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Primer Note

Development and characterization of EST-SSR markers via transcriptome sequencing in *Brainea insignis* (Aspleniaceae s.l.)[1]

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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 *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 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 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

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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 R: GCGCTCTATTTTCGTCGTTC	$(GAGAGG)_6$	56	193	6 (175–205)	ASR protein [*Ginkgo biloba*]	MF150401
BS07	F: GGGGATCAAGTTTGGGTCTT R: AAGCGCTTTCAACCAACCTA	$(GGGAA)_5$	58	201	10 (171–216)	Hypothetical protein PGUG_05234 [*Meyerozyma guilliermondii*]	MF150402
BS13	F: AATGGAAGGGGGAGAGAGAA R: CTGTGGATGTTGCAGCTGTT	$(AGC)_8$	58	273	9 (237–288)	VAMP family protein [*Arabidopsis thaliana*]	MF150403
BS14	F: TTTTAGAGGGGGAAGGGCTA R: CAGTAAAGGCCCGAAGAACA	$(GTG)_8$	60	149	4 (140–149)	ENT domain-containing protein [*Arabidopsis thaliana*]	MF150404
BS24	F: AAAACCGGCATTGAGATCAG R: TACTGAAATGCCACACCGAA	$(CATA)_6$	60	115	5 (139–155)	RNA-dependent RNA polymerase [Hantavirus L99]	MF150405
BS33	F: TGTGAAGAAAGCATTGACGC R: TTGGCCTCATCATCACTCAC	$(GCA)_7$	56	101	6 (95–110)	BolA-like family protein [*Arabidopsis thaliana*]	MF150406
BS35	F: CACAAGAGCCAGCACCATAA R: GGAAAAACACTTCGGGTGAA	$(CCA)_7$	58	222	11 (222–252)	RNA-binding protein precursor [*Nicotiana tabacum*]	MF150407
BS38	F: AGTCGGAGCCAAGCTACAGA R: CATGAGAGTCACCATGTGGG	$(TGA)_7$	58	234	7 (234–267)	Protein kinase family protein [*Arabidopsis thaliana*]	MF150408
BS43	F: GGCTCTACATGCATCCTCTTG R: CAGTGGGCCCTTACACACTT	$(CAT)_7$	60	141	5 (135–147)	No hits	MF150409
BS46	F: CTCTCCTTCTCCGATTGCAC R: GACTCGATCTCGTACTGGGC	$(CTC)_7$	58	128	4 (125–134)	Chloroplast 29k Da ribonucleoprotein [*Oryza sativa*]	MF150410
BS47	F: CGCCTGGTATAGCTGCTCTT R: GGCATCATTCTTTGGCAGTT	$(TGA)_7$	60	194	5 (188–206)	Potassium transporter HAK4 [*Hordeum vulgare*]	MF150411
BS48	F: GATGGGAGCAAGCAAATGAT R: ATCTCTCCCAAGACCCTCGT	$(GAT)_7$	60	252	6 (249–288)	ATP binding/DNA-directed DNA polymerase [*Arabidopsis thaliana*]	MF150412
BS51	F: CATAGCCTCGGCATAGCTTC R: CCCTTCTTCTGGATGGCATA	$(TTG)_7$	60	263	6 (260–275)	RNA methyltransferase family protein [*Arabidopsis thaliana*]	MF150413
BS58	F: ACCATGATGACGATGGTGAA R: CGATTTGCTGTAGTTGCTCG	$(GAT)_7$	58	168	10 (156–183)	DEAD/DEAH box helicase, putative (RH28) [*Arabidopsis thaliana*]	MF150414
BS61	F: TGAGCAAAGCTGTTGGATTG R: CATGCCGCATACACAAAAAC	$(TGA)_7$	58	249	4 (237–246)	Phospholipase A1 [*Capsicum annuum*]	MF150415
BS68	F: AGGCAGCAAAAACGAAGCTA R: TCTATATACGCCCCACAGCC	$(GAA)_7$	58	159	5 (150–162)	No hits	MF150416
BS69	F: TGAGAGAACTAAGGGGCCAA R: ATGTGCATGGATGCATGAGT	$(TAC)_7$	58	183	6 (168–183)	Apocytochrome b [*Helianthus petiolaris*]	MF150417
