



The development of 204 novel EST-SSRs and their use for genetic diversity analyses in cultivated alfalfa



Qiang Zhou, Tianlong Chen, Yanrong Wang, Zhipeng Liu*

State Key Laboratory of Grassland Agro-ecosystems, School of Pastoral Agricultural Science and Technology, Lanzhou University, Lanzhou 730020, China

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ABSTRACT

Cultivated alfalfa (*Medicago sativa* L.) is one of the most important forage legume in the world. Here, we report the development of 204 novel polymorphic expressed sequence tag simple sequence repeat (EST-SSRs) markers from transcript sequences via global Illumina sequencing for cultivated alfalfa. Among the synthesized 750 pairs of primers, 204 EST-SSR primer pairs showed polymorphisms among 10 alfalfa accessions (including five individual plants in each accession) generating a total of 1222 alleles, with the number of alleles per locus, the observed heterozygosity (H_o), the corrected heterozygosity (H_e) and the Shannon-Wiener diversity index (H') averaging at 5.99, 0.73, 0.71 and 1.14, respectively. Of the 204 novel EST-SSRs in alfalfa, 120 can be *in silico* mapped onto the eight *Medicago truncatula* chromosomes (Mt3.5.2). Considering the high polymorphism, these EST-SSRs can be applied to assess genetic diversity, population structure, relatedness, evolution, linkage mapping and cultivar protection of cultivated alfalfa to facilitate alfalfa breeding programs.

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1. Introduction

Cultivated alfalfa (*Medicago sativa* L.) is one of the most important forage legume in the world and the third most valuable crop in the United States. In addition to being a valuable forage crop for ruminants, alfalfa possesses considerable potential as a biofuel crop for a sustainable feedstock for ethanol production. This species is a perennial, cross-pollinated and autotetraploid ($2n = 4x = 32$) plant with a genome size of 800–900 Mb (Choi et al., 2004). Extensive efforts have been made to develop alfalfa transcriptome data, including 454 sequencing (Han et al., 2011) and Illumina sequencing (Li et al., 2012; Liu et al., 2013; Postnikova et al., 2013; Yang et al., 2011), which have facilitated the development of transcript-based molecular markers for alfalfa, such as expressed sequence tag simple sequence repeat markers (EST-SSRs).

SSR markers are very useful for a spectrum of genetic and breeding because of their codominant nature, abundance in genomes, high reproducibility, hyperpolymorphism and high rates of transferability. EST-SSRs always exhibit lower levels of polymorphism than the genomic sequence-based SSRs. However, they are predicted to possess advantages like easy access, presence in gene-rich regions and high level of transferability to related species, and they can often be used as anchor markers for comparative mapping and evolutionary studies (Zhou et al., 2014). Despite the growing availability of single nucleotide polymorphism (SNP) markers, SSRs are still the most used molecular markers by many breeders that might not have access to expensive SNP-genotyping platforms for their species (Tyrka et al., 2008).

* Corresponding author. Tel.: +86 9318914051.

E-mail address: lzp@lzu.edu.cn (Z. Liu).

Extensive studies have reported the usage of SSR markers in alfalfa (Diwan et al., 1997; Eujayl et al., 2004; Robins et al., 2008; Sakiroglu et al., 2010; Sledge et al., 2005). But all of them were derived from *Medicago truncatula*. To date, 61 polymorphic genomic SSRs (He et al., 2009), 27 EST-SSRs confirmed by individual alfalfa plants in each accession (Liu et al., 2013), and 401 EST-SSRs confirmed by mixing plants in each accession have been developed (Wang et al., 2013, 2014), which is still very insufficient for genetic research when compared with other plants, such as 1281 polymorphic EST-SSR markers available for peanut (*Arachis hypogaea* L.) (Koilkonda et al., 2012) and 2240 polymorphic SSR markers for rice (*Oryza sativa* L.; McCouch et al., 2002). Moreover, considering the autotetraploid trait of alfalfa, it is more precise to validate the polymorphism of EST-SSRs by the DNA of individual plants instead of mixing plants in each accession.

