Development and Characterization of EST-SSR Markers via Transcriptome Sequencing in Brainea insignis (Aspleniaceae s.l.)

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• Premise of the study: Brainea insignis (Aspleniaceae) is an endangered tree fern in China whose wild populations have been seriously damaged due to overexploitation. Expressed sequence tag–simple sequence repeat (EST-SSR) primers were developed to investigate its genetic diversity and provide resources for future conservation studies.
• Methods and Results: We obtained 72,897 unigenes of B. insignis using transcriptome sequencing and detected 15,006 SSRs in 12,058 unigenes. Based on these results, we designed 100 EST-SSR primer pairs and successfully amplified 52 of them in six individuals; 27 demonstrated polymorphisms after amplification against 72 individuals across three populations. Allele numbers ranged from three to 10, and the observed and expected heterozygosities ranged from 0.105 to 1.00 and from 0.523 to 0.865, respectively, in the tested populations. Most of these primers could be successfully amplified in two other fern species (Blechnum orientale and Chieniopteris harlandii).
• Conclusions: These selected EST-SSRs are valuable for genetic diversity and conservation studies in B. insignis and other related fern species.

Key words: Aspleniaceae s.l.; Brainea insignis; EST-SSR; transcriptome sequencing.

Brainea insignis (Hook.) J. Sm. (Aspleniaceae) is a tree fern that thrived in the Tertiary period (De Gasper et al., 2016) and belongs to the monotypic genus Brainea J. Sm. Currently, it is widely distributed across tropical and subtropical areas of Asia and found on damp and exposed hillsides (300–1700 m a.s.l.) with high light availability and low soil water content (Wang et al., 2013). Brainea insignis has been listed as a protected species (Category II) in China (Order of the Forestry Bureau and Ministry of Agriculture of China, 1999), as well as a near-threatened (NT) species in India (Fraser-Jenkins, 2012). Furthermore, some wild populations near cities in the Pearl River Delta, such as the Huichen population near Huizhou and the Yinpinzui population near Dongguan, have been seriously affected by urbanization in southern China over the past 50 yr. Thus, microsatellite markers, which have been shown to be beneficial to the conservation of other fern species such as Blechnum orientale L. and Chieniopteris harlandii (Hook.) Ching, would be valuable as a first step in assessing the genetic structure and diversity of wild populations of B. insignis.

To date, simple sequence repeat (SSR) markers have only been developed in several fern species, such as Isoetes sinensis Palmer (Gichira et al., 2016), Athyrium distentifolium Tausch ex Opiz (Woodhead et al., 2005), Neottopteris nidus (L.) J. Sm. ex Hook. (Jia et al., 2016), and Huperzia serrata (Thunb.) Trevis. (Luo et al., 2010). No efficient molecular markers have been reported for B. insignis, and no genetic studies have been performed for this species. Brainea insignis, which first appeared during the Devonian period of the Paleozoic Era, is an important relic and endangered fern species that has played a significant role in the origin and evolution of palaeoflora and other ferns (Liao and Zhang, 1994; Liu et al., 2016). The development of reliable SSR markers would be beneficial to studies on genetic diversity, reproductive biology, and phyleogeography of B. insignis and related species.

In this study, the transcriptome of B. insignis was sequenced using the Illumina platform and was de novo assembled into 85,415 transcripts (72,897 unigenes after removing redundant transcripts), which were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) and Transcriptome Shotgun Assembly (TSA) databases (SRR583471 and GFUE00000000; BioProject: PRJNA396460). Based on these sequences, 27 novel polymorphic expressed sequence tag (EST)–SSR primer pairs were developed, their polymorphisms were characterized across three populations.
of *B. insignis*, and their transferability was also inspected with respect to three other ferns.

**METHODS AND RESULTS**

One seedling of *B. insignis* was sampled from Tiantou Mountain in Shenzhen, Guangdong Province, China (Appendix 1), and planted in the greenhouse of Sun Yat-sen University (Guangzhou, Guangdong Province, China). Fresh leaves were collected from the seedling for RNA extraction via the modified cetyltrimethylammonium bromide (CTAB) method (Fu et al., 2004; Chen et al., 2011), and the subsequent protocols for transcriptome sequencing were as follows: mRNAs were extracted from the total RNA using Oligotex-dT30 (TaKaRa Biotechnology Co., Dalian, China) and ultrasonically fragmented and converted to double-stranded cDNAs. After adding an “A” nucleotide at the 5′-end of the cDNAs, adapters were ligated to both ends, and the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) was used to purify and collect the cDNAs of approximately 215 bp in length. Finally, each amplified molecule was cloned, and the inserts were sequenced to identify ESTs.

![Table 1. Characteristics of 27 EST-SSR markers developed for *Brainea insignis*.](https://www.bioone.org/loi/apps)
was sequenced using Illumina sequencing technology (Illumina, San Diego, California, USA) to obtain short reads of 90 bp from both ends. A total of 23.9 million 125-bp paired-end reads were obtained and de novo assembled into 72,897 unigenes using Trinity version 2.3.2 (Grabherr et al., 2011), with a minimum length of 201 bp and an average length of 799 bp. The MISA tool (Thiel et al., 2003) was used with the default parameters except that settings for mononucleotide repeats were removed from analysis; 15,006 SSRs were detected from 12,058 unigenes. Among these SSR loci, dinucleotide repeats (74.66%) were the most common, followed by single nucleotide (13.8%), trinucleotide (10.82%), tetranucleotide (0.63%), hexanucleotide (0.07%), and pentanucleotide (0.03%) repeats. With the help of the online perl scripts p3-in and p3-out (http://pgrc.ipk-gatersleben.de/misa/primer3.html) and Primer3 (Rozen and Skaletsky, 1999), a total of 4,928 primer pairs were successfully designed.

