Development of SSR markers via de novo transcriptome assembly in *Akebia trifoliata* (Thunb.) Koidz.

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1 **Development of SSR markers via *de novo* transcriptome assembly in *Akebia trifoliata* (Thunb.) Koidz.**

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Abstract: Owing to its high nutritive, economic, and medicinal values, *Akebia trifoliata* has received increased attention, making worthy of being used as a new fruit crop for further domestication and commercialization in China. However, molecular research of *A. trifoliata* has lagged as investigations of its genomic resources and molecular markers are rare. In this study, a cDNA library of *A. trifoliata* leaves was sequenced using the Illumina NovaSeq 6000 sequencing system. 101 417 transcripts, 63 757 unigenes, and 9494 simple sequence repeats were assembled and identified from the transcriptome datasets. The majority of the SSRs were di- and trinucleotide repeats. Length and number of SSR motifs ranged from 15 to 66, and 5 to 48 bp, respectively. Of which, the A/T mononucleotide motif and AG/TC and CT/GA dinucleotide motifs were the most abundant. Furthermore, 100 SSR primers were randomly selected to validate amplification and polymorphism, and 88 *A. trifoliata* accessions were definitively distinguished by 49 primers. With the Qinling mountains and Huaihe River line as the boundaries, the northern and southern accessions were clustered into different groups, but no clear geographical patterns (city or origin) were observed in the southern accessions. These newly identified molecular markers may provide a foundation for the genetic identification and diversity analysis and marker-assisted selection breeding in *Akebia* species.

Key words: SSR. Accession identification. Transcriptome. *Akebia trifoliata*.

24 **Introduction**

25 *Akebia trifoliata* (Thunb.) Koidz ($2n = 16$) is a wild perennial woody liana, and the *A. trifoliata* plants are monoecious with flowers functionally unisexual and
26 self-incompatible (Zou et al. 2018; Li et al. 2019). It belongs to the Lardizabalaceae family, that is mainly distributed throughout China and Japan, and is also
27 commonly known in China as August melon, August bar, Bayuezha, and wild banana (Liu et al. 2007; Du et al. 2012). *A. trifoliata* has been used in traditional
28 Chinese medicine (TCM) for more than 2000 years (Wang et al. 2014). Previous research has shown that its various parts, such as the roots, stems, leaves, flowers,
29 and fruits, may be edible, medicinal, and ornamental, making it a desirable plant for consumers (Zhou et al. 2017). For example, its fruits and stems are used in TCM
30 to promote diuresis, and as an antiphlogistic to stimulate blood circulation (Kawata et al. 2007); its dried young leaves can be used as tea leaves, and the fresh fruits,
31 which are rich in saccharides, vitamins, and amino acids and serve as a kind of high-value and health fruit (Du et al. 2012; Wang et al. 2015), can be consumed
32 directly or used to make juice, sugar, and vinegar (Jiang et al. 2012). Furthermore, the oil of seeds exhibits an abundance of nutritional properties and is commonly
33 used as an edible oil in southern China (Kitaoka et al. 2009). Owing to its highly nutritive, economic, and medicinal values, *A. trifoliata* is currently being widely
34 cultivated and rapidly developed as a commercial species in many regions of China (Li et al. 2010a; Li et al. 2010b).

35 However, many of the natural *A. trifoliata* resources have been lost over the past several decades because of overexploitation and habitat degradation, making the
36 natural populations scarce (Li et al. 2010a). It is thus important to understand the genetic variation and structure, conservation, and sustainability of this species.

Owing to their high polymorphism, reliability, and rapid and ready detection, simple sequence repeat markers (SSR) have proven to be powerful tools in conservation biology and molecular ecological studies (Ohbayashi et al. 2019). However, until now, a mere 145 nucleotide sequences and no expressed sequence tags (EST) sequences had been deposited for *A. trifoliata* in the National center for biotechnology information (NCBI) database, and only 125 unreviewed sequences have been added to the UniProt database. Furthermore, only 11 microsatellite loci were reported from an AC-enriched genomic library of *A. trifoliata* ssp. *australis*, to be utilized for population genetic diversity analysis of different *Akebia* species (Li et al. 2009; Li et al. 2019). As the 11 reported markers were developed from partial genomic DNA sequences, their isolation was time-consuming, laborious, and expensive. Previous studies indicated that most of the reported genomic SSR markers have lower rate of transferability than EST-SSR markers to related species, and were difficult to correlate with the target agronomic traits (Shi et al. 2017). The low percentage of polymorphic markers identified for *A. trifoliata* has greatly limited research into its genetic diversity, accession preservation, and molecular breeding. Additionally, although the use of transcriptome sequencing has recently been reported in *A. trifoliata* (Yang et al. 2016), very few molecular markers have been developed.

Therefore, more highly polymorphic microsatellite loci are urgently needed to elucidate the genetic diversity distribution, breeding, and genetic improvement of *A. trifoliata*. With the development of next-generation sequencing (NGS) technologies, large-scale SSRs can now be easily generated for a variety of species (Deng et al. 2018; Ma et al. 2019). In the present study, using Illumina RNA-seq technology, we have characterized the transcriptome of *A. trifoliata* and developed a large

50 number of SSR markers from the assembled transcripts. We randomly selected 100 SSR primers to test for their utilization in the study of the genetic diversity of *A.*
51 *trifoliata* accessions. These findings could provide new insights for genetic research and marker-assisted selection (MAS) breeding of *Akebia* plants.

52

53 **Materials and methods**

54 **Plant materials**

55 The Qinling mountains and Huaihe River line (Qinling-Huaihe line) form the geographical dividing line between the northern and southern regions of China. They
56 run west to east, stretching across the Gansu, Shaanxi, and Henan Provinces. To the north of the Qinling-Huaihe line, the climate is temperate, rivers freeze, and the
57 leaves seasonally turn yellow and fall from the trees. To the south of the Qinling-Huaihe line, there is a subtropical monsoon climate, the rivers do not freeze, and the
58 trees stay green all year round (Li et al. 2015). Nanyang city in Henan Province is on the Qinling Huaihe line boundary, but it has a subtropical monsoon climate and
59 is grouped with the southern region. For this study, a total of 88 wild accessions of *A. trifoliata* were collected from 8 provinces including 16 regions of China and
60 their topographic altitudes ranged from 33 to 1646 m above sea level (Fig. 1). According to the divisions of the Qinling-Huaihe line, the accessions collected from the
61 cities of Luoyang and Dengfeng were categorized as northern resources. While the accessions collected from Changsha, Changde, Zhangjiajie, Zhuzhou, Nanchang,

Ganzhou, Dazhou, Sanming, Hanzhong, Shangrao, Ningguo, Nanyang city, Enshi, and the Xiangxi autonomous prefecture were categorized as southern resources. All the accessions collected ($2n = 16$) of *A. trifoliata* were wild, monoecious with flowers functionally unisexual and self-incompatible. The accessions from 1-36 were cultivated in preservation sites, including Hunan academy of agricultural sciences (HAAS) and Jiangxi academy of forestry (JXAF), and the accessions 37-88 were planted in the resources nursery of Institute of bast fiber crops (IBFC). However, owing to a lack of systematic planting maps and uncertain recordings, the accessions collected from HAAS and JXAF (accounting for 37.5 % of all accessions) was not systematically recorded. The geographical origin and biological characteristics of the accessions in these regions was also not available. In addition, for the purple peel and higher fruit quality of the accession, the fresh young leaves from ‘Dazhou No.3’ were used to construct a transcriptome library. Furthermore, 88 accessions were used to examine the polymorphisms of the detected SSR markers in our study (Table 1). All the fresh young leaves were collected and immediately frozen in liquid nitrogen and stored at -80 °C, until further analysis.

