Development of EST-SSR Markers in *Albizia odoratissima* and Their Interspecific Transferability: Postprint

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Abstract

Albizia odoratissima is a precious timber tree species endemic to southern China. Conducting population genetic studies on its germplasm resources is of great significance for their conservation and utilization. This study designed and developed EST-SSR primers based on transcriptome sequencing data of *A. odoratissima*, and evaluated their transferability in related species including *Albizia lebbeck*, *Falcataria moluccana*, *Acacia melanoxylon*, and *Erythrophleum fordii*. The results showed that among 243 primer pairs developed, 171 successfully amplified target bands, with effective amplification rates of 63.79%, 33.75%, 45.68%, 41.56%, and 14.81% in *A. odoratissima*, *A. lebbeck*, *F. moluccana*, *A. melanoxylon*, and *E. fordii*, respectively; polymorphism ratios were 23.87%, 12.20%, 9.01%, 3.96%, and 2.78%, respectively; and 18 primer pairs were universally applicable across all five species. Validation yielded 37 polymorphic SSR markers for *A. odoratissima*, 10 each for *A. lebbeck* and *F. moluccana*, 4 for *A. melanoxylon*, and 1 for *E. fordii*. The EST-SSR markers developed for *A. odoratissima* can meet the requirements for population genetic studies on this species and demonstrate good transferability and research utility in related species such as *A. lebbeck* and *F. moluccana*. In summary, these EST-SSR markers will provide reliable tools for genetic diversity assessment of germplasm resources, construction of fingerprinting profiles for breeding materials, and analysis of population mating systems in *A. odoratissima* and related species.

Full Text

Development and Interspecific Transferability of EST-SSR Markers for *Albizia odoratissima*

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Abstract

Albizia odoratissima is a precious timber tree species endemic to southern China. Population genetics research on its germplasm resources is of great significance for the conservation and utilization of this species. This study designed and developed EST-SSR primers based on transcriptome sequencing data of *A. odoratissima* and analyzed their transferability in related species including *Albizia procera*, *Falcataria moluccana* (formerly *Albizia falcataria*), *Acacia melanoxylon*, and *Erythrophloeum fordii*. Among the 243 primer pairs developed, 171 successfully amplified target bands. The effective amplification rates in *A. odoratissima*, *A. procera*, *F. moluccana*, *A. melanoxylon*, and *E. fordii* were 63.79%, 33.75%, 45.68%, 41.56%, and 14.81%, respectively, while polymorphism ratios were 23.87%, 12.20%, 9.01%, 3.96%, and 2.78%, respectively. Eighteen primer pairs were universally applicable across all five species. In total, 37 polymorphic SSR markers were obtained for *A. odoratissima*, 10 each for *A. procera* and *F. moluccana*, 4 for *A. melanoxylon*, and 1 for *E. fordii*. The developed EST-SSR markers can meet the requirements for population genetics research on *A. odoratissima* and demonstrate good transferability and practical utility in related species such as *A. procera* and *F. moluccana*. These EST-SSR markers will provide reliable tools for genetic diversity evaluation, fingerprinting of breeding materials, and population mating system analysis in *A. odoratissima* and its related species.

Keywords: *Albizia odoratissima*, EST-SSR, molecular marker, transferability, polymorphism

Introduction

Genetic diversity is a core component of conservation biology research and represents the product of long-term evolution. Studies on population genetic diversity are essential for evaluating a population' s adaptive capacity to environmental changes, revealing the mechanisms underlying biodiversity and ecosystem function maintenance, identifying threatening factors, and formulating effective conservation strategies for germplasm resources (Meng et al., 2020).

Albizia odoratissima (L.f.) Benth. is a large evergreen tree belonging to the subfamily Mimosoideae of the family Leguminosae. It is distributed across Fujian, Guangdong, Guangxi, Guizhou, Yunnan, Sichuan, and Hainan provinces in China (Wei et al., 2020). The species exhibits rapid growth, high timber yield, and strong natural regeneration capacity, making it a high-value afforestation species with substantial development potential. Additionally, *A. odoratissima* possesses significant medicinal value; its roots are used in traditional medicine to treat rheumatism, traumatic injuries, bleeding, skin diseases, and insomnia

(Jiang, 2003; Cai, 1996). Despite attracting scholarly attention since the 1990s due to its high economic value, research has primarily focused on community composition, medicinal properties, and cultivation techniques, with limited studies on population genetics and a lack of available molecular markers.