BS70	F: ATGTGTGGAGAGCCATCCTT R: TTGTCATGGCAATCACCACT	$(TCC)_7$	58	223	6 (217–232)	Proton-dependent oligopeptide transport (POT) family protein [*Arabidopsis thaliana*]	MF150418
BS75	F: CGCAGCATAACACTGAGAGC R: TGATGATGGGCATGTAGACG	$(CAT)_7$	60	170	4 (176–185)	ADP-ribosylation/Crystallin J1 [*Beggiatoa* sp.]	MF150419
BS78	F: TGCAACAAAAATCGCAGAAG R: GTGAGAGAGGATCGCACCAT	$(CAA)_7$	60	252	7 (246–267)	Leukocyte surface antigen CD47 [*Anthurium amnicola*]	MF150420
BS79	F: CCGTACGAGAAGCCTCAAAG R: GAAGATGATGCCTGACCCAT	$(CAT)_7$	58	238	9 (235–259)	Solute carrier family 35 member B3, related [*Medicago truncatula*]	MF150421
BS80	F: GCCTGCACATTAACAGGTGA R: CCCGCGTTTTTAAGATCAGA	$(GAG)_7$	60	232	6 (229–244)	Exodeoxyribonuclease V, alpha subunit [*Burkholderia pseudomallei*]	MF150422
BS81	F: CCACTAGAATGCTTGCACGA R: CCGTCAGCCTCAAAGTCTTC	$(ATG)_7$	58	254	8 (248–269)	RIKEN cDNA 4930427A07 [*Mus musculus*]	MF150423
BS83	F: AATTCCAATAGCCGGAGGAG R: GAGGACGCAGGTATGGTTGT	$(CTG)_7$	58	194	7 (182–200)	Vacuolar processing enzyme 2 [*Glycine max*]	MF150424
BS85	F: TACTGCACATGCAGAAAGGC R: CATCTTCCTCTTCCTCGTCG	$(TTC)_7$	58	165	9 (153–177)	No hits	MF150425
BS90	F: AGCGCGCAGAGATAGCTTAG R: CTCACATTATAATCGGTGCCC	$(ATG)_7$	58	177	5 (162–174)	No hits	MF150426
BS94	F: GGATTTCGAGCTACCTGCTG R: CTTGCTGATCCTTCAAGCGT	$(CAT)_7$	60	107	5 (104–116)	Circumsporozoite protein [*Plasmodium falciparum*]	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

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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 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-μL reaction volumes, containing 25 ng of genomic DNA, 2 μL 10× buffer (with Mg^{2+}), 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, 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*.[a]

	DG (n = 32)				HD (n = 19)				SZ (n = 21)			
Locus	A	H_o	H_e	HWE	A	H_o	H_e	HWE	A	H_o	H_e	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_e = expected heterozygosity; H_o = observed heterozygosity; HWE = P value of Hardy–Weinberg test; n = number of individuals collected for each population.

[a]Locality and voucher information are provided in Appendix 1.

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TABLE 3. Cross-amplification of 27 *Brainea insignis* EST-SSR markers in other ferns.[a]

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
Blechnum orientale	—	+	—	+	—	+	+	+	—	+	+	+	+	—	—	—	—	—	+	—	—	—	—	+	±	±	—
Chieniopteris harlandii	—	+	—	+	—	+	+	—	—	+	+	+	+	+	—	—	—	—	—	±	—	—	—	±	±	±	—

Note: + = primers could be successfully amplified in all individuals; — = primers could not be amplified in any individual; ± = primers could be amplified in few individuals.
[a]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[a]
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*
	Jiulong 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.

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