RNA and DNA extraction

The total RNA was isolated from ‘Dazhou No.3’ using the cetyltrimethylammonium bromide (CTAB) methods, as described by Niu et al. (2018). Total genomic DNA was extracted from each of the 88 leaf samples using the CTAB method with minor modifications, according to Xu et al. (2013). The quality and the concentration of the total RNA and DNA was evaluated by electrophoresis using a 1.0 % agarose gel and a NanoDrop 2000C Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

75 **Library construction and Illumina sequencing**

76 First, the total RNA of the sample ‘Dazhou No.3’ was collected and oligo (dT) beads were used to enrich the poly (A) mRNA. The enriched mRNA was then broken
77 into short fragments using a fragmentation buffer. Subsequently, the first-strand cDNA was synthesized by random hexamers using short fragments as templates, and
78 then the second-strand cDNA was synthesized by using buffer, dNTPs, RNase H, and DNA polymerase I. The synthesized double-stranded cDNA fragments were
79 then purified by magnetic beads and eluted with EB buffer for end-repair and the addition of poly (A). The short fragments were connected with sequencing adapters,
80 and suitable fragments were selected as templates with magnetic beads for PCR amplification and agarose gel electrophoresis. Finally, cDNA libraries were built and
81 sequenced using the Illumina NovaSeq 6000 platform of Anhui Microanaly Genetech Company (Anhui, China).

82 ***De novo* assembly of the transcriptome**

83 To ensure the accuracy of the *de novo* assembly and subsequent analysis, the raw sequence data reads were processed prior to the assembly of the *A. trifoliata*
84 transcriptome. The raw reads were initially processed using custom Perl scripts in fastq format and the filtering process was divided into 3 parts: (1) the excess “N”
85 nucleotides, with more than 10 % of the bases, and reads with low-quality bases ($Q < 30$), were filtered out; (2) reads with less than 15 bases or more than 40 % poor
86 bases were removed; (3) the adaptor sequences were filtered out. Then the clean reads were obtained by filtering out these adapter and low-quality sequences from

the raw reads. The assembly of clean reads was performed using the Trinity software package (<https://github.com/trinityrnaseq/trinityrnaseq/releases>) (Grabherr et al. 2011). The Trinity software used three independent modules, including Inchworm, Chrysalis, and Butterfly, and default parameter settings; transcripts shorter than 200 bp were discarded. The sequences without redundancy, containing the longest transcripts of the cluster extracted from the transcript could be regarded as unigenes.

Gene annotation

All of the assembled unigene sequences were aligned to the TrEMBL protein database (<http://www.ebi.ac.uk/trEMBL/index.html>), the Protein families database (Pfam) (<http://pfam.xfam.org/>), the Evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG) (<http://eggnogdb.embl.de/#/app/home>), the Swiss-Prot protein database (<http://www.expasy.ch/sprot>), Gene Ontology (GO) database (<http://www.geneontology.org>), and Kyoto Encyclopedia of Genes and Genomes pathway database (KEGG) (<http://www.genome.jp/kegg>), using the Basic Local Alignment Search Tool (BLAST) search (<https://www.arabidopsis.org/cgi-bin/Blast/TAIRblast.pl>) with an *E*-value of 10^{-5} .

Simple sequence repeat (SSR) identification

SSR sites were detected using the microsatellite identification tool MicroSatellite (MISA; <http://pgrc.ipk-gatersleben.de/misa/misa.html>). The sequences were

99 searched for perfect mononucleotide, dinucleotide, trinucleotide, tetranucleotide, pentanucleotide, and hexanucleotide motifs, with a minimum of fifteen, eight, five,
100 four, three, and three repeats, respectively. Primer pairs were randomly selected and designed in a further study using Primer Premier 3.0
101 (<http://sourceforge.net/projects/primer3>) in the flanking regions of the SSRs. In order to acquire a high probability of amplification, the primer design parameters
102 were set as follows: the primer length was 18 - 23 bp with an optimal length of 21 bp; the PCR products were 100 - 300 bp, the annealing temperature was 52 – 58 °C
103 with an optimal temperature of 55 °C, and the GC percentage was 40 – 60 %, with an optimal GC content of 50 %. Primers were synthesized by Qingke Biotech
104 (Beijing, China).

105 **EST-SSR marker characterization and validation**

106 To analyze the SSR polymorphisms, the identified SSR markers were amplified and validated with 88 accessions of *A. trifoliata*. PCR was performed in a total
107 reaction volume of 20 µL containing 1 U Taq DNA polymerase (TIANGEN Biotech, Beijing, China), 1 × PCR Buffer, 1.5 mM MgCl₂, 0.52 mM dNTPs (TIANGEN
108 Biotech, Beijing, China), 0.4 µM of each primer, and 20 ng of genomic DNA. PCR was carried out on a Bio-Rad T100™ Thermal Cycler (Bio-Rad Laboratories,
109 Hercules, CA, USA) as follows: 94 °C for 5 min; 31 cycles of 94 °C for 30 s, 52 – 56 °C for 40 s, and 72 °C for 1 min; and a final extension at 72 °C for 10 min
110 followed by a final hold at 4 °C. PCR products were verified using 8.0 % non-denaturing polyacrylamide gel electrophoresis (PAGE) and stained using 0.1 % silver
111 nitrate as described by Huang et al. (2014).

SSR reproducible fragments were scored following methods described in Ni et al. (2018); so, the band types of the allele maximal molecular weights were recorded as the capital letter A, followed by B, C, D, etc., and different letters were used to record different morphological variants. If only one band type was found by a pair of primers, the accession was recorded as homozygous. The major allele frequency (MAF), the number of alleles (N_a), genetic distance, observed heterozygosity (H_o), and the polymorphic information content (PIC) were calculated and constructed using PowerMarker v3.25 (Liu et al. 2005). The clustering tree was constructed based on Nei's genetic distance, which was calculated by PowerMarker and visualized with the MEGA version 7.0 and iTOL (<https://itol.embl.de/>) (Luo et al. 2018). The population structure of the accessions was determined using the model with the admixture and the allele variation frequency correlation model of the software program Structure (version 2.3.4) (Pritchard et al. 2000). The software program Structure Harvester V6.0 was used to determine the optimal K value (Earl et al. 2012).