Beyond *A. odoratissima*, many valuable timber species exist within the genus *Albizia* and related taxa, including *Albizia procera* (siris), *Acacia melanoxylon* (blackwood), *Falcataria moluccana* (Moluccan albizia, previously *Albizia falcataria*), and *Erythrophloeum fordii* (Ford' s erythrophleum). Research on these species has similarly concentrated on cultivation techniques, community characteristics, and medicinal properties, while population genetics remains understudied. Currently, only 82 SSR primer pairs have been published for the genus *Acacia*, with no SSR primers reported for *Albizia*, *Falcataria*, or *Erythrophloeum*, thereby limiting in-depth molecular genetic research on these species. Appropriate molecular markers are imperative for conducting genetic diversity evaluation, comparative genomics, and gene expression mapping in these species.

Simple sequence repeats (SSRs), also known as microsatellites, are tandem repeats of 1–6 nucleotide motifs spanning dozens of nucleotides (Tautz, 1989). SSRs are abundant throughout eukaryotic genomes and are widely applied in genetic diversity studies, genetic linkage map construction, gene localization, and molecular marker-assisted breeding due to their high polymorphism, reproducibility, codominant inheritance, and specificity (Powell et al., 1996). SSRs are classified as genomic SSRs (G-SSRs) or expressed sequence tag SSRs (EST-SSRs). G-SSR development is complex, costly, and inefficient, whereas EST-SSRs are derived from expressed sequences and exhibit higher sequence conservation and greater transferability among plant species (Wang and Yang, 2017; Zhang and Tang, 2010; Preethi et al., 2020). Consequently, this study developed molecular markers based on transcriptome sequencing data of *A. odoratissima*.

Numerous studies have demonstrated that SSR primers exhibit certain transferability among species and even between distantly related taxa (Zhong et al., 2012). Zhang et al. (2020) reported transferability rates of sisal EST-SSR primers in *Agave*, *Yucca*, and *Furcraea* at 68%, 52%, and 52%, respectively. Zhang et al. (2019) found that 66.67% of peach EST-SSR primers amplified polymorphic products in Rosaceae species. Fang et al. (2018) observed 53.5% transferability of cotton SSR markers to kenaf. These findings confirm that investigating EST-SSR primer transferability among related species is effective and feasible.

This study designed EST-SSR primers based on *A. odoratissima* transcriptome sequences and evaluated their transferability in *A. odoratissima*, *A. procera*, *A. melanoxylon*, *F. moluccana*, and *E. fordii*. Transferring existing primers to these related species can reduce development costs, improve primer utilization efficiency, and provide reliable molecular markers for germplasm resource conservation and genetic diversity studies in these species.

1.1 Experimental Materials

Thirty *A. odoratissima* germplasm accessions were selected for polymorphism analysis. Additionally, four accessions each of *A. odoratissima*, *A. procera*, *A. melanoxylon*, *F. moluccana*, and *E. fordii* were selected for transferability studies (Table 1). *Erythrophloeum fordii* and *A. melanoxylon* materials were collected from the Nanning Forestry Research Institute of Guangxi. The four *A. odoratissima* accessions represented local varieties from Tiandong County and Xilin County in Baise City, Guangxi, as well as from Jianfengling and Bawangling in Hainan Province. Two *F. moluccana* accessions were collected from the Tree Garden of Guangdong Academy of Forestry and two from Guangxi Institute of Forestry Science. Four *A. procera* accessions were collected from Guangxi Institute of Forestry Science. Materials of *E. fordii*, *A. melanoxylon*, and *A. procera* were immediately stored in sampling boxes after collection due to convenient sampling locations, then transferred to -80°C freezers. *Albizia odoratissima* and *F. moluccana* materials were dried in silica gel immediately after collection due to distant sampling locations and difficult access.