In addition, 72 individuals of Brainea insignis were collected from three populations in Guangdong Province, China. Voucher specimens for these populations were deposited at the Herbarium of Sun Yat-sen University (SYS; Appendix 1). The genomic DNA was extracted from silica-dried leaves using a modified CTAB method (Doyle and Doyle, 1987). The top 100 primer pairs with the highest SSR repeat motifs were synthesized, and PCR amplification was performed on six individuals that were randomly selected from the three populations of Brainea insignis (two individuals for each population). PCR amplifications were performed in 20-μL reaction volumes, containing 25 ng of genomic DNA, 2 μL 10× PCR buffer (with Mg²⁺), 0.25 mM of dNTPs, 0.2 μM of each primer, and 1 unit of Easy-Taq DNA polymerase (TransGen Biotech Co. Ltd., Beijing, China). PCR reactions were conducted with the following conditions: initial denaturing at 94°C for 2 min; followed by 35 cycles of 94°C for 30 s, appropriate annealing temperature (Table 1) for 30 s, and 72°C for 40 s; and a final extension at 72°C for 5 min (Fan et al., 2013). The PCR products were electrophoresed and visualized in 2% agarose gel. The results showed that 52 primer pairs were successfully amplified in six individuals with the expected product sizes. After amplification, the PCR products were further inspected with capillary gel electrophoresis (Fragment Analyzer; Advanced Analytical Technologies) was used to analyze the sample size, and 27 primer pairs showed polymorphisms among the six tested individuals (GenBank accession number: MF150401–MF150427; Table 1). To determine and annotate the putative function, 27 EST-SSRs were compared with the public sequence database, contrasting BLASTX with the nonredundant (NR) protein database. The results showed that 22 primer pairs had significant BLASTX hits to the protein database and that one was annotated as a plastid gene.

The 27 primer pairs were then amplified across all 72 individuals in the three populations to assess their polymorphism levels (Table 2). The number of alleles, observed heterozygosity, and expected heterozygosity were calculated using GenAlEx 6.501 software (Peakall and Smouse, 2012). Null alleles were checked using the program MICRO-CHECKER version 2.2.3 (van Oosterhout et al., 2004). Linkage disequilibrium testing and deviation from Hardy–Weinberg equilibrium (HWE) were carried out using GENEPOP 4.3 (Rousset, 2008). Signs of null alleles were detected on loci BS61 and BS69 in three populations and loci BS80 in one population. The results showed that the number of alleles ranged from three to 10 in all three populations of B. insignis. Observed and expected heterozygosity ranged, respectively, from 0.469 to 1.000 and from 0.539 to 0.840 in population DG, from 0.105 to 1.000 and from 0.566 to 0.842 in population HD, and from 0.286 to 1.000 and from 0.523 to 0.865 in population SZ. The linkage disequilibrium test showed no significantly linked pairs of primers after a Bonferroni correction. HWE tests showed that all three populations significantly deviated from HWE in most loci (Table 2).

Furthermore, individuals of B. orientale (Aspleniaceae) and C. harlandii (Aspleniacae) were collected to test the transferability of these primers. The results showed that 14 primer sets could be amplified in B. orientale, while 13 could be amplified in C. harlandii (Table 3).

**CONCLUSIONS**

In our study, we obtained 72,897 unigenes of B. insignis via transcriptome sequencing and developed 27 novel EST-SSRs for the species. Some of these primers could be amplified in...
B. orientale and C. harlandii, showing good transferability to other fern species. These polymorphic markers are valuable for genetic conservation studies in the endangered B. insignis and other related fern species.

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**Appendix 1.** Location and voucher information of *Brainea insignis* and other related species used in this study.

| Species                  | Location                        | Population code | Geographic coordinates       | Altitude (m) | N  | Voucher specimen |
|--------------------------|---------------------------------|-----------------|------------------------------|--------------|----|------------------|
| *Brainea insignis* (Hook.) J. Sm. | Tiantou Mountain, Shenzhen, Guangdong Province | SZ   | 22°39'25.31"N, 114°25'04.41"E | 243          | 21 | Q. Fan 14447    |
|                          | Jiuulong Mountain, Huidong, Guangdong Province | HD   | 22°57'27.48"N, 114°47'59.30"E | 223          | 19 | Q. Fan 14449    |
|                          | Yinping Mountain, Dongguan, Guangdong Province | DG   | 22°54'14.66"N, 114°13'18.82"E | 138          | 32 | Q. Fan 14456    |
| *Blechnum orientale* L.  | Heishiding Mountain, Fengkai, Guangdong Province | —   | 23°27'53.20"N, 111°54'15.10"E | 180          | 6  | Q. Fan 15495    |
| *Chieniopteris harlandii* (Hook.) Ching | Heishiding Mountain, Fengkai, Guangdong Province | —   | 23°27'49.06"N, 111°53'48.41"E | 335          | 5  | Q. Fan 15497    |

*Note: N = number of individuals.*

*a Vouchers have been deposited at the Herbarium of Sun Yat-sen University (SYS), Guangzhou, Guangdong Province, China.