Availability of sequencing data

All raw sequencing data from this study have been deposited in the NCBI Sequence Read Archive (SRA; <http://www.ncbi.nlm.nih.gov/sra>). The BioProject and SRA accession numbers are PRJNA525587 and SRP187511, respectively.

124 **Results**

125 **Sequencing and *de novo* assembly of the *A. trifoliata* transcriptome**

126 A total of 50 364 426 raw reads (7.555 Gb) were produced for *A. trifoliata* using Illumina sequencing, and 25 049 450 clean reads were obtained after filtering and
127 trimming of the low quality and adaptor sequences, and the ambiguous (“N”) nucleotides. The clean reads totaled 7.205 Gb with a mean length of 143.81 bp, the GC
128 and Q30 content was 45.10 % and 92.56 %, respectively. A total of 101 417 transcripts were assembled from the clean reads after the *de novo* assembly, with an N50
129 of 1692 bp, and an average length of 983.28 bp. The length distributions of the unigenes are shown in Fig. S1. A total of 63 757 unigene sequences were generated
130 from the transcriptome library, with an N50 of 1487 bp, and an average length of 769.89 bp (Table 2). Moreover, based on the BLASTx algorithm with an *E*-value
131 threshold of 10⁻⁵, 19 661 (30.84 %) unigenes were annotated in the Pfam database, 27 772 (43.56 %) in TrEMBL, 20 917 (32.81 %) in eggNOG, 19 096 (29.95 %) in
132 Swiss-Prot, 22 798 (35.76 %) in GO, and 27 309 (42.83 %) in KEGG (Table 3); suggesting that the TrEMBL database provided the greatest number of annotations.
133 Moreover, 28 504 unigenes were shown to correspond with sequences from at least one of the public databases, while the remainder (55.29 %) were unannotated in
134 *A. trifoliata*. Additionally, *Arabidopsis thaliana* (14 552, 63.80 %) provided most of the hits for the *A. trifoliata* unigenes in the Swiss-Prot database, followed by
135 *Nelumbo nucifera* (13 247, 47.70 %) in the TrEMBL database.

136 **Frequency and distribution of EST- SSRs in *A. trifoliata***

137 A total of 9494 SSRs were identified from 63 757 unigene sequences using the MISA tool. The frequencies of these SSR repeat types were different; the dinucleotide
138 motifs were the most common type of repeat motif, with a frequency of 42.35 %, followed by the trinucleotide (30.02 %), mononucleotide (24.65 %), and
139 tetranucleotide (2.18 %) motifs (Fig. 2A, Table 4). The lengths of the SSR motifs ranged from 15 to 66 bp, with 15 to 19 bp (60.80 %) being the most abundant SSR
140 motif lengths, followed by SSR motifs with lengths ranging from 20 to 24 bp (27.63 %). However, SSR motifs with lengths that ranged from 25 to 29 bp, 30 to 34
141 bp, and those composed of greater than 35 bp, accounted for only 4.14, 4.22, and 3.21 % of the total number of SSRs, respectively (Fig. 2B).

142 The number of repeats in the SSR motifs ranged from 5 to 48, and 10-14 was the most abundant number of repeats, accounting for 48.43 % of the SSRs, followed
143 by 5-9 repeats (37.12 %). The next most common numbers of repeats were 15-19 (11.00 %) and 20-24 (2.68 %) (Fig. 3A). The largest number of repeats for each
144 repeat length was 48, 33, 20, 12, 10, and 8 for the mono, di-, tri-, tetra-, penta-, and hexanucleotides, respectively.

145 A total of 155 types of repeats were identified, where 2, 6, 30, 29, 34, and 33 different types of repeat motifs were detected within the mono-, di-, tri-, tetra-,
146 penta-, and hexanucleotide repeats, respectively. In the mononucleotide repeats, A/T was the most abundant, and accounted for 24.39 % of all motifs. The AG/TC
147 repeat was dominant in the di-motifs and accounted for 19.04 % of all motifs. Furthermore, each of the repeats CT/GA (15.10 %), AC/TG (3.27 %), AT/TA

(2.85 %), AGA/TCT (3.21 %), TTC/AAG (2.86 %) and GAA/CTT (2.70 %) represented at least 2 % of the total number of repeats detected (Fig. 3B). Among the 2340 mononucleotide motifs, 2316 (98.97 %) were composed of A/T repeats. Among the 4021 dinucleotide motifs, 1808 (44.96 %) and 1434 (35.66 %), were composed of AG/TC, and CT/GA repeats, respectively. No CG/GC motifs were present. Among the 2850 trinucleotide motifs, 305 (10.70 %), 272 (9.54 %), and 256 (8.98 %) were composed of AGA/TCT, AAG/TTC and CTT/GAA repeats, respectively. (Table S1). Among the 207 tetranucleotide motifs, 22 (10.63 %) and 15 (7.25 %) were composed of AGAA/TCTT and AAAG/TTTC, respectively.

Validation of SSR markers in *A. trifoliata*

The ID, length of the unigenes, SSR type, SSR loci, and the sequences of the SSR loci are shown in Table S2. To validate the amplification efficacy and polymorphisms of the putative SSR markers, 100 primers were randomly selected, synthesized, and screened based on the SSRs. Of these, 72 primers successfully produced clear bands with polymorphisms, and 28 primers produced banding patterns without polymorphisms in 4 accessions, as visualized by PAGE. Moreover, 49 of the 72 primers produced the expected bands and showed polymorphisms in all 88 of the *A. trifoliata* accessions. A total of 136 alleles were detected, with an average of 3 alleles per locus, where the number of alleles per locus ranged from 2 to 4. H_0 ranged from 0 to 0.94 (with a mean of 0.22), and the PIC values varied from 0.09 to 0.63 (with a mean of 0.43). MAF varied from 0.36 to 0.95, with an equilibration of 0.60, and the gene diversity ranged from 0.09 to 0.69, with an average of 0.50. All characteristics of these polymorphic microsatellite loci are listed in Table 5, and the results of the PCR amplification using primers P009 and

P023 are shown as examples (Fig. S2). These results indicate that the polymorphic SSRs derived from the RNA-seq behave effectively as markers and are suitable for inferring population genetic structure in *A. trifoliata*.

