to ILP, SSR is another important and preferred genetic marker widely used for marker-assisted selection, population diversity studies, genetic map construction and gene mapping (Liu et al. 2015). The previous comparative analysis of these two molecular markers in rice and tobacco indicated that the SSR markers could produce higher PIC value and number of alleles than that of ILP markers (Huang et al. 2010, Huang et al. 2013). In this study, the average of the PIC value and number of alleles per locus generated by our alfalfa ILP markers was 0.60 and 4.7, which is also a bit lower than that obtained with alfalfa SSR markers (0.608 and 6.8 for PIC value and number of alleles per locus, respectively) (Wang et al. 2013). Although ILP markers produced less genetic information than SSR markers, considering the mechanisms of the polymorphisms generated by these two marker systems are different, these two marker systems could be combined use for further research into alfalfa breeding.

UPGMA cluster analysis was performed to analyze the genetic diversity of 21 alfalfa accessions using the 36 polymorphic MsILP markers. The cluster results revealed that the 21 alfalfa accessions could be clustered into four major groups. The accession "Gannong3" did not exhibit much similarity with cluster II or cluster III and was thus considered ungrouped (Fig. 4). Among the 21 alfalfa accessions, the association between the clustering pattern and the geographical distribution was less significant. This intermixing of accessions may have been due to the small number of the markers or the small number of accessions from each geographical location used in this study. Similar observations have also been reported in alfalfa (Liu et al. 2015; Wang et al. 2013) and other plant species (Kumar Ganesan et al. 2014; Singh et al. 2014). These findings also confirm that genetic distance cannot be the only criterion for the genetic divergence of the population (Verma and Rana 2011).

Conclusions

To the best of our knowledge, the ILP markers developed in this study are the large-scale novel set of makers in alfalfa. In the present study, a total of 502 alfalfa ILP markers were successfully developed. The validation, cross-species transferability and genetic diversity studies demonstrated expediency of these ILP markers in genetic relationship assessments, comparative genomic studies and marker-assisted breeding in leguminous and non-leguminous species.

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Compliance with ethical standards

Conflict of interests The authors declare that they have no conflict of interest.

References

Anderson JA, Churchill GA, Autrique JE, Tanksley SD, Sorrells ME (1993) Optimizing parental selection for genetic-linkage maps. Genome 36:181–186

Arruda MP, Lipka AE, Brown PJ, Krill AM, Thurber C, Brown-Guedira G, Dong Y, Foresman BJ, Kolb FL (2016) Comparing genomic selection and marker-assisted selection for Fusarium head blight resistance in wheat (*Triticum aestivum* L.) Mol Breed 36:84

Badoni S, Das S, Sayal YK, Gopalakrishnan S, Singh AK, Rao AR, Agarwal P, Parida SK, Tyagi AK (2016) Genome-wide generation and use of informative intron-spanning and intron-length polymorphism markers for high-throughput genetic analysis in rice. Sci Rep 6:23765

Chen HL, Liu LP, Wang LX, Wang SH, Wang ML, Cheng XZ (2015) Development of SSR markers and assessment of genetic diversity of adzuki bean in the Chinese gerplams collection. Mol Breeding 35:191

Dien BS, Miller DJ, Hector RE, Dixon RA, Chen F, McCaslin M, Reisen P, Sarath G, Cotta MA (2011) Enhancing alfalfa conversion efficiencies for sugar recovery and ethanol production by altering lignin composition. Bioresour Technol 102:6479–6486

Ferreira AO, Cardoso HG, Macedo ES, Breviario D, Arnholdt-Schmitt B (2009) Intron polymorphism pattern in *AOX1b* of wild St John's wort (*Hypericum perforatum*) allows discrimination between individual plants. Physiol Plantarum 137: 520–531

Gonzalez-Garcia S, Moreira MT, Feijoo G (2010) Environmental performance of lignocellulosic bioethanol production from alfalfa stems. Biofuels Bioprod Biorefin 4:118–131

Grover A, Sharma PC (2016) Development and use of molecular markers: past and present. Crit Rev Biotechnol 36:290–302

Guo WZ, Wang W, Zhou BL, Zhang TZ (2006) Cross-species transferability of *G. arboreum*-derived EST-SSRs in the diploid species of *Gossypium*. Theor Appl Genet 112:1573–1581

Gupta S, Kumari K, Das J, Lata C, Puranik S, Prasad M (2011) Development and utilization of novel intron length polymorphic markers in foxtail millet (*Setaria italica* (L.) P. Beauv.) Genome 54:586–602

Gupta SK, Bansal R, Gopalakrishna T (2012) Development of intron length polymorphism markers in cowpea [*Vigna unguiculata* (L.) Walp.] and their transferability to other *Vigna* species. Mol Breed 30:1363–1370

Huang L, Cao H, Yang L, Yu Y, Wang Y (2013) Large-scale development of PIP and SSR markers and their complementary applied in *Nicotiana*. Russ J Genet 49:827–828

Huang M, Xie FM, Chen LY, Zhao XQ, Jojee L, Madonna D (2010) Comparative analysis of genetic diversity and structure in rice using ILP and SSR markers. Rice Sci 17:257–268

Kita T, Komatsu K, Zhu S, Iida O, Sugimura K, Kawahara N, Taguchi H, Masamura N, Cai SQ (2016) Development of intron length polymorphism markers in genes encoding diketide-CoA synthase and curcumin synthase for discriminating *Curcuma* species. Food Chem 194:1329–1336