1.2 SSR Marker Development

Transcriptome sequencing was performed on *A. odoratissima* leaves. Using the sequencing data, Novofinder software was employed to search for SSR loci among 38,107 unigenes. The screening criteria included: repeat motifs of 3–5 nucleotides with $ $5 repeats, and sequence lengths of 18–26 bp. Primers were synthesized by Guangzhou Aiji Biotechnology Co., Ltd.

1.3 DNA Extraction

Genomic DNA was extracted from fresh leaves of *A. odoratissima* and related species. Leaves were ground into powder using liquid nitrogen, and DNA was extracted using the Ezup Column Plant Genomic DNA Extraction Kit. DNA concentration was measured using a microspectrophotometer, and samples were stored at -20°C.

1.4 PCR Amplification

PCR amplification was performed in a 10 μL reaction volume containing 1 μL DNA ($60\ \mathrm{ng \cdot \mu L^{-1}}$), 0.2 μL dNTPs, 0.25 μL each of forward and reverse primers, 1 μL 10×PCR Buffer, 0.1 μL Taq DNA Polymerase ($5\ \mathrm{U \cdot \mu L^{-1}}$), and 7.2 μL ddH_2O. Amplification was conducted in a thermal cycler with the following program: initial denaturation at 94°C for 4 min; 25 cycles of denaturation at 94°C for 15 s, annealing at 58°C for 15 s, and extension at 72°C for 30 s; final extension at 72°C for 20 min; and storage at 12°C.

1.5 Detection of Amplification Products

Amplification products were separated by 8% polyacrylamide gel electrophoresis at 240 V constant voltage (typically 50-55 min), with electrophoresis time adjusted based on bromophenol blue indicator position. Gels were washed twice with ddH_2O, fixed for 10 min, washed twice with distilled water (2 min each), stained in 0.15% $AgNO_3$ solution for 6-7 min, washed twice with ddH_2O (2 min each), developed in developer solution until bands were clear, washed twice with ddH_2O, and then photographed.

1.6 Data Statistics and Processing

Bands were scored manually. For each primer’ s amplification products, bands were labeled A, B, C···in descending order of size. Popgene 1.32 software was used to calculate genetic diversity indices for the 30 samples. Polymorphism information content (PIC) was calculated using the formula from Yang (2004), where PIC represents polymorphism information content, Pi and Pj are frequencies of the ith and jth alleles, and n is the number of alleles. Effective amplification rate was defined as the ratio of primers that successfully amplified to the total number of primers tested.

Results

2.1 SSR Repeat Motif Types in *Albizia odoratissima* Transcriptome

RNA transcriptome sequencing of nine *A. odoratissima* samples identified 33,335 SSR loci. Statistical analysis of repeat motif types revealed six major categories: mono-, di-, tri-, tetra-, penta-, and hexa-nucleotide repeats (Table 2). Mononucleotide repeats accounted for 64.73% of total SSR loci, dinucleotide repeats for 20.64%, trinucleotide repeats for 12.30%, and tetra-, penta-, and hexa-nucleotide repeats for 1.62%, 0.28%, and 0.42%, respectively.

Among mononucleotide repeats, T/A (45.07%) was most frequent, followed by A/T (44.73%). For dinucleotide repeats, AT (16.97%) was most common, followed by TA (14.34%) and AG/CT (12.42%). Among trinucleotide repeats, AAT/ATT (4.85%) was most frequent, followed by GAA/TTC (4.59%) and TTC/GAA (4.24%). For tetranucleotide repeats, AAAT/ATTT (9.98%) was most common, followed by TTTA/TAAA (9.06%). A total of 399 nucleotide repeat types were identified: 4 mononucleotide, 12 dinucleotide, 60 trinucleotide, 123 tetranucleotide, 65 pentanucleotide, and 135 hexanucleotide types.

SSR repeat numbers ranged from 5 to 68 (Table 3). Repeats of 5-11 times were most abundant, accounting for 54.95% of total SSR loci; 12-18 repeats comprised 34.48%; 19-25 repeats comprised 7.74%; and repeats >25 times were rare (2.83%). Among all repeat numbers, 10 repeats were most common (5,792 loci, 17.38%), followed by 11 repeats (3,441 loci, 10.32%) and 6 repeats (2,926 loci, 8.78%).

2.2 Effectiveness of *Albizia odoratissima* EST-SSR Primers

As shown in Table 4 , among 243 EST-SSR primer pairs, 155 successfully amplified in *A. odoratissima*. Effective amplification rates varied among different core repeat types, with trinucleotide repeat primers showing higher success (66.34%) than pentanucleotide (64.71%) and tetranucleotide (61.11%) repeat primers.

Thirty-seven primer pairs (23.87%) amplified polymorphic bands in *A. odoratissima*. Ten primer pairs with clear, reproducible, and highly polymorphic bands were selected to genotype 30 *A. odoratissima* accessions, amplifying 26 bands total (Figure 1 [Figure 1: see original paper]), with an average of 2.6 bands per primer. Genetic diversity indices for these 10 primers were analyzed using Popgene 1.32 (Table 5). Effective allele numbers ranged from 1.1497 to 2.4557 (mean = 1.8164). Nei’ s gene diversity index (H) ranged from 0.1302 to 0.5928 (mean = 0.4209). Shannon’ s index (I) ranged from 0.2897 to 0.9840 (mean = 0.6771). According to Botstein et al. (1980), PIC > 0.5 indicates high polymorphism, 0.25 < PIC < 0.5 indicates moderate polymorphism, and PIC < 0.25 indicates low polymorphism. PIC values for the 10 primers ranged from 0.1249 to 0.5184 (mean = 0.3517). Except for primers AO-130 and AO-133 (low polymorphism), all primers showed moderate to high polymorphism, indicating their suitability for genetic diversity analysis and fingerprinting of *A. odoratissima* germplasm.

2.4 Transferability Analysis of *Albizia odoratissima* EST-SSR Primers

Among the 171 successfully amplified primer pairs, trinucleotide repeat primers showed effective amplification rates of 34.65%, 42.57%, 44.55%, and 15.84% in *A. procera*, *F. moluccana*, *A. melanoxylon*, and *E. fordii*, respectively. Corresponding rates for tetranucleotide repeats were 32.41%, 44.44%, 41.67%, and 12.04%, while pentanucleotide repeats showed rates of 38.24%, 52.94%, 35.29%, and 20.59%. Eighteen primer pairs were universally applicable across all five species. Species-specific markers included 26 pairs for *A. odoratissima*, 8 for *A. procera*, 5 for *A. melanoxylon*, and 1 for *E. fordii*; no *F. moluccana*-specific markers were identified.

Ten primer pairs amplified polymorphic bands in *A. procera* (12.20% polymorphism ratio), 10 in *F. moluccana* (9.01%), 4 in *A. melanoxylon* (3.96%), and 1 in *E. fordii* (2.78%). Two primer pairs amplified polymorphic bands in three or more species. Amplification results for representative primers are shown in Figure 2 [Figure 2: see original paper]. The average transferability rates of *A. odoratissima* EST-SSR primers were 71.29% for trinucleotide repeats, 70.37% for tetranucleotide repeats, and 67.65% for pentanucleotide repeats, indicating that trinucleotide repeat EST-SSR primers exhibited the highest transferability.

Discussion

3.1 Transferability of *Albizia odoratissima* EST-SSR Primers

Numerous studies have demonstrated that trinucleotide EST-SSR primers show higher transferability than other repeat types in plants (Wen et al., 2011; Chen et al., 2005; Li et al., 2008). This study confirmed that trinucleotide repeat primers had the highest transferability rate (71.29%) compared to tetranucleotide (70.37%) and pentanucleotide (67.65%) repeats in *A. odoratissima*.

The overall effective amplification rate of the 243 designed EST-SSR primers was 70.37%, consistent with previous reports of 60–90% effective amplification rates for EST-SSR primers (Gao et al., 2003; Qi et al., 2004; Wei et al., 2008). Several factors may contribute to amplification failure: (1) potential assembly errors in next-generation sequencing data used for primer design, and (2) high GC content in primer sequences leading to dimer or hairpin structures that interfere with polymerase chain reactions (Zhong, 2012).