Analysis of genetic diversity

The genetic relationships of the 88 *A. trifoliata* accessions were analyzed based on the allele polymorphisms of the 49 SSR primers, and the accessions were grouped into four main clades, using Powermarker software. Clade I contained 27 accessions, including LuoyangNo.1-No.11, DengfengNo.2-No.14, JF05, DazhouNo.1, and No.3. Clade II contained 12 accessions, including ZhangjiajieNo.1, HanzhongNo.1-No.5, JF06, HA1, HA10, JF02, JF04, and JF11. Clade III contained 18 accessions, including HA06-HA08, HA12-HA15, HA18, ShangraoNo.1-No.2, NingguoNo.1, JF08-JF10, and JF14-JF17. Clade IV contained 31 accessions, including HA02-HA05, HA09, HA11, HA16-HA17, HA19, JF01, JF07, ChangdeNo.1-No.4, DengfengNo.1, NanyangNo.1, DazhouNo.2, DazhouNo.4-No.6, ZhangjiajieNo.2, SanmingNo.1, EnshiNo.1-No.5, GanzhouNo.1, ZhuzhouNo.1, and XiangxiNo.1 (Fig. 4). The 88 accessions were clustered into different groups. With the Qinling-Huaihe line as the boundary, the accessions from Clade I were grouped into the northern region, which was consistent with their geographical distribution, but no clear geographical patterns (city or origin) were observed among Clades II-IV. Additionally, the accessions from the same locations did not always cluster into the same group, while those from different locations did sometimes cluster together.

173 The population structure of the 88 accessions was also examined, based on the SSR markers using the Structure software program. The results of the inferred
174 clustering, completed using a K -value (population number) of 2-10, revealed that the most significant change in likelihood occurred when the K value increased from
175 2 to 4, and that the highest Delta $K(\Delta K)$ value was observed between $K = 2$ and $K = 4$. Moreover, when $K = 4$, the northern accessions could be completely separated
176 from the southern accessions. The results from the dendrogram analysis were similar, as four main groups were cultured, and all parameters suggested that the
177 four-clade model sufficiently explained the genetic structures of the 88 accessions (Fig. 5).

178

179 **Discussion**

180 **Unigene assembly and annotation**

181 *A. trifoliata* is rich in nutrients, has health benefits, and other newly discovered fruit properties in the primary stages of its domestication, that make it worthy of
182 exploitation as a new high-value crop (Li et al. 2010b). However, only 11 SSR markers had previously been reported for it (Li et al. 2009; Li et al. 2019). The current
183 shortage of SSR markers limits our understanding of the genetic basis for its desirable features, accession conservation, effective genetic mapping, and MAS
184 breeding in *A. trifoliata*.

High-throughput, widely available, relatively fast and low cost, transcriptome sequencing has played a vital role in gene discovery (Nguyen et al. 2018), gene expression (Hou et al. 2018), developing species molecular markers, and phylogenetic relationships (Choudhary et al. 2018; Yang et al. 2018). In the present study, a total of 25 049 450 clean reads were obtained using transcriptome sequencing, and the GC content and Q30 were 45.10 % and 96.31 %, respectively. Moreover, 63 757 unigenes with an N50 of 1487 bp, and an average length of 769.89 bp were successfully assembled from the *A. trifoliata* transcriptome (Table 2). The lengths obtained in this study were longer than those of other Ranunculales, such as *Dysosma versipellis* (938 bp and 409 bp, respectively) (Guo et al. 2014), *Sargentodoxa cuneata* (an average length of 255 bp) (Sun et al. 2016), and *D. aurantiocaulis* (807 bp and 531 bp, respectively) (Mao et al. 2016). These differences indicate the high-quality nature of the transcriptome sequencing and *de novo* assembly performed in the present study on *A. trifoliata*.

Additionally, approximately 44.71 % of the unigenes were annotated and matched to known proteins from at least one of the six public databases (Table 3). These results are similar to values reported for *D. versipellis* (27.98 %) (Guo et al. 2014) and *Rhododendron* (43.22 %) (Xing et al. 2017), but less than the values reported for *D. aurantiocaulis* (56.87 %) and *D. versipellis* (66.48 %), with at least one significant match to the public databases (Mao et al. 2016). The results indicate that they may be limited by the lack of genomic information, relevant research reports, or novel genes that are unique to *A. trifoliata*. The sequence database of *A. trifoliata* may provide useful reference data for the molecular research of *Akebia* species.

Markers identification and characterization of SSRs

198 As one of the most widely used molecular markers in crops and fruits, the clear advantages of SSR markers (i.e., a high degree of polymorphism, good repeatability,
199 and ease of use) play a key role in their broad application for studies on population genetic structure and diversity, evolution, genetic mapping, comparative
200 genomics, etc. (Hu et al. 2017). Despite these advantages, few SSR markers and nucleotide sequences—and no EST sequences—have been reported in the GenBank
201 database for *A. trifoliata*, which is a major obstacle for the development of molecular marker-assisted selection in this species. Transcriptome sequences have become
202 the preferred method for the development of molecular markers, particularly in non-model species, such as *A. trifoliata*. A total of 9494 SSRs were identified in the
203 present study, where the dinucleotide motifs were the most common type of repeat motif, with a frequency of 42.35 %, followed by trinucleotides (30.02 %). By
204 contrast, tetra-, penta-, and hexa-nucleotide repeats were far less common (2.18, 0.45, and 0.35 %, respectively) (Table 4, Fig. 2A). These results are consistent with
205 the SSR results observed in the transcriptome of *D. versipellis* (Guo et al. 2014). In addition, the distributions of the types of microsatellite repeats were also similar
206 to those of other plants, such as in *D. versipellis* and sweet potato, suggesting that the AG/TC, CT/GA, and AGA/TCT, AAG/TTC repeats may be the predominant
207 motifs in dicotyledonous plants (Guo et al. 2014; Wang et al. 2010). Additionally, there was no GC/CG motif, which was consistent with the observation that
208 GC-rich repeats of any class are extremely rare in dicots, such as *Arabidopsis* and papaya. A possible explanation for this is that GC tends to form highly stable
209 hairpins structures (Eustice et al. 2008).

210 **Validation of the predicted SSR markers**

211 In order to validate the amplification quality and polymorphisms of the SSRs in the present study, a total of 100 randomly generated primers were validated across 88
212 accessions. Of these, 72 primers (72 %) successfully produced clear bands with polymorphisms, 28 (28 %) primers produced bands without polymorphism in 4
213 accessions. Forty-nine of the 72 primers generated the expected bands (49 %) with polymorphism in 88 *A. trifoliata* accessions. The polymorphic ratio (49 %) of the
214 primers was similar to the results found in species of the subfamily Podophylloideae (51.0 %) (Mao et al. 2016) but higher than the ratios observed in *D. versipellis*
215 (46.3 %) (Guo et al. 2014) and *S. cuneata* (36.2 %) (Sun et al. 2016). This may be explained by the presence of introns and deletions within the amplicons, assembly
216 errors, or low specificity because of unintentional selection of these primers (Wu et al. 2013; Chen et al. 2017). Moreover, the corresponding value for the average
217 alleles per locus was 3 of *A. trifoliata*, which is like the average values for EST-SSR markers reported in previous studies on *D. versipellis* (3.32) and *D. pleiantha*
218 (3.50). The PIC values, which varied from 0.09 to 0.63 (with a mean of 0.43) (Table 4), were lower than the range observed in populations of *D. versipellis* (from
219 0.009 to 0.750; mean 0.496) reported by Guo et al. (2014). The lower level of allelic diversity at the sites of the SSRs could be the result of the random selection of
220 primers, that were not intentionally selected for high degrees of polymorphism, or the functional constraints of the transcribed regions of the *A. trifoliata* genome
221 (Liang et al. 2015). In summary, the newly identified SSR markers will provide a valuable resource for future genetic studies across a wider range of taxa in
222 Lardizabalaceae and Ranunculales.