Kumar Ganesan S, Singh R, Choudhury DR, Bharadwaj J, Gupta V, Singode A (2014) Genetic diversity and population structure study of drumstick (*Moringa oleifera* lam.) using morphological and SSR markers. Ind Crop Prod 60:316–325

Liu HL, Lin YA, Chen GB, Shen Y, Liu J, Zhang SZ (2012) Genome-scale identification of resistance gene analogs and the development of their intron length polymorphism markers in maize. Mol Breed 29:437–447

Liu WX, Jia XT, Liu ZM, Zhang ZS, Wang YR, Liu ZP, Xie WG (2015) Development and characterization of transcription factor gene-derived microsatellite (TFGM) markers in *Medicago truncatula* and their transferability in leguminous and non-leguminous species. Molecules 20:8759–8771

Liu WX, Zhang ZS, Chen SY, Ma LC, Wang HC, Dong R, Wang YR, Liu ZP (2016) Global transcriptome profiling analysis reveals insight into saliva-responsive genes in alfalfa. Plant Cell Rep 35:561–571

Mouttet R, Escobar-Gutierrez A, Esquibet M, Gentzbittel L, Mugniery D, Reignault P, Sarniguet C, Castagnone-Sereno P (2014) Banning of methyl bromide for seed treatment: could *Ditylenchus dipsaci* again become a major threat to alfalfa production in Europe? Pest Manag Sci 70:1017–1022

Muthamilarasan M, Venkata Suresh B, Pandey G, Kumari K, Parida SK, Prasad M (2014) Development of 5123 intron-length polymorphic markers for large-scale genotyping applications in foxtail millet. DNA Res 21:41–52

O'Rourke JA, Fu F, Bucciarelli B, Yang SS, Samac DA, Lamb JF, Monteros MJ, Graham MA, Gronwald JW, Krom N, Li J, Dai X, Zhao PX, Vance CP (2015) The *Medicago sativa* gene index 1.2: a web-accessible gene expression atlas for investigating expression differences between *Medicago sativa* subspecies. BMC Genomics 16:502

Rohlf F, NTSYS-pc NT (2000) Multivariate analysis system, version 2.10 e. Applied Biostatistics Inc, New York

Scoles G, Gupta S, Prasad M (2009) Development and characterization of genic SSR markers in *Medicago truncatula* and their transferability in leguminous and non-leguminous species. Genome 52:761–771

Sharma V, Rana M, Katoch M, Sharma P, Ghani M, Rana J, Sharma T, Chahota R (2015) Development of SSR and ILP markers in horsegram (*Macrotyloma uniflorum*), their characterization, cross-transferability and relevance for mapping. Mol Breed 35:1–22

Shu YJ, Li Y, Zhu YM, Zhu ZL, Lv D, Bai X, Cai H, Ji W, Guo DJ (2010) Genome-wide identification of intron fragment insertion mutations and their potential use as SCAR molecular markers in the soybean. Theor Appl Genet 121:1–8

Singh R, Narzary D, Bhardwaj J, Singh AK, Kumar S, Kumar A (2014) Molecular diversity and SSR transferability studies in Vetiver grass (*Vetiveria zizanioides* L. Nash). Ind Crop Prod 53:187–198

Verma S, Rana TS (2011) Genetic diversity within and among the wild populations of *Murraya koenigii* (L.) Spreng., as revealed by ISSR analysis. Biochem Syst Ecol 39:139–144

Voorrips RE (2002) MapChart:software for the graphical presentation of linkage maps and QTLs. J Hered 93:77–78

Wang H, Devos KM, Bennetzen JL (2014) Recurrent loss of specific introns during angiosperm evolution. PLoS Genet 10:e1004843

Wang XS, Zhao XQ, Zhu J, Wu WR (2005) Genome-wide investigation of intron length polymorphisms and their potential as molecular markers in rice (*Oryza sativa* L.) DNA Res 12: 417–427

Wang YY, Chen J, Francis DM, Shen HL, Wu TT, Yang WC (2010) Discovery of intron polymorphisms in cultivated tomato using both tomato and *Arabidopsis* genomic information. Theor Appl Genet 121:1199–1207

Wang Z, Yan HW, Fu XN, Li XH, Gao HW (2013) Development of simple sequence repeat markers and diversity analysis in alfalfa (*Medicago sativa* L.) Mol Biol Rep 40:3291–3298

Yang L, Jin GL, Zhao XQ, Zheng Y, Xu ZH, Wu WR (2007) PIP: a database of potential intron polymorphism markers. Bioinformatics 23:2174–2177

Ye J, Fang L, Zheng HK, Zhang Y, Chen J, Zhang ZJ, Wang J, Li ST, Li RQ, Bolund L, Wang J (2006) WEGO: a web tool for plotting GO annotations. Nucleic Acids Res 34:W293–W297

Zhou SF, Runge TM (2015) Mechanism of improved cellulosic bio-ethanol production from alfalfa stems *via* ambient-temperature acid pretreatment. Bioresour Technol 193:288–296

Zhou SF, Yang Q, Runge TM (2015) Ambient-temperature sulfuric acid pretreatment to alter structure and improve enzymatic digestibility of alfalfa stems. Ind Crop Prod 70:410–416

Zivkovic B, Radovic J, Sokolovic D, Siler B, Banjanac T, Strbanovic R (2012) Assessment of genetic diversity among alfalfa (*Medicago sativa* L.) genotypes by morphometry, seed storage proteins and RAPD analysis. Ind Crop Prod 40:285–291