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Source: Applications in Plant Sciences, 5(10)

Published By: Botanical Society of America

URL: <https://doi.org/10.3732/apps.1700067>

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## DEVELOPMENT AND CHARACTERIZATION OF EST-SSR MARKERS VIA TRANSCRIPTOME SEQUENCING IN *BRAINEA INSIGNIS* (ASPLENIACEAE S.L.)<sup>1</sup>

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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 1.000 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 *Chienopteris 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 *Chienopteris*

*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 relict 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 phylogeography 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 (SRR5883471 and GFUE000000000; 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

<sup>1</sup> Manuscript received 28 June 2017; revision accepted 22 August 2017.

This work was supported by the Administration Bureau of Neilingding-Futian National Nature Reserve in Guangdong (4206874); the Urban Management Bureau of Shenzhen Municipality (71020106 and 71020140); the Basic Work Special Project of the National Ministry of Science and Technology of China (2013FY111500); the Natural Science Foundation of Guangdong Province, China (2016A030313326); and the Science and Technology Planning Project of Guangdong Province, China (2015A030302020).

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doi:10.3732/apps.1700067

*Applications in Plant Sciences* 2017 5(10): 1700067; <http://www.bioone.org/loi/apps> © 2017 Liu et al. Published by the Botanical Society of America. This is an open access article distributed under the terms of the Creative Commons Attribution License (CC-BY-NC-SA 4.0), which permits unrestricted noncommercial use and redistribution provided that the original author and source are credited and the new work is distributed under the same license as the original.

TABLE 1. Characteristics of 27 EST-SSR markers developed for *Brainea insignis*.

| Locus | Primer sequences (5′–3′)                            | Repeat motif          | $T_a$ (°C) | Expected allele size (bp) | A (Allele size range, bp) | Putative function [Organism]                                                                 | GenBank accession no. |
|-------|-----------------------------------------------------|-----------------------|------------|---------------------------|---------------------------|----------------------------------------------------------------------------------------------|-----------------------|
| BS04  | F: TTGTTGCTCGTGGTGATGAT<br>R: GCGCTCTATTTTCGTCGTTC  | (GAGAGG) <sub>6</sub> | 56         | 193                       | 6 (175–205)               | ASR protein [ <i>Ginkgo biloba</i> ]                                                         | MF150401              |
| BS07  | F: GGGGATCAAGTTTGGGTCTT<br>R: AAGCGCTTTCAACCAACCTA  | (GGGAA) <sub>5</sub>  | 58         | 201                       | 10 (171–216)              | Hypothetical protein PGUG_05234 [ <i>Meyerozyma guilliermondii</i> ]                         | MF150402              |
| BS13  | F: AATGGAAGGGGAGAGAGAA<br>R: CTGTGGATGTTGCGGTGTT    | (AGC) <sub>8</sub>    | 58         | 273                       | 9 (237–288)               | VAMP family protein [ <i>Arabidopsis thaliana</i> ]                                          | MF150403              |
| BS14  | F: TTTTAGAGGGGGAAGGGCTA<br>R: CAGTAAAGGCCGGAAGAACA  | (GTG) <sub>8</sub>    | 60         | 149                       | 4 (140–149)               | ENT domain-containing protein [ <i>Arabidopsis thaliana</i> ]                                | MF150404              |
| BS24  | F: AAAACCGGCATTGAGATCAG<br>R: TACTGAAATGCCACCGGAA   | (CATA) <sub>6</sub>   | 60         | 115                       | 5 (139–155)               | RNA-dependent RNA polymerase [ <i>Hantavirus L99</i> ]                                       | MF150405              |
| BS33  | F: TGTGAAGAAAGCATTGACGC<br>R: TTGGCCTCATCATCACTCAC  | (GCA) <sub>7</sub>    | 56         | 101                       | 6 (95–110)                | BolA-like family protein [ <i>Arabidopsis thaliana</i> ]                                     | MF150406              |
| BS35  | F: CACAAGAGCAGCACCATAA<br>R: GGAAAAACACTTCCGGTGAA   | (CCA) <sub>7</sub>    | 58         | 222                       | 11 (222–252)              | RNA-binding protein precursor [ <i>Nicotiana tabacum</i> ]                                   | MF150407              |
| BS38  | F: AGTCGGAGCCAAGCTACAGA<br>R: CATGAGAGTCACCATGTGGG  | (TGA) <sub>7</sub>    | 58         | 234                       | 7 (234–267)               | Protein kinase family protein [ <i>Arabidopsis thaliana</i> ]                                | MF150408              |
| BS43  | F: GGCTCTACATGCATCCTCTTG<br>R: CAGTGGGCCCTTACACACTT | (CAT) <sub>7</sub>    | 60         | 141                       | 5 (135–147)               | No hits                                                                                      | MF150409              |
| BS46  | F: CTCTCCTTCTCCGATTGCAC<br>R: GACTCGATCTCGTACTGGGC  | (CTC) <sub>7</sub>    | 58         | 128                       | 4 (125–134)               | Chloroplast 29k Da ribonucleoprotein [ <i>Oryza sativa</i> ]                                 | MF150410              |
| BS47  | F: CGCCTGGTATAGCTGCTCTT<br>R: GGCAATCATCTTTTGGCAGTT | (TGA) <sub>7</sub>    | 60         | 194                       | 5 (188–206)               | Potassium transporter HAK4 [ <i>Hordeum vulgare</i> ]                                        | MF150411              |
| BS48  | F: GATGGGAGCAAGCAATGAT<br>R: ATCTCTCCCAAGACCTCGT    | (GAT) <sub>7</sub>    | 60         | 252                       | 6 (249–288)               | ATP binding/DNA-directed DNA polymerase [ <i>Arabidopsis thaliana</i> ]                      | MF150412              |
| BS51  | F: CATAGCCTCGGCATAGCTTC<br>R: CCCTTCTTCTGGACATA     | (TTG) <sub>7</sub>    | 60         | 263                       | 6 (260–275)               | RNA methyltransferase family protein [ <i>Arabidopsis thaliana</i> ]                         | MF150413              |