223 **Phylogenetic analyses of *A. trifoliata***

224 The long-term over-harvesting and habitat degradation of the natural *A. trifoliata* populations has resulted in their dramatic decline and thus, reasonable conservation
225 strategies and restoration of the accessions is urgently needed (Li et al. 2010a). Furthermore, the lack of systematic planting maps or reliably recorded data makes it
226 difficult to identify the available accessions collected from HAAS and JXAF. Their geographical origins and biological characteristics were also not available. In
227 order to improve the conservation and sustainable use of this species, it is necessary to analyze the phylogenetic relationships of these accessions. The 88 *A. trifoliata*
228 accessions were found to be grouped within four main clades by the clustering analysis, which is in accord with the results of the population structure analyses (Fig. 4
229 and Fig. 5). According to the divisions of the Qinling-Huaihe line, the northern and southern accessions were clustered into different groups, but no clear
230 geographical patterns (city or origin) were observed among the southern accessions. These results showed that not only the geographical environment had a certain
231 effect on the differences of the accessions, but other factors such as altitude, climatic, differences biological characteristics, gene flow, and genetic drift may also
232 affect the genetic diversity of the accessions (Wróblewska 2014).

233 The relationships of these accessions were clarified and could contribute to their protection, as well as the industrial development of *A. trifoliata*. The present
234 results not only provide a valuable analysis of polymorphic molecular markers in this species, but also improve the utilization of *A. trifoliata* accessions collections in
235 conservation genetics, linkage mapping, genetic breeding, and MAS breeding for this commercially and medicinally valuable species.

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332 **Tables**

333 **Table 1.** Sampling details of 88 *A. trifoliata* accessions from different regions used in the present study for SSR development and characterization.

334 **Table 2.** Summary of sequencing data of *A. trifoliata* transcriptome

335 **Table 3.** The statistical data of unigenes annotation

336 **Table 4.** Summary of simple sequence repeats (SSRs) identified from the *A. trifoliata* material

337 **Table 5.** Characteristics of 49 SSR primers that amplified successfully and exhibited polymorphism in *A. trifoliata*

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344 **Figure captions**

345 **Fig. 1.** Geographical distribution of collected *A. trifoliata* accessions

346 **Fig. 2.** The frequencies of SSR repeat motif, and the length of repeats in *A. trifoliata*. (A) The frequencies of the length of repeats. (B) The distribution of the SSR
347 repeat motif.

348 **Fig. 3.** Distribution of the number of repeats and principal type in SSR motif in *A. trifoliata*. (A) The distribution of the number of repeats. (B) The number of
349 principal type in SSR motif

350 **Fig. 4.** Phylogenetic tree of 88 *A. trifoliata* accessions. Blue lines: Clade I; Green lines: Clade II; Purple lines: Clade III; Red lines: Clade IV

351 **Fig. 5.** Population structure analysis of 88 *A. trifoliata* accessions. (A) Population structure based on K = 2 and 4. When K = 4, Red: Group 1; green: Group 2; blue:
352 Group 3; yellow: Group 4. (B) Delta K based on the rate of change of L (K) between successive K values. (C) Evanno table output.

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355 **Supplementary material**

356 **Supplementary Fig. 1** The size distribution of the assembled transcript and unigene sequences of *A. trifoliata*

357 **Supplementary Fig. 2** Polymorphism validation of microsatellite primer P009 and P023 in 88 individuals of *A. trifoliata* Lane M 100bp DNA marker.

358 **Supplementary Table S1** Main repeat motif in the different type of repeat

359 **Supplementary Table S2** Characterization of all microsatellite loci, described by ID, forward and reverse primer sequences, T_m, size, product size (bp), start and
360 end of product size.

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Table 1. Sampling details of 88 *A. trifoliolate* accessions from different regions used in the present study for SSR development and characterization

No.	Name	collection site	The geographical place of origin (China)	preservation site (China)	Unit abbreviation
1	HA01	Changsha city, Hunan province	Unknown	Hunan academy of agricultural sciences	HAAS
2	HA02	Changsha city, Hunan province	Unknown	Hunan academy of agricultural sciences	HAAS
3	HA03	Changsha city, Hunan province	Unknown	Hunan academy of agricultural sciences	HAAS
4	HA04	Changsha city, Hunan province	Unknown	Hunan academy of agricultural sciences	HAAS
5	HA05	Changsha city, Hunan province	Unknown	Hunan academy of agricultural sciences	HAAS
6	HA06	Changsha city, Hunan province	Unknown	Hunan academy of agricultural sciences	HAAS
7	HA07	Changsha city, Hunan province	Unknown	Hunan academy of agricultural sciences	HAAS
8	HA08	Changsha city, Hunan province	Unknown	Hunan academy of agricultural sciences	HAAS
9	HA09	Changsha city, Hunan province	Unknown	Hunan academy of agricultural sciences	HAAS
10	HA10	Changsha city, Hunan province	Unknown	Hunan academy of agricultural sciences	HAAS
11	HA11	Changsha city, Hunan province	Unknown	Hunan academy of agricultural sciences	HAAS
12	HA12	Changsha city, Hunan	Unknown	Hunan academy of agricultural sciences	HAAS

		province				
13	HA13	Changsha city, Hunan	Unknown	Hunan academy of agricultural sciences		HAAS
		province				
14	HA14	Changsha city, Hunan	Unknown	Hunan academy of agricultural sciences		HAAS
		province				
15	HA15	Changsha city, Hunan	Unknown	Hunan academy of agricultural sciences		HAAS
		province				
16	HA16	Changsha city, Hunan	Unknown	Hunan academy of agricultural sciences		HAAS
		province				
17	HA17	Changsha city, Hunan	Unknown	Hunan academy of agricultural sciences		HAAS
		province				
18	HA18	Changsha city, Hunan	Unknown	Hunan academy of agricultural sciences		HAAS
		province				
19	HA19	Changsha city, Hunan	Unknown	Hunan academy of agricultural sciences		HAAS
		province				
20	JF01	Nanchang city, Jiangxi province	Unknown	Jiangxi academy of forestry		JXAF
21	JF02	Nanchang city, Jiangxi province	Unknown	Jiangxi academy of forestry		JXAF
22	ShangraoNo.1	Shangrao city, Jiangxi province	Shangrao city, Jiangxi province	Jiangxi academy of forestry		JXAF
23	JF04	Nanchang city, Jiangxi province	Unknown	Jiangxi academy of forestry		JXAF
24	JF05	Nanchang city, Jiangxi province	Unknown	Jiangxi academy of forestry		JXAF
25	JF06	Nanchang city, Jiangxi province	Unknown	Jiangxi academy of forestry		JXAF

