Development of expressed sequence tag-based microsatellite markers for the critically endangered *Isoëtes sinensis* (Isoetaceae) based on transcriptome analysis

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ABSTRACT. *Isoëtes sinensis* is a critically endangered quillwort. To facilitate studies on the conservation genetics of this species, we developed expressed sequence tag-simple sequence repeat (EST-SSR) markers. A total of 50,063 unigenes were predicted by transcriptome sequencing, 5294 (10.6%) of which significantly matched 3011 Gene Ontology annotations and 2363 were assigned to Kyoto Encyclopedia of Genes and Genomes metabolic pathways. Most of these (2297) were involved in metabolism. A total of 1982 SSR motifs were identified, with trinucleotides being the dominant repeat motif, and 1438 (72.6%) SSR primers were designed. Eighteen randomly selected primer pairs were used to genotype 24 *I. sinensis* accessions, which confirmed the suitability of these novel markers for molecular studies of *I. sinensis*.

The heterozygosity index value ranged between 0.0799 and 0.9106, while the Shannon-Wiener diversity index value ranged between 0.1732 and 2.5589. The EST-SSRs reported in this study are linked to genic sequences, and are therefore ideal for investigating the evolutionary history of *I. sinensis*. These markers, together with the large EST dataset generated in this study, will greatly facilitate conservation genetic studies of *I. sinensis*.

Key words: *De novo* assembly; EST-SSR; *Isoëtes sinensis*; Microsatellite; RNA-sequencing; Transcriptome

INTRODUCTION

Isoëtes sinensis Palmer is a heterosporous, tetraploid aquatic quillwort that is endemic to East Asia (Liu et al., 2002; He et al., 2004). In China, *I. sinensis* is found in freshwater intertidal zones, valley marshes and abandoned fields in the southeast region. It often grows close to farmland, and consequently is subject to intense competition from other hydrophyte species because of increased fertilizer input (Pang et al., 2003). Recently, *I. sinensis* populations have declined steadily (Kang et al., 2005; Liu et al., 2005; Zhu, 2006), and only two populations remain in China (Chen et al., 2012).

I. sinensis is now considered rare and critically endangered, and is listed in the first category of the national key that protects wild plants (Yu, 1999; Liu et al., 2005) and in the International Union for Conservation of Nature (IUCN) Red List (IUCN, 2008). It is an evolutionarily relevant species, because it belongs to a genus that has been in existence since the Devonian geological period, and phylogenetic studies have classified it among basal vascular plants (Pigg, 2001). *I. sinensis* provides environmental services such as air purification, and is a good bioremediation agent. The continuous reduction of its population has been associated with water quality deterioration in regions where it has ceased to grow (Wen et al., 2003). Therefore, appropriate means of conservation should be formulated to save this species from extinction, and to achieve this, proper molecular analysis is essential.

Consequently, in recent years, genetic variation in *I. sinensis* has been studied using a variety of genetic markers, e.g., allozymes (Chen et al., 2009), random-amplified polymorphic DNA (Chen et al., 2004), inter-simple sequence repeats (Chen and Wang, 2006), amplified fragment length polymorphisms (AFLPs) (Kang et al., 2005), and simple sequence repeats (SSRs) (Chen et al., 2012). However, the results obtained by these studies do not adequately elucidate the effects of genetic variation and natural selection on populations of *I. sinensis*. For example, no molecular markers that are linked to specific functional genes, such as expressed sequence tag (EST)-SSRs, have been developed for this critically endangered species. EST-SSRs, also referred to as genic simple sequence repeats, have gained popularity in plant molecular research due to their efficacy in evaluating functional diversity in natural populations. They are obtained from transcribed regions of DNA, and are used in dataset homology searches in order to ascertain their putative functions (Varshney et al., 2005).

The main objectives of our study were as follows: 1) to construct a transcriptome dataset in order to facilitate the discovery of novel genes and to study the comparative genomics of *I. sinensis*; 2) to develop EST-SSR markers using high-throughput RNA sequencing (RNA-Seq); and 3) to evaluate the viability of the EST-SSR markers developed by testing randomly selected primers.