| BS58  | F: ACCATGATGACGATGGTGAA<br>R: CGATTTGCTGTAGTTGCTCG  | (GAT) <sub>7</sub>    | 58         | 168                       | 10 (156–183)              | DEAD/DEAH box helicase, putative (RH28) [ <i>Arabidopsis thaliana</i> ]                      | MF150414              |
| BS61  | F: TGAGCAAAGCTGTTGGATTG<br>R: CATGCCGATACACAAAAC    | (TGA) <sub>7</sub>    | 58         | 249                       | 4 (237–246)               | Phospholipase A1 [ <i>Capsicum annuum</i> ]                                                  | MF150415              |
| BS68  | F: AGGCAGCAAAAACGAAGCTA<br>R: TCTATATACGCCCCACAGCC  | (GAA) <sub>7</sub>    | 58         | 159                       | 5 (150–162)               | No hits                                                                                      | MF150416              |
| BS69  | F: TGAGAGAACTAAGGGGCCAA<br>R: ATGTGCATGGATGCATGAGT  | (TAC) <sub>7</sub>    | 58         | 183                       | 6 (168–183)               | Apocytochrome b [ <i>Helianthus petiolaris</i> ]                                             | MF150417              |
| BS70  | F: ATGTGTGGAGAGCCATCCTT<br>R: TTGTCATGGCAATCACCCT   | (TCC) <sub>7</sub>    | 58         | 223                       | 6 (217–232)               | Proton-dependent oligopeptide transport (POT) family protein [ <i>Arabidopsis thaliana</i> ] | MF150418              |
| BS75  | F: CGCAGCATAAAGCTGAGAGC<br>R: TGATGATGGGCATGTAGACG  | (CAT) <sub>7</sub>    | 60         | 170                       | 4 (176–185)               | ADP-ribosylation/Crystallin J1 [ <i>Beggiatoa</i> sp.]                                       | MF150419              |
| BS78  | F: TGCAACAAAATCGCAGAAG<br>R: GTGAGAGAGGATCGCACCAT   | (CAA) <sub>7</sub>    | 60         | 252                       | 7 (246–267)               | Leukocyte surface antigen CD47 [ <i>Anthurium amnicola</i> ]                                 | MF150420              |
| BS79  | F: CCGTACGAGAAGCCTCAAAG<br>R: GAAGATGATGCCTGACCCAT  | (CAT) <sub>7</sub>    | 58         | 238                       | 9 (235–259)               | Solute carrier family 35 member B3, related [ <i>Medicago truncatula</i> ]                   | MF150421              |
| BS80  | F: GCCTGCACATTAACAGGTGA<br>R: CCCGCGTTTTTAAGATCAGA  | (GAG) <sub>7</sub>    | 60         | 232                       | 6 (229–244)               | Exodeoxyribonuclease V, alpha subunit [ <i>Burkholderia pseudomallei</i> ]                   | MF150422              |
| BS81  | F: CCACTAGAATGCTTGCACGA<br>R: CCGTCAGCCTCAAAGTCTTC  | (ATG) <sub>7</sub>    | 58         | 254                       | 8 (248–269)               | RIKEN cDNA 4930427A07 [ <i>Mus musculus</i> ]                                                | MF150423              |
| BS83  | F: AATTCCAATAGCCGGAGGAG<br>R: GAGGACGCAGGTATGGTTGT  | (CTG) <sub>7</sub>    | 58         | 194                       | 7 (182–200)               | Vacuolar processing enzyme 2 [ <i>Glycine max</i> ]                                          | MF150424              |
| BS85  | F: TACTGCACATGCAGAAAGGC<br>R: CATCTTCTCTTCTCGTCG    | (TTC) <sub>7</sub>    | 58         | 165                       | 9 (153–177)               | No hits                                                                                      | MF150425              |
| BS90  | F: AGCGCGCAGAGATAGCTTAG<br>R: CTCACATTATAATCGGTGCCC | (ATG) <sub>7</sub>    | 58         | 177                       | 5 (162–174)               | No hits                                                                                      | MF150426              |
| BS94  | F: GGATTTGAGCTACCTGCTG<br>R: CTTGCTGATCCTTCAAGCGT   | (CAT) <sub>7</sub>    | 60         | 107                       | 5 (104–116)               | Circumsporozoite protein [ <i>Plasmodium falciparum</i> ]                                    | MF150427              |

Note: A = number of alleles;  $T_a$  = annealing temperature.

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 3′-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 cDNAs of approximately 215 bp in length. Finally, each amplified molecule

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 mono-nucleotide 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 Skalaetsky, 1999), a total of 4928 paired primers were successfully designed.

In addition, 72 individuals of *B. 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 *B. insignis* (two individuals for each population). PCR amplifications were performed in 20- $\mu$ L reaction volumes, containing 25 ng of genomic DNA, 2  $\mu$ L 10 $\times$  buffer (with Mg<sup>2+</sup>), 0.25 mM of dNTPs, 0.2  $\mu$ M 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, Ankeny, Iowa, USA) using the Quant-iT PicoGreen dsDNA reagent kit (35–500 bp; Invitrogen, Carlsbad, California, USA). PROSize 2.0 software (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* (Aspleniaceae) 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

TABLE 2. Polymorphism of the 27 EST-SSRs in three populations of *Brainea insignis*.<sup>a</sup>

| Locus | DG (n = 32) |                |                |       | HD (n = 19) |                |                |       | SZ (n = 21) |                |                |       |
|-------|-------------|----------------|----------------|-------|-------------|----------------|----------------|-------|-------------|----------------|----------------|-------|
|       | A           | H <sub>o</sub> | H <sub>e</sub> | HWE   | A           | H <sub>o</sub> | H <sub>e</sub> | HWE   | A           | H <sub>o</sub> | H <sub>e</sub> | HWE   |
| BS04  | 5           | 0.969          | 0.652          | 0.000 | 6           | 0.947          | 0.715          | 0.009 | 5           | 0.952          | 0.639          | 0.002 |
| BS07  | 8           | 0.938          | 0.811          | 0.000 | 9           | 0.947          | 0.805          | 0.001 | 10          | 1.000          | 0.865          | 0.000 |
| BS13  | 8           | 1.000          | 0.779          | 0.000 | 8           | 1.000          | 0.778          | 0.002 | 7           | 1.000          | 0.804          | 0.001 |
| BS14  | 3           | 1.000          | 0.618          | 0.000 | 4           | 1.000          | 0.626          | 0.001 | 4           | 1.000          | 0.626          | 0.000 |
| BS24  | 4           | 0.813          | 0.670          | 0.000 | 5           | 1.000          | 0.730          | 0.002 | 4           | 1.000          | 0.621          | 0.000 |
| BS33  | 4           | 0.906          | 0.539          | 0.000 | 5           | 0.947          | 0.615          | 0.001 | 6           | 1.000          | 0.676          | 0.000 |
| BS35  | 10          | 1.000          | 0.797          | 0.000 | 10          | 1.000          | 0.816          | 0.000 | 6           | 0.952          | 0.770          | 0.000 |
| BS38  | 6           | 1.000          | 0.767          | 0.000 | 6           | 1.000          | 0.794          | 0.001 | 6           | 1.000          | 0.754          | 0.000 |
| BS43  | 5           | 1.000          | 0.698          | 0.000 | 4           | 1.000          | 0.655          | 0.000 | 3           | 1.000          | 0.543          | 0.000 |
| BS46  | 3           | 0.875          | 0.656          | 0.001 | 4           | 0.842          | 0.722          | 0.043 | 4           | 0.952          | 0.656          | 0.000 |
| BS47  | 4           | 1.000          | 0.621          | 0.000 | 5           | 1.000          | 0.727          | 0.001 | 4           | 1.000          | 0.686          | 0.000 |
| BS48  | 6           | 1.000          | 0.771          | 0.000 | 7           | 1.000          | 0.810          | 0.000 | 6           | 0.952          | 0.783          | 0.000 |
| BS51  | 5           | 0.719          | 0.623          | 0.000 | 6           | 0.737          | 0.668          | 0.385 | 3           | 0.810          | 0.563          | 0.035 |
| BS58  | 8           | 1.000          | 0.840          | 0.000 | 10          | 1.000          | 0.842          | 0.000 | 9           | 1.000          | 0.798          | 0.000 |
| BS61  | 4           | 0.531          | 0.694          | 0.004 | 3           | 0.105          | 0.566          | 0.000 | 3           | 0.286          | 0.557          | 0.003 |
| BS68  | 5           | 0.938          | 0.751          | 0.000 | 5           | 0.842          | 0.737          | 0.001 | 5           | 0.952          | 0.761          | 0.000 |
| BS69  | 5           | 0.469          | 0.764          | 0.000 | 5           | 0.474          | 0.762          | 0.001 | 5           | 0.381          | 0.719          | 0.000 |
| BS70  | 5           | 0.844          | 0.690          | 0.125 | 5           | 0.789          | 0.715          | 0.778 | 6           | 0.905          | 0.747          | 0.007 |
| BS75  | 3           | 0.938          | 0.617          | 0.000 | 4           | 0.789          | 0.694          | 0.001 | 3           | 1.000          | 0.523          | 0.000 |
| BS78  | 6           | 0.938          | 0.608          | 0.000 | 4           | 0.842          | 0.644          | 0.000 | 4           | 0.810          | 0.552          | 0.000 |
| BS79  | 8           | 0.750          | 0.709          | 0.136 | 7           | 0.842          | 0.783          | 0.003 | 4           | 0.762          | 0.671          | 0.526 |
| BS80  | 6           | 0.781          | 0.606          | 0.134 | 5           | 0.579          | 0.602          | 0.289 | 5           | 0.714          | 0.641          | 0.157 |
| BS81  | 5           | 0.906          | 0.687          | 0.090 | 8           | 0.842          | 0.730          | 0.070 | 5           | 0.905          | 0.709          | 0.003 |
| BS83  | 6           | 0.938          | 0.710          | 0.000 | 6           | 0.842          | 0.784          | 0.000 | 5           | 1.000          | 0.669          | 0.000 |
| BS85  | 8           | 0.969          | 0.788          | 0.017 | 9           | 1.000          | 0.806          | 0.515 | 8           | 1.000          | 0.808          | 0.669 |
| BS90  | 4           | 0.938          | 0.704          | 0.000 | 5           | 0.789          | 0.715          | 0.000 | 4           | 0.857          | 0.698          | 0.000 |
| BS94  | 5           | 0.969          | 0.611          | 0.000 | 4           | 0.947          | 0.626          | 0.000 | 4           | 1.000          | 0.681          | 0.000 |

Note: A = number of alleles; H<sub>e</sub> = expected heterozygosity; H<sub>o</sub> = observed heterozygosity; HWE = P value of Hardy–Weinberg test; n = number of individuals collected for each population.

<sup>a</sup>Locality and voucher information are provided in Appendix 1.

| TABLE 3. Cross-amplification of 27 <i>Brainea insignis</i> EST-SSR markers in other ferns. <sup>a</sup>                                                                               |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
| Species                                                                                                                                                                               | BS04 | BS07 | BS13 | BS14 | BS24 | BS33 | BS35 | BS38 | BS43 | BS46 | BS47 | BS48 | BS51 | BS58 | BS61 | BS68 | BS69 | BS70 | BS75 | BS78 | BS79 | BS80 | BS81 | BS83 | BS85 | BS90 | BS94 |
| <i>Blechnum orientale</i>                                                                                                                                                             | —    | +    | —    | +    | —    | +    | +    | +    | —    | +    | +    | +    | +    | —    | —    | —    | —    | —    | —    | —    | —    | —    | —    | —    | +    | ±    | ±    |
| <i>Chienopteris harlandii</i>                                                                                                                                                         | —    | +    | —    | +    | —    | +    | +    | —    | —    | +    | +    | +    | +    | +    | —    | —    | —    | —    | —    | ±    | —    | —    | —    | —    | ±    | ±    | —    |
| <i>Note:</i> + = primers could be successfully amplified in all individuals; — = primers could not be amplified in any individual; ± = primers could be amplified in few individuals. |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| <sup>a</sup> Locality and voucher information are provided in Appendix 1.                                                                                                             |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |

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

Note: N = number of individuals.

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