		Jiangxi province					
26	JF07	Nanchang	city,	Unknown	Jiangxi academy of forestry		JXAF
		Jiangxi province					
27	JF08	Nanchang	city,	Unknown	Jiangxi academy of forestry		JXAF
		Jiangxi province					
28	JF09	Nanchang	city,	Unknown	Jiangxi academy of forestry		JXAF
		Jiangxi province					
29	JF10	Nanchang	city,	Unknown	Jiangxi academy of forestry		JXAF
		Jiangxi province					
30	JF11	Nanchang	city,	Unknown	Jiangxi academy of forestry		JXAF
		Jiangxi province					
31	ShangraoNo.2	Shangrao	city, Jiangxi	Shangrao	city, Jiangxi	Jiangxi academy of forestry	JXAF
		province		province			
32	NingguoNo.1	Nanchang	city,	Ningguo	city, Anhui	Jiangxi academy of forestry	JXAF
		Jiangxi province		province			
33	JF14	Nanchang	city,	Unknown	Jiangxi academy of forestry		JXAF
		Jiangxi province					
34	JF15	Nanchang	city,	Unknown	Jiangxi academy of forestry		JXAF
		Jiangxi province					
35	JF16	Nanchang	city,	Unknown	Jiangxi academy of forestry		JXAF
		Jiangxi province					
36	JF17	Nanchang	city,	Unknown	Jiangxi academy of forestry		JXAF
		Jiangxi province					
37	ChangdeNo.1	Changde	city, Hunan	Changde	city, Hunan	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC
		province		province			
38	ChangdeNo.2	Changde	city, Hunan	Changde	city, Hunan	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC

39	ChangdeNo.3	province Changde city, Hunan province	province Changde city, Hunan province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC
40	ChangdeNo.4	province Changde city, Hunan province	province Changde city, Hunan province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC
41	LuoyangNo.1	province Luoyang city, Henan province	province Luoyang city, Henan province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC
42	LuoyangNo.2	province Luoyang city, Henan province	province Luoyang city, Henan province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC
43	LuoyangNo.3	province Luoyang city, Henan province	province Luoyang city, Henan province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC
44	LuoyangNo.4	province Luoyang city, Henan province	province Luoyang city, Henan province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC
45	LuoyangNo.5	province Luoyang city, Henan province	province Luoyang city, Henan province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC
46	LuoyangNo.6	province Luoyang city, Henan province	province Luoyang city, Henan province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC
47	LuoyangNo.7	province Luoyang city, Henan province	province Luoyang city, Henan province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC
48	LuoyangNo.8	province Luoyang city, Henan province	province Luoyang city, Henan province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC
49	LuoyangNo.9	province Luoyang city, Henan province	province Luoyang city, Henan province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC
50	LuoyangNo.10	province Luoyang city, Henan province	province Luoyang city, Henan province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC
51	Luoyang No.11	Luoyang city, Henan	Luoyang city, Henan	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC

		province	province			
52	DengfengNo.1	Dengfeng city, Henan province	Dengfeng city, Henan province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC	
53	DengfengNo.2	Dengfeng city, Henan province	Dengfeng city, Henan province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC	
54	DengfengNo.3	Dengfeng city, Henan province	Dengfeng city, Henan province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC	
55	DengfengNo.4	Dengfeng city, Henan province	Dengfeng city, Henan province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC	
56	DengfengNo.5	Dengfeng city, Henan province	Dengfeng city, Henan province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC	
57	DengfengNo.6	Dengfeng city, Henan province	Dengfeng city, Henan province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC	
58	DengfengNo.7	Dengfeng city, Henan province	Dengfeng city, Henan province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC	
59	DengfengNo.8	Dengfeng city, Henan province	Dengfeng city, Henan province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC	
60	DengfengNo.9	Dengfeng city, Henan province	Dengfeng city, Henan province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC	
61	DengfengNo.10	Dengfeng city, Henan province	Dengfeng city, Henan province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC	
62	DengfengNo.11	Dengfeng city, Henan province	Dengfeng city, Henan province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC	
63	DengfengNo.12	Dengfeng city, Henan province	Dengfeng city, Henan province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC	
64	DengfengNo.13	Dengfeng city, Henan province	Dengfeng city, Henan province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC	

65	DengfengNo.14	province Dengfeng city, Henan province	province Dengfeng city, Henan province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC
66	DazhouNo.1	Dazhou city, Sichuan province	Dazhou city, Sichuan province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC
67	DazhouNo.2	Dazhou city, Sichuan province	Dazhou city, Sichuan province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC
68	DazhouNo.3	Dazhou city, Sichuan province	Dazhou city, Sichuan province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC
69	DazhouNo.4	Dazhou city, Sichuan province	Dazhou city, Sichuan province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC
70	DazhouNo.5	Dazhou city, Sichuan province	Dazhou city, Sichuan province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC
71	DazhouNo.6	Dazhou city, Sichuan province	Dazhou city, Sichuan province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC
72	ZhangjiajieNo.1	Zhangjiajie city, Hunan province	Zhangjiajie city, Hunan province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC
73	ZhangjiajieNo.2	Zhangjiajie city, Hunan province	Zhangjiajie city, Hunan province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC
74	SanmingNo.1	Sanming city, Fujian province	Sanming city, Fujian province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC
75	EnshiNo.1	Enshi autonomous prefecture, Hubei province	Enshi autonomous prefecture, Hubei province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC
76	EnshiNo.2	Enshi autonomous	Enshi autonomous	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC

		prefecture, province	Hubei	prefecture, province	Hubei		
77	GanzhouNo.1	Ganzhou city, Jiangxi province		Ganzhou city, Jiangxi province		Institute of bast fiber crops, Chinese academy of agricultural science	IBFC
78	ZhuzhouNo.1	Zhuzhou city, Hunan province		Zhuzhou city, Hunan province		Institute of bast fiber crops, Chinese academy of agricultural science	IBFC
79	XiangxiNo.1	Xiangxi autonomous prefecture, province	Hunan	Xiangxi autonomous prefecture, province	Hunan	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC
80	EnshiNo.3	Enshi autonomous prefecture, province	Hubei	Enshi autonomous prefecture, province	Hubei	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC
81	NanyangNo.1	Nanyang city, Henan province		Nanyang city, Henan province		Institute of bast fiber crops, Chinese academy of agricultural science	IBFC
82	EnshiNo.4	Enshi autonomous prefecture, province	Hubei	Enshi autonomous prefecture, province	Hubei	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC
83	HanzhongNo.1	Hanzhong city, Shanxi province		Hanzhong city, Shanxi province		Institute of bast fiber crops, Chinese academy of agricultural science	IBFC
84	HanzhongNo.2	Hanzhong city, Shanxi province		Hanzhong city, Shanxi province		Institute of bast fiber crops, Chinese academy of agricultural science	IBFC
85	HanzhongNo.3	Hanzhong city, Shanxi province		Hanzhong city, Shanxi province		Institute of bast fiber crops, Chinese academy of agricultural science	IBFC
86	HanzhongNo.4	Hanzhong city, Shanxi province		Hanzhong city, Shanxi province		Institute of bast fiber crops, Chinese academy of agricultural science	IBFC