MATERIAL AND METHODS

RNA extraction

Total RNA was extracted from leaf tissues of *I. sinensis.* Samples were collected from four accessions cultivated at Wuhan Botanical Garden, and were immediately frozen in liquid nitrogen. One milliliter of TRIzol reagent (Invitrogen, CA, USA) was added for every 100 mg leaf tissue and treated with RNase-free DNase 1 (TaKaRa Bio, Shandong, China) for 1 h at 37°C. RNA was then dissolved in RNase-free water (Ambion, USA). A 1-µL aliquot of each sample was used to check RNA quality and concentration with NanoDrop 2000 Spectrophotometer (Thermo Fischer Scientific Inc., DE, USA) and Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). To maximize the quality of the transcriptional units and improve the analytical process, a pool was created by mixing equal volumes of total RNA from each sample.

cDNA synthesis and sequencing

A working concentration of 50 ng/µL of total RNA was used for cDNA synthesis. mRNA was isolated from the total RNA pool and purified using a MicroPoly(A)Purist™ mRNA Purification Kit (Ambion™) following the manufacturer's protocol. cDNA was then synthesized from the mRNA fragments according to the protocol developed by Ng et al. (2005), with slight modifications. First-strand cDNA was synthesized from 10 µg mRNA templates using Gsu1-oligo(dT) and 1000 U SuperScript™ II Reverse Transcriptase (Invitrogen). The mixture was incubated at 42°C for 1 h. The cDNA was then treated with sodium periodate (Sigma, USA) and mRNA 5' oxide caps were biotinylated and connected using Dynabeads™ M-280 beads (Invitrogen). The alkaline lysis method was used to release first-strand cDNA from the biotin-linked mRNA/cDNA. Second-strand cDNA was synthesized using *Ex Taq*™ Polymerase (TaKaRa, Japan), and the *Gsu*I restriction enzyme was used to cut-off poly(A) tails. Double-stranded cDNA was purified using a QIAquick® Polymerase Chain Reaction (PCR) Extraction Kit (Qiagen, Hilden, Germany), and the sequencing adaptors were ligated onto the fragments. Fragments whose sizes were between 300 and 500 bp were selected and purified using AMPure® beads (Agencourt, USA), and then enriched by PCR to construct a library for transcriptome sequencing and to improve the quality of the sequencing process by ensuring fragment uniformity. Finally, the library was sequenced using an Illumina HiSeq™ 2000 platform (Illumina Inc., USA).

De novo assembly and functional annotation of unigenes

Prior to assembly, stringent filtering was conducted, and only high-quality reads were assembled using the Trinity software (Grabherr et al., 2011). This was followed by the prediction and identification of putative open reading frames and untranslated regions within the unigenes using the EMBOSS software (Rice et al., 2000). The predicted protein-coding sequences were compared using the Swiss-Prot and TrEMBL protein databases, with a BLASTp alignment and an E-value set at less than 1E-5.

We used the Perl-based GoPipe program (Chen et al., 2005) to assign Gene Ontology (GO) annotations; in BLASTp, the E-value was again set at less than 1E-5. Metabolic pathway

construction was conducted using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa et al., 2010). The E-value for a bi-directional BLAST was also set at less than 1E-5. The unigenes were assigned KEGG Orthology numbers and their involvement in the metabolic pathway was ascertained.

SSR mining, primer design, and marker validation

SSR motifs were detected using the program MiSa (http://pgrc.ipk-gatersleben.de/misa/); mononucleotides were excluded from the search. We used the Primer 3.0 software (Rozen and Skaletsky, 2000) to design the SSR primers. *I. sinensis* samples were collected from 24 individuals from natural populations in Zhejiang Province, China. About 5 g fresh leaves was harvested from each plant and immediately dried in a Zip lock plastic bag containing 70 g silica gel. Samples were stored at room temperature until the DNA was isolated in the laboratory. Total genomic DNA was extracted from each individual using a MagicMag Genomic DNA Micro Kit (Sangon Biotech Co., Shