Postprint of Genetic Diversity Analysis of Star Anise Germplasm Resources in Guangxi Based on SLAF-Seq

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Abstract

Star anise (Illicium verum), an important characteristic economic forest tree species in Guangxi, exhibits rich genetic variation. To reveal the genetic diversity of star anise germplasm resources in Guangxi, this study employed SLAF-Seq technology to conduct comprehensive identification of single nucleotide polymorphism (SNP) loci in 53 population samples from different geographical regions of Guangxi and 42 artificially selected elite germplasm samples; based on SNP polymorphisms, population genetic structure and genetic diversity analyses were performed on these star anise samples. The results showed that: (1) From the 95 star anise samples, a total of 1,588 Mb of sequencing data and 643,690 SLAF tags were obtained, including 74,434 polymorphic SLAF tags, and after filtering, 2,690,564 population SNPs were obtained. (2) The 95 star anise samples could be divided into two main clusters, with population samples from northern Guangxi, western Guangxi, and parts of central Guangxi clustering into one group; the 42 artificially selected elite germplasm and population samples from southern Guangxi, eastern Guangxi, and parts of central Guangxi clustered into another group. (3) The genetic diversity of populations in northern Guangxi was the highest, followed in order by population samples from eastern Guangxi, central Guangxi, western Guangxi, and southern Guangxi, while the genetic diversity of artificially selected elite star anise germplasm was the lowest. In summary, the SNP molecular markers developed based on SLAF-Seq in this study can effectively analyze the genetic diversity of population samples from different regions of Guangxi and artificially selected elite germplasm, providing an important theoretical reference basis for the conservation and utilization of star anise germplasm resources and the screening of elite germplasm in Guangxi.

Genetic Diversity Analysis of *Illicium verum* Germplasm Resources in Guangxi Using SLAF-Seq

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Abstract: Star anise (*Illicium verum* Hook. f.), as one of the significant characteristic economic forestry species in Guangxi, exhibits rich genetic variation. To elucidate the genetic diversity of star anise germplasm resources in Guangxi, this study employed SLAF-Seq technology to conduct an in-depth investigation of single nucleotide polymorphism (SNP) loci across 53 star anise population samples collected from different geographical regions of Guangxi, along with 42 samples of artificially selected superior germplasm. Based on SNP polymorphisms, population genetic structure and genetic diversity analyses were performed on these star anise samples. The results were as follows: (1) From the 95 star anise samples, a total of 1,588 Mb of sequencing data and 643,690 SLAF tags were obtained, including 74,434 polymorphic SLAF tags, which yielded 2,690,564 population SNPs after filtering. (2) The 95 star anise samples were classified into two major groups: one group comprised population samples from northern, western, and some central Guangxi regions, while the other group included the 42 artificially selected superior germplasm samples together with population samples from southern, eastern, and portions of central Guangxi. (3) Populations from northern Guangxi exhibited the highest genetic diversity, followed sequentially by those from eastern, central, western, and southern Guangxi, while the artificially selected superior germplasm showed the lowest genetic diversity. In summary, this study demonstrates that SNP molecular markers developed from SLAF-Seq can effectively analyze the genetic diversity of regional population samples and artificially selected superior germplasm in Guangxi, providing important theoretical guidance for the conservation, utilization, and superior germplasm selection of star anise resources.

Keywords: *Illicium verum*, SLAF-Seq, SNP, genetic structure, genetic diversity

Star anise (*Illicium verum* Hook.f.), also known as Chinese anise or star aniseed, belongs to the family Illiciaceae and genus *Illicium*. From a phylogenetic perspective, star anise represents an ancient lineage within the angiosperms, revealing its close affinity with Magnoliales and representing an early evolutionary branch of flowering plants (Editorial Committee of China Flora of Chinese Academy of Sciences, 2004; Chen et al., 2019). The Illiciaceae family is characterized by aromatic odors and distinctive fruit structures, highlighting its special taxonomic status (Sun et al., 2011; Zeng et al., 2014). Star anise is not only an important spice and medicinal resource but also holds significant economic and pharmaceutical value (Wang et al., 2011). The dried mature fruits are rich in essential oils and represent a crucial spice component in the food industry. With its unique sweet and pungent flavor profile, star anise essential oil is widely applied in baked goods, alcoholic beverages, drinks, confectionery, and other food sectors (He et al., 2024). In traditional Chinese medicine, star anise is extensively used to treat cold hernia abdominal pain and cold pain in the waist and knees due to its warming yang, dispelling cold, and regulating qi to relieve pain properties (Chinese Pharmacopoeia Commission, 2020). Guangxi is China’ s largest origin and main production area for star anise, accounting for over 85% of the national planting area and total output (Liao et al., 2023). In 2023, the star anise planting industry achieved an annual output value of 4 billion yuan, and as one of the “Top Ten Guangxi Medicinal Materials,” its development prospects are highly anticipated (Zou et al., 2023).

Germplasm resource evaluation and superior variety selection are critical steps for enhancing the economic and medicinal value of star anise. In Guangxi, benefiting from government policy support, growers have employed grafting and crown replacement techniques to improve low-yield star anise forests and increase productivity. Current research on star anise primarily focuses on its biological characteristics, active components, and pharmacological effects. For instance, Lu et al. (2024) investigated the effects of light-nitrogen interactions on the growth and physiological characteristics of star anise seedlings, finding that light intensity is crucial for seedling development. Additionally, comprehensive reviews on the chemical constituents and pharmacological research progress of star anise have demonstrated that its biological activity depends on valuable secondary metabolites such as monoterpenoids, sesquiterpenoids, phenylpropanoids, and flavonoids (Patra et al., 2020; Sharafan et al., 2022). Meanwhile, Li et al. (2022) identified 20 compounds in star anise fruits and evaluated their antiviral and antioxidant activities, providing a scientific foundation for developing new natural products. However, molecular genetic studies on star anise remain relatively scarce, directly affecting the progress of superior germplasm selection and, to some extent, hindering the scientific development of the star anise industry. Previous studies have primarily relied on morphological traits such as flower color, leaf shape, and fruit external characteristics to classify germplasm resources, lacking genetic evidence support (Sun et al., 2011; Dai and Jiang, 2019). The genetic background of star anise germplasm resources from different Guangxi regions has not been systematically studied, and the genetic variation and population structure among regional populations and artificially selected superior germplasm remain insufficiently elucidated.

SLAF-Seq (Specific Length Amplified Fragment sequencing) is a high-throughput sequencing method combining restriction enzyme digestion and PCR amplification, primarily used for genome-wide single nucleotide polymorphism (SNP) identification and genetic variation analysis (Zhao et al., 2021). This technology can efficiently identify genetic variation sites in plant germplasm resources, deeply revealing genetic mechanisms, evolutionary history, and germplasm relationships, providing theoretical support for superior germplasm breeding and significantly shortening the breeding cycle for new varieties. Due to its efficiency, cost-effectiveness, and suitability for large-scale studies, SLAF-Seq demonstrates broad application prospects in plant genetics, crop improvement, and germplasm resource conservation (Cui et al., 2023). For example, Liu et al. (2024) successfully distinguished 25 different ecological types of *Isatis tinctoria* and revealed their genetic relationships using SLAF-Seq combined with leaf phenotypic characteristic analysis. Additionally, Shen et al. (2023) investigated the genetic structure and diversity of 19 *Gentiana rigescens* populations from different regions using SLAF-Seq, concluding that environmental isolation is one of the main factors causing population differentiation. These studies demonstrate that SLAF-Seq plays an important role in diversity research of traditional medicinal plant germplasm resources. However, this technology has not yet been applied to genetic diversity analysis of star anise germplasm resources.

Addressing the bottleneck of insufficient systematic research on the genetic background of star anise germplasm resources in Guangxi, some scholars have conducted preliminary analyses using second-generation molecular markers and DNA barcoding. Yu et al. (2022) analyzed eight star anise germplasms with different flower colors using SSR molecular marker technology, while Wang et al. (2023) conducted phylogeographic studies on 13 star anise populations in Guangxi based on ITS2 and psbA-trnH sequences. However, due to the limited number of star anise germplasm resources collected in previous studies and the technical limitations of second-generation markers such as low marker density and throughput, research on the genetic diversity of star anise in Guangxi remains insufficiently deep and systematic. This study collected a total of 95 star anise samples from multiple regional populations and artificially selected superior germplasm in Guangxi. Using SLAF-Seq technology, we successfully obtained abundant SNP marker data. With these molecular markers, we conducted in-depth phylogenetic analysis, population structure resolution, and genetic relationship exploration. This work not only reveals the genetic diversity and phylogenetic relationships between regional populations and artificially selected superior germplasm in Guangxi but also provides a solid theoretical foundation and scientific basis for germplasm resource evaluation and new variety breeding of star anise in Guangxi. Therefore, this study holds extremely important theoretical guidance value for the scientific classification, standardized nomenclature, accurate evaluation of germplasm resources, and superior variety breeding of star anise.

1.1 Experimental Materials

Star anise materials were collected from major distribution areas in Guangxi, totaling 95 samples. These included 53 population samples from different regions

(Table 1), comprising 17 samples from southern Guangxi (GS), with seven from Pubei, Qinzhou (PB) and ten from Fangchenggang (FCG); eight samples from central Guangxi (GM), all from Shanglin, Nanning (SL); 16 samples from western Guangxi (GW), with eight from Fengshan, Hechi (FS) and eight from Baise (BS); six samples from northern Guangxi (GN), all from Guilin (GL); and six samples from eastern Guangxi (GE), all from Teng County, Wuzhou (TX). All mentioned local population samples were adult trees grown from local seeds for over 10 years. To avoid collecting duplicate clones from the same individual, single star anise plants spaced more than 50 m apart were collected from each population based on field surveys. The remaining materials consisted of superior germplasm (GV) currently used as scions for star anise grafting and variety replacement, including three samples each of TM, CP, LM, MW, DR, EN, SS, GJ, ME, and HY germplasm, and two samples each of HZ, BH, HZ-1, RG, FJJ, and SJ germplasm, totaling 42 samples. These germplasms all originated from Teng County, Wuzhou, and were artificially selected as commonly used superior scion germplasm. All 95 samples were identified as *Illicium verum* by Professor Huang Rongshao from Guangxi University of Chinese Medicine. Young leaves were collected with longitude and latitude recorded, and the tender leaves of all 95 germplasm samples were snap-frozen in liquid nitrogen and stored at -80°C for future use.

1.2 Star Anise Genomic DNA Preparation

This study used the CTAB method to extract total DNA from the 95 star anise samples. The quality of extracted DNA was assessed via electrophoresis, and DNA concentration and purity were measured using a NanoDrop spectrophotometer. Qualified DNA samples were used for subsequent SLAF-Seq library construction and sequencing.

1.3 High-Throughput Sequencing

First, the genome of *Liriodendron*, a close relative of star anise, was selected as a reference for in silico enzyme digestion prediction to determine suitable enzyme combinations that could generate randomly distributed fragments with low proportions of repetitive sequences in the genome. In this study, following these criteria, the selected restriction enzyme combination was HaeIII and HinCII. This enzyme combination was used to digest the genomic DNA of sequencing samples to construct SLAF sequencing libraries, which were then subjected to PE150 sequencing on the Illumina HiSeq system.

1.4 Data Processing and Variant Detection

To obtain valid data, sequences contaminated with adapters, low-quality reads, and primers were removed. High-quality reads were clustered using LAST (lastal 759) to obtain SLAF tags and construct a “pseudo” reference genome. Reference sequences were selected for each site based on the maximum sequencing depth of the corresponding SLAF tag. High-quality sequencing reads were aligned to the reference genome using bwa software, and local realignment was performed using GATK software. To ensure accurate variant detection results, samtools and GATK were used for detection, and consistent SNP sites (integrity > 0.8 and MAF > 0.05) were obtained for subsequent analysis.

1.5 Data Analysis

Following data mining and SNP detection, SNP information was used for genetic evolution analysis. MEGA X software and the neighbor-joining algorithm (Kimura 2-parameter model, 1,000 bootstrap replicates) were used to construct phylogenetic trees. Population structure analysis of the 95 star anise samples was performed using admixture software based on the maximum likelihood method with K values ranging from 1 to 10, and cross-validation error rates for K values were analyzed. Principal component analysis (PCA) was conducted on the 95 star anise materials using EIGENSOFT software. Genetic diversity analysis was performed based on SNP information for each population using perl scripts written by Biomarker Technologies Co., Ltd.

2.1 Enzyme Digestion Scheme Evaluation and Sequencing Results

Given that no reference genome for star anise is currently available, this study selected the genome of *Liriodendron*, a close relative of star anise (Chen et al., 2019), as a reference. Based on in silico enzyme digestion prediction, the restriction enzymes HaeIII and HinCII were selected, with digested fragment lengths of 364-464 bp designated as SLAF tags. To evaluate sequencing data quality, statistics were compiled for the sequencing data (read numbers, GC content, and Q30) from 95 star anise individuals. The results showed that a total of 1,588 Mb of reads data were obtained from the 95 sequencing samples, with Q30 values ranging from 88.97% to 96.62% (average 92.88%) and GC content ranging from 43.22% to 45.61% (average 44.29%). These results indicate that the sequencing data obtained in this study were of high quality and reliability, meeting the requirements for subsequent analysis.

2.2 SLAF Tag and SNP Statistics

Through bioinformatics analysis, 643,690 SLAF tags (average sequencing depth: 9.71×) were obtained from the 95 star anise germplasm resources, of which 74,434 were polymorphic SLAF tags, generating 2,690,564 population SNPs. The integrity of these SNPs ranged from 14.33% to 47.67% (average 29.35%), while heterozygosity ranged from 2.06% to 7.78% (average 4.46%) (Table 2). To investigate genetic relationships among different star anise germplasms, 229,017 high-quality SNP markers were selected based on the above population SNP results.

2.3 Principal Component and Phylogenetic Analysis

To clarify the phylogenetic relationships among star anise population samples from different Guangxi regions and artificially selected superior germplasm, this study performed principal component analysis (PCA) and phylogenetic analysis on the 95 star anise germplasms based on the selected highly consistent effective SNP variation sites. As shown in Figure 1 [Figure 1: see original paper], PCA analysis divided the 95 star anise germplasms into two clusters (I and II), with cumulative variance contributions of PC1 and PC2 at 8.49%. Cluster I comprised star anise population samples from northern, western, and some central Guangxi regions, distributed on the right side of the principal component coordinate axis with relatively concentrated distribution. Cluster II comprised star anise population samples from southern, eastern, and some central Guangxi regions, along with the 42 artificially selected superior germplasm samples, distributed on the left side of the principal component coordinate axis. Compared with Cluster I, samples in Cluster II showed a more dispersed distribution in PCA space, suggesting that this group of star anise germplasm had diverse origins and relatively higher genetic diversity than Cluster I.

Consistent with the PCA results, phylogenetic tree analysis divided the 95 star anise germplasms into two major groups. Group I consisted of population samples from northern and western Guangxi and three central Guangxi samples (NNSL3, NNSL4, NNSL5). Group II comprised star anise population samples from eastern, southern, and remaining central Guangxi regions, along with the 42 artificially selected superior germplasm materials (Figure 2 [Figure 2: see original paper]). Further analysis of the phylogenetic tree structure allowed subdivision of Group II samples into three subgroups. Most star anise population samples from southern, eastern, and central Guangxi each clustered into separate subgroups (II-1, II-2, II-3), while the 42 artificially selected superior germplasm materials were interspersed among the three subgroups. Specifically, 14 artificially selected superior germplasm samples (HZ1-2, HZ-1-2, EN2-3, SJ1-2, FJJ1-2, RG1-2, BH1-2) clustered with southern Guangxi population samples in subgroup II-1; 21 samples (LM1-3, ME1-3, HY1-3, CP1,3, GJ1,3, MW1-3, SS1-3, MT1-2) clustered with eastern Guangxi population samples in subgroup II-2; and seven samples (CP2, MT3, EN1, GJ2, DR1-3) clustered with central Guangxi population samples in subgroup II-3. These results indicate that the genetic structure of star anise population samples from different regions shows obvious geographic characteristics. Moreover, the artificially selected superior germplasm materials have closer phylogenetic relationships with star anise population samples from southern, eastern, and central Guangxi.

2.4 Population Genetic Structure

To further understand the genetic background relationships of star anise in Guangxi, this study used the above highly consistent SNP molecular markers to analyze the population genetic structure of the 95 star anise germplasms through admixture analysis (Figure 3 [Figure 3: see original paper]). Cross-validation clustering results showed that when K=2, the cross-validation error rate was almost similar to that at K=1, indicating that dividing these samples into either one or two groups was reasonable. When K=2, the 95 star anise samples were divided into two distinct populations (Figure 3A), which was highly consistent with the classification results from PCA and phylogenetic tree analysis. Therefore, K=2 was considered a more reasonable grouping method than K=1. Based on the K=2 grouping results (Figure 3B), Group I samples were predominantly blue genotype, though some samples were mixed with partial red genotype. Group II samples were dominated by red genotype, with some artificially selected superior germplasm in subgroups II-1 and II-2 showing nearly half blue genotype, indicating certain gene flow between these samples and Group I samples.

2.5 Genetic Diversity Analysis

Analyzing genetic diversity can reveal gene flow and genetic variation among different local populations, helping to elucidate the effects of geographic isolation and environmental factors on species genetic structure (Wu et al., 2024). In this study, we calculated genetic diversity parameters for star anise population samples from five Guangxi regions and artificially selected superior germplasm materials (Table 3). The results showed that observed heterozygosity (Ho) among star anise populations ranged from 0.130 to 0.318 (average 0.231), while expected heterozygosity (He) ranged from 0.262 to 0.364 (average 0.320). Notably, observed heterozygosity was lower than expected heterozygosity in all samples, suggesting possible inbreeding within populations. At the population level, minor allele frequency (MAF) ranged from 0.18 to 0.27 (average 0.23). Nei' s diversity index (H) ranged from 0.268 to 0.411 (average 0.350). Shannon-Wiener index (I) ranged from 0.417 to 0.543 (average 0.489). Polymorphism information content (PIC) ranged from 0.219 to 0.291 (average 0.260), indicating that all six populations showed moderate polymorphism (PIC values between 0.25 and 0.5), suggesting certain genetic structure within populations, such as possible subpopulation structure or genetic drift effects. Specifically, star anise populations from northern Guangxi showed the highest values for MAF, H, I, and PIC, followed by populations from eastern, central, western, and southern Guangxi, while artificially selected superior germplasm populations showed the lowest genetic diversity values. These results demonstrate significant differences in genetic diversity among star anise populations from different regions among the 95 samples, with northern Guangxi populations showing the highest genetic diversity and artificially selected superior germplasm populations showing the lowest.

3.1 Advantages of SLAF-Seq in Genetic Diversity Analysis

Star anise has a long cultivation history in Guangxi, with abundant germplasm resources and diverse phenotypes manifested in both external traits and internal quality (Pan et al., 2007). Phenotypic diversity is the product of interaction between genetic diversity and environmental adaptation, exhibiting both stability and variability (Li et al., 2018). Although phenotype-based classification methods are intuitive, their main limitation lies in the difficulty of eliminating environmental effects on morphological characteristics. For example, anthocyanin content in star anise flowers is influenced by various environmental factors such as light and temperature, which significantly regulate color expression (Zeng et al., 2024). Therefore, revealing the genetic diversity of different star anise germplasm resources in Guangxi holds important theoretical significance for germplasm classification, superior germplasm selection, development, and conservation. Compared with traditional molecular marker technologies, SLAF-Seq can provide more genetic markers without reference genome limitations and can be applied to medicinal plant germplasm evaluation, genetic map construction, and phylogenetic analysis (Cui et al., 2023). This study used SLAF-Seq technology for high-throughput sequencing of 53 star anise population samples from five Guangxi regions and 42 artificially selected superior germplasm samples, achieving an average sequencing depth of 9.71× and average Q30 of 92.88%. The study obtained 1,588 Mb reads, 643,690 SLAF tags (including 74,434 polymorphic SLAF tags), 2,690,564 population SNPs, and 229,017 high-consistency SNP sites. Therefore, using these high-throughput SNPs for genetic diversity analysis of 95 star anise samples will substantially improve the resolution and accuracy compared with previous studies.

3.2 Genetic Structure Analysis of Star Anise in Guangxi

Germplasm resources are the foundation for genetic breeding work. Population genetics analysis helps us understand intra-species genetic diversity and structure, thereby guiding germplasm resource conservation and utilization and providing important information for superior germplasm selection and genetic improvement (Yan et al., 2024). In this study, PCA, phylogenetic, and population structure analyses consistently showed that star anise population samples from different Guangxi regions could be divided into two groups with distinct geographic characteristics. Geographic distribution is an important factor affecting population genetic structure (Li et al., 2022). In this study, the clustering of western and northern Guangxi population samples separately from eastern and southern Guangxi samples suggests that under large environmental and climatic differences between introduction regions, star anise may have gradually developed heritable geographic variation during long-term adaptation, eventually differentiating into two major groups, giving star anise genetic structure in Guangxi obvious regional characteristics (Huang, 1994). Star anise germplasm from central Guangxi was distributed in both major groups, possibly because Nanning, as an important economic center and transportation hub along the Beibu Gulf coast, has greatly facilitated star anise introduction and cultivation in this region, increasing gene flow. Furthermore, the genetic background of the superior germplasm collected in this study was closer to star anise populations from southern and eastern Guangxi, indicating that various superior star anise germplasms currently circulating in the market have mainly been selected from planting origins in southern and eastern Guangxi.

3.3 Genetic Diversity Analysis of Star Anise in Guangxi

This study also evaluated the population genetic diversity of different local population samples and artificially selected superior germplasm. The results showed that star anise populations from northern Guangxi exhibited the highest genetic diversity, while local population samples from southern Guangxi showed the lowest. The reasons are as follows: First, northern Guangxi is not an optimal planting region for star anise, and higher genetic diversity helps plant populations adapt to environmental changes. Second, star anise introduction and cultivation in this region have a relatively short history, resulting in minimal artificial selection. Additionally, geographic isolation may be another reason for the high genetic diversity in northern Guangxi (Huang, 1994). In contrast, other regions, especially southern Guangxi (Fangchenggang), have a relatively long star anise cultivation history and may have undergone prolonged artificial selection, potentially reducing genetic diversity. The genetic diversity of artificially selected superior germplasm was lower than that of all regional populations, further supporting this speculation. Expected heterozygosity is a key indicator for measuring population genetic diversity. In this study, observed heterozygosity was lower than expected heterozygosity in all star anise population samples, a phenomenon possibly caused by several factors: (1) gene flow may introduce foreign genes, affecting original genetic diversity; (2) inbreeding may increase homozygote frequency and reduce heterozygote frequency; and (3) natural selection may favor certain alleles, increasing the proportion of specific homozygous genotypes. Considering the actual situation of star anise in Guangxi, we believe that inbreeding (selfing) may be the main factor causing observed heterozygosity to be lower than expected, similar to the findings on genetic diversity of *Artemisia argyi* by Chen et al. (2024). Additionally, the research results of Wang et al. (2023) on frequent gene flow among star anise populations in Guangxi also support this viewpoint.

In summary, this study used SLAF-Seq technology to analyze in detail the genetic structure and diversity among different local population samples and artificially selected superior germplasm in Guangxi. The study provides theoretical references for identifying the genetic background and scientifically systematic classification of these artificially selected superior star anise germplasms, offering a scientific research foundation for advancing star anise variety identification, germplasm classification, and molecular-assisted breeding in Guangxi.

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