87	HanzhongNo.5	Hanzhong city, Shanxi province	Hanzhong city, Shanxi province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC
88	EnshiNo.5	Enshi autonomous prefecture, Hubei province	Enshi autonomous prefecture, Hubei province	Institute of bast fiber crops, Chinese academy of agricultural science	IBFC

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Table 2. Summary of sequencing data of *A. trifoliata* transcriptome

Summary of the transcriptome	Number/length
Raw reads	50 364 426
Total bases (Gb)	7.555
Clean reads	25 049 450
Clean bases (Gb)	7.205
Average size of clean reads (bp)	143.81
GC content (%)	45.10%
Q30 percentage (%)	92.56%
Transcripts	101 417
N50 length (bp)	1692
Mean length (bp)	983.28
Unigenes	63 757
N50 length (bp)	1487
Mean length (bp)	769.89

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Table 3. The statistical data of unigenes annotation

Annotated databases	Number and ratio of annotated unigenes	The species provided most of the annotation
Pfam	19 661 (30.84 %)	
Tremble	27 772 (43.56 %)	<i>Arabidopsis thaliana</i> (14 552, 63.8 %)
eggNOG	20 917 (32.81 %)	
Swissport	19 096 (29.95 %)	<i>Nelumbo nucifera</i> (13 247, 47.70 %)
GO	22 798 (35.76 %)	
KEGG	27 309 (42.83 %)	
All	28 504	
Annotation ratio	44.71 %	

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Table 4. Summary of simple sequence repeats (SSRs) identified from the *A. trifoliata* material

SSR information	Number
Total Number of sequences examined	63 757
Total number of identified SSRs	20 169
Number of sequences containing SSRs	14 519
Number of sequences containing more than 1 SSR	4036
Number of SSRs with high quality	9494
Mono-nucleotide repeats	2340
Di-nucleotide repeats	4021
Tri-nucleotide repeats	2850
Tetra-nucleotide repeats	207
Penta-nucleotide repeats	43
Hexa-nucleotide repeats	33

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Table 5. Characteristics of 49 SSR primers that amplified successfully and exhibited polymorphism in *A. trifoliata*

Name	Gene_ID	Repeat motif	Primer pair (5'-3')	Size (bp)	MAF ^[1]	N _a ^[2]	Gene diversity	H _o ^[3]	PIC value ^[4]
P003	c14599_c1_g3	(GAC)5	AACGCCTCCAAAGGGATGAG GAGGAGATGAGGTGAAGCCG	252	0.7273	3	0.4307	0.3636	0.3875
P004	c6766_c0_g1	(TTC)5	GCGCTCTTCTCTTCTGTGGT GGTAGTTCGGTGCCAGTTGA	231	0.5977	3	0.5586	0.0000	0.4954
P005	c11874_c0_g1	(AG)7 (G)11	CAACCAATTGCTGCCCATGG GCGCTCTATCTTGGCTGGAT	177	0.8750	2	0.2188	0.0000	0.1948
P009	c13416_c3_g1	(TC)12	AGAAGGGAGGACGTATGCCT TGGGAGGGAAATCGTGTGTT	216	0.4070	3	0.6433	0.3721	0.5671
P013	c1927_c0_g1	(TC)11	ATGGGTTGTGAAGCGCCATA ACACCAGCCTGTTCTAGTGC	215	0.3614	4	0.6881	0.4699	0.6271
P019	c37961_c0_g1	(TG)6(TC)6	AGTGCCTAACCAACCCATGT GCGCAGAGCTTCGATTCTTG	191	0.5556	3	0.5328	0.0000	0.4342
P020	c38769_c0_g1	(AAAT)5	ACCATTGAAACCAGTTGACCA ATGTGGGTTGGGCTTGGTAG	206	0.4070	3	0.6585	0.1628	0.5848
P021	c14906_c0_g2	(T)10 (A)12 (T)17	AATGGATGGCGTTTCTCAGT CTGTTTCCACCACCCCATGA	252	0.7073	2	0.4140	0.4390	0.3283
P022	c14528_c1_g1	(CCA)5	AGCTGGGCTAAGTTGGCAAA GTGTCATCGCTCTCGCTGTA	216	0.4706	4	0.6626	0.2000	0.6046

^[1] Major allele frequency
^[2] Number of alleles
^[3] Observed heterozygosity
^[4] Value of polymorphism information content

P024	c5508_c0_g1	(CT)8 (AG)7	GGCTGAGCCCTCATACACTT GCTTTGGGCATAAACGACGA	280	0.6566	3	0.5003	0.1687	0.4400
P025	c6825_c0_g1	(TC)19	GAGGCCAGCTTTACAGAGCA GTCAAGAGAGAGGTGGTGCA	242	0.4489	3	0.6291	0.9432	0.5518
P026	c13046_c0_g1	(GAT)6	CCAGCGCGGTAAAGAAACTG GCTCACCACCATTCCCA	170	0.7045	2	0.4163	0.0000	0.3297
P027	c13580_c0_g1	(CAT)8	GCCCAAGTGTCGAATTGTCC CGTACCCAACCACGTACCAA	230	0.4940	3	0.5789	0.0000	0.4893
P028	c13090_c1_g2	(TC)9	GAGGTGGAGATGGAGGAGGA AACCCTAGTTCTCTCTGCGT	216	0.8125	3	0.3070	0.3750	0.2635
P029	c13943_c3_g1	(AG)10	GAGAGCAGAACTGGAGCCA GCCACAGAGCCATACTTCGA	196	0.6221	3	0.5378	0.4302	0.4773
P030	c14482_c0_g3	(AG)16	AGCAACCGAAGATTCTGCCA CTTCAGACGCTCACACTCGT	158	0.6188	3	0.5445	0.3875	0.4862
P032	c6144_c0_g1	(AG)11	TGTGCGAAGGAGGAAGTGTC CTGCCCAAGTACTAACCAGGT	163	0.5909	3	0.5655	0.3750	0.5026
P034	c13887_c6_g2	(TTTTG)5	TGGAGATGTTGAAATCCCACA GCTCCGAACTGACTAGATCCA	278	0.4226	3	0.6533	0.3571	0.5796
P036	c14068_c2_g1	(CT)9	GCCAACACCTCATCCCTTCA GGGGGACTATTTTGGGTGGG	216	0.7841	2	0.3386	0.0000	0.2813
P039	c14048_c1_g1	(AAAC)5	AAACACAAGGCTGCCACAAC AGGTGGTGCTTTCCATGCTC	245	0.7529	2	0.3720	0.0000	0.3028
P040	c13725_c4_g1	(CCA)7	AAATACACTGTGCACTTCCGA TCCCTCTCTGCTTCGAGTGA	217	0.6860	2	0.4308	0.0000	0.3380
P042	c11753_c0_g1	(AGA)5	ACCAGCAAAGGAAATCCACA AGCGTTCCAGGTTGGTTGAT	233	0.5714	2	0.4898	0.1905	0.3698