Based on our previous study, here, we investigated the sequence identity and presence of EST-SSRs in the effective unigenes on a large scale. Our objective was to increase the number of EST-SSR markers for cultivated alfalfa by further mining the current unigene resources for polymorphisms. An additional 750 EST-SSR primer pairs were designed and synthesized, and 204 novel EST-SSR primers showed polymorphisms using 50 individual plants. Totally, 120 polymorphic EST-SSR loci were *in silico* mapped to the *M. truncatula* chromosomes.

2. Materials and methods

2.1. EST-SSRs detection and primer design

EST-SSRs were detected in 40,433 alfalfa unigenes with the simple sequence repeat identification tool (SSRIT) program. Only unigenes longer than 1 kb were included in the EST-SSR detection. To avoid possible duplicates of published EST-SSRs (Liu et al., 2013; Wang et al., 2013, 2014), a local BLASTX was performed using the published EST-SSR sequences against the 1494 SSR-containing unigene sequences (E-value <10E-10). The parameters were adjusted to identify perfect di-, tri-, tetra-, penta- and hexa-nucleotide motifs with a minimum of 6, 5, 5, 5 and 5 repeats, respectively. The EST-SSR primers were designed using BatchPrimer3. The primer design parameters were set as follows: length range, 18–23 nucleotides with 21 as optimum; PCR product size range, 100–250 bp; optimum annealing temperature, 55 °C; and GC content, 40–60%, with an optimum content of 50%.

After EST-SSR identification, 750 non-redundant EST-SSR primer pairs were newly designed and synthesized. Five different plants, one plant from each accession (StarQueen, AmeriStand IV, Longdong I, Xinjiangdaye and Qingyang; Table S1), were employed for optimizing Mg^{2+} concentration and melting temperatures (T_m) for polymerase chain reaction (PCR) and for investigating the sequence identity between the primers and assembled unigenes.

2.2. Plant materials and EST-SSRs amplification

A total of 10 alfalfa accessions, including AmeriStand IV, Ladak DL, AmeriStand III, Resis, Longdong I, Algonquin, Xinjiangdaye, StarQueen, Qingyang, and Longdong III were selected for polymorphism analyses with the EST-SSRs (Table S1). To accurately validate the polymorphisms of EST-SSRs, young leaves of five individual plants in each accession were used to extract genomic DNA via a modified cetyltrimethylammonium bromide (CTAB) method (Liu et al., 2013). PCR amplifications were conducted in a final volume of 10 μ L containing 40 ng template DNA, 1 \times PCR buffer, 2.0 mM $MgCl_2$, 2.5 mM dNTPs, 4 μ M each primer and 0.8 U Taq polymerase (TaKaRa, Kyoto, Japan). The PCR reactions were performed using the following conditions: 4 min at 94 °C, 35 cycles of 30 s at 94 °C, 35 s at the annealing temperature (Table 1 and Table S2) and 1 min at 72 °C, with a final extension step of 5 min at 72 °C. The PCR products were subjected to electrophoresis on an 8.0% non-denaturing polyacrylamide gels and stained by ethidium bromide (Liu et al., 2013). The PCR product sizes were identified by comparison with the DL500 DNA maker (TaKaRa, Kyoto, Japan).

2.3. Diversity analysis

The observed heterozygosity (H_o) was calculated as previously shown (Liu et al., 2007), and the corrected heterozygosity (H_e , corrected for sample size) and Shannon–Wiener diversity index (H' , corrected for sample size) were analysed using the ATETRA 1.2.a software program. Only specific bands that could be unambiguously scored across all individual plants were used in this study. A clustering analysis was used to generate a dendrogram using the unweighted pair-group method with arithmetic mean (UPGMA) and Nei's unbiased genetic distance with NTSYSPC 2.0 software package (Nei, 1978).

Table 1

Characterization of 204 EST-SSRs in 10 alfalfa accessions.

Primer number	Number of alleles \pm standard derivation	$H_o \pm$ standard derivation	$H_e \pm$ standard derivation	$H' \pm$ standard derivation
204	5.99 \pm 1.53	0.73 \pm 0.19	0.71 \pm 0.09	1.14 \pm 0.19

Note: H_o , observed heterozygosity; H_e , corrected heterozygosity; H' , Shannon–Wiener diversity index.

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