Among the 171 successfully amplified primer pairs, 26 were not transferable to the other four species, similar to findings by Zhong et al. (2012) who identified 216 primer pairs specific to mung bean. These *A. odoratissima*-specific EST-SSR loci may represent unique sequences valuable for classification and identification of wild or related species.

SSR primer transferability depends on conservation of microsatellite flanking sequences and microsatellite stability during evolution. Coding regions exhibit higher sequence conservation than non-coding regions. Since EST-SSRs originate from coding sequences associated with functional genes, they show higher conservation and transferability than genomic SSRs, with transferability generally correlating with genetic distance (Fu et al., 2011; Liewlaksaneeyanawin et al., 2004; Wen et al., 2011). Li and Sun (2012) reported 42.7% transferability and 56.6% polymorphism for 124 chestnut EST-SSR markers in *Castanopsis fargesii*. Feng et al. (2014) identified 15, 10, and 10 polymorphic markers from 318 masson pine SSR primers in *Pinus elliottii*, *P. caribaea*, and *P. yunnanensis*, respectively. Xu et al. (2016) similarly demonstrated high transferability of *P. yunnanensis* EST-SSR markers to related pine species.

In this study, transferability rates followed the pattern: *A. odoratissima* (63.79%) > *F. moluccana* (45.68%) > *A. melanoxylon* (41.56%) > *A. procera* (33.75%) > *E. fordii* (14.81%). This may reflect that *A. procera*, *F. moluccana*, and *A. melanoxylon* belong to Mimosoideae, while *E. fordii* belongs to Caesalpinioideae, making the former three more closely related to *A. odoratissima* with more homologous sequences and higher amplification success. Interestingly, transferability was higher in *F. moluccana* and *A. melanoxylon* than in *A. procera*, suggesting greater transcriptome similarity between *A. odoratissima* and the former two species—a discrepancy between phenotypic taxonomy and genomic relationships warranting further investigation. The limited number of primers designed in this study, which did not cover the complete EST-SSR repertoire of *A. odoratissima*, may have influenced these results. More comprehensive studies with additional primers tested across congeneric, confamilial, and conspecific plants are needed to validate transferability patterns.

3.2 Polymorphism of *Albizia odoratissima* EST-SSR Primers

Polymorphism information content values for EST-SSR primers vary with species, sample size, and marker number (Wei et al., 2008). This study identified 37 polymorphic primer pairs (23.87%) in *A. odoratissima*. Analysis of 10 highly polymorphic, reproducible SSR primers across 30 *A. odoratissima* accessions revealed an average of 2.6 polymorphic bands per primer, with only primers AO-130 and AO-133 showing low polymorphism. This may reflect the limited genetic background diversity among the 30 accessions, all of which were local varieties from Longlin County, Baise City, Guangxi. Future work should expand collection and utilization of *A. odoratissima* germplasm from diverse geographic regions.

Polymorphic primers were also identified in related species: 10 pairs (12.20%) in *A. procera*, 10 pairs (9.01%) in *F. moluccana*, 4 pairs (3.96%) in *A. melanoxylon*, and 1 pair (2.78%) in *E. fordii*. These markers can basically meet research needs for genetic diversity studies in *A. odoratissima*, *A. procera*, and *F. moluccana*. To further analyze polymorphism in *A. melanoxylon* and *E. fordii*, larger sample sizes from different provinces or countries should be employed.

Current understanding of genetic information for these high-value leguminous timber species remains limited, with molecular genetics research on Mimosoideae and Caesalpinioideae lagging behind other fields. The transferable markers developed in this study will facilitate continued research on genetic diversity and germplasm conservation in *A. odoratissima* and promote collection, conservation, identification, and genetic diversity analysis in related species, particularly *A. procera*, *F. moluccana*, *A. melanoxylon*, and *E. fordii*.

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Note: Figure translations are in progress. See original paper for figures.