P043	c13784_c1_g3	(TG)6 (GA)6	TGGGGATGATCTGGGAGGAA CTTTCCCTCTTGACGCTCC	111	0.5114	2	0.4997	0.1136	0.3749
P045	c13495_c2_g9	(GAG)5	CTTCAATTCGCCTCCGCAAC AAGCAGAGGACGGATTGCTC	263	0.4943	3	0.5515	0.1379	0.4503
P046	c1000_c0_g1	(CT)9	TACAAGTGGGCGGAGATGAC ACACATGCTAAAAGGCCGGT	191	0.6131	3	0.5484	0.5000	0.4887
P048	c12057_c0_g1	(CTT)8	GATGTGAGCCAACCAACACG GCACGGAAGGGTACATGGAA	188	0.6098	2	0.4759	0.1707	0.3627
P050	c14479_c0_g2	(TCA)5	GGACTCGTTGGAACAGACGT TCTCAACACCTTCCTCCCCA	266	0.5747	3	0.5443	0.0230	0.4600
P052	c13640_c0_g1	(GA)10	CACCAATCACAGGCGTTGC AACCCGGGTACATGTTTGCA	141	0.5411	3	0.6019	0.2329	0.5347
P057	c14561_c4_g3	(TC)8	CTCTCTCGTCTTTGCTGCCA CGGTTTGGTCGTAAATCGCA	181	0.5145	3	0.5689	0.4348	0.4784
P059	2584_c0_g1	(CT)11	AGGTGTAGACGAGGTCCTCC TCGCCATCCAAATCTACCATT	198	0.5515	3	0.5855	0.3235	0.5143
P063	c13600_c1_g2	(ACG)5	GGCTCTGAACAAGGAGACGA CCCAATGCTTGTGAGTTCGC	170	0.7467	2	0.3783	0.2133	0.3068
P065	c13206_c1_g2	(AAT)6	GCCCCGATACCCTTCGTTAT TGGTGTCTGACAGAGGAGGT	212	0.6500	2	0.4550	0.1000	0.3515
P067	c6691_c0_g1	(GAA)8	GAGATCCACAAGCGAGCGAA AGCCGCGTTATTCAATCCCT	196	0.5529	4	0.5722	0.0000	0.4962
P068	c10956_c0_g1	(TC)13 (TA)6	GCATGTAACACCCCATCCCA ACGTGCCATTCCAAGGAGAA	254	0.5130	3	0.5236	0.2987	0.4111
P070	c12176_c0_g1	(AG)11 (TAGA)7	AGGAGTAAGCAAACCTCAGCAGT TCACGCCCCCTTTACGATCTC	135	0.4013	4	0.6791	0.4079	0.6180

P071	c12176_c0_g1	(AAG)5	TTCAGACCAGGAGGGACCAT GGCCTCGGAAATCACCCCTTA	229	0.6329	2	0.4647	0.0253	0.3567
P072	c13823_c4_g3	(TGC)7	AGCTTTGATGGGGATGGGTG CTCTCAGGTTAGCCCGCATT	250	0.6081	3	0.5008	0.3108	0.4048
P074	c13519_c0_g1	(TC)14	GGCGACTTACCTGATCCGAG GCGATCATCTTTCGGCAACC	160	0.4695	3	0.6232	0.5000	0.5465
P077	c15128_c1_g1	(AGG)5	CAGCAACACCAAGAAGCCAC ATCTAAGGGCGGACCAGAGT	240	0.6148	3	0.5357	0.4754	0.4686
P078	c15128_c1_g1	(TTA)5	GCGACCATGAAACCCTAGCT CCCTCACATCTCAACTAGCGG	267	0.5575	2	0.4934	0.0345	0.3717
P084	c14885_c1_g4	(AG)9	ACAGGTACATCAACGCGGTT GCAAACCCAATCCCCATCCT	203	0.6105	4	0.5571	0.1860	0.5035
P085	c14885_c1_g4	(ACA)8	CGGAGATCCACTCGTTCGAG TCGCCAGTCATCGATTTCGT	159	0.5390	3	0.5196	0.2987	0.4090
P086	c14018_c1_g2	(AG)7 (T)13	GGGAAAAATCGTAACGCCGT TCGAACGCCATCGATTCTCT	242	0.7278	2	0.3962	0.0886	0.3177
P089	c15100_c2_g1	(AG)10	GGTTTGGTGCCTGATGACCT CTTCAGCCCAGTCGTGACTT	185	0.9529	2	0.0897	0.0000	0.0857
P090	c12812_c2_g2	(CCA)5	TGGTTCATCTTGAGTGCGT TCTGGAAGGAAGGGAGGGAG	208	0.8023	2	0.3172	0.0000	0.2669
P091	c12812_c2_g2	(TGC)5	TGGTTCATCTTGAGTGCGT AGACTTCTCAGGCACACGTG	194	0.6829	3	0.4786	0.0000	0.4262
P092	c12812_c2_g2	(AAGA)5	CACGTGTGCCTGAGAAGTCT CACAGCCGATCCTTCCAAC	250	0.7841	3	0.3596	0.1705	0.3278
P096	c15106_c0_g3	(CAT)6	GCGGTACATTCCCCGGTAAA ACTCGTAATCCTGTTGGCCG	241	0.5647	2	0.4916	0.1412	0.3708

P100	c13025_c2_g1	(CAA)6	GTTCGCGTGGGAAGAACAAC ACCTTCTGATGCGCTTCGAA	217	0.4651	4	0.6170	0.4070	0.5416
Mean					0.6033	3	0.5020	0.2210	0.4261

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297x210mm (300 x 300 DPI)

254x190mm (300 x 300 DPI)

254x190mm (300 x 300 DPI)

338x190mm (300 x 300 DPI)

254x190mm (300 x 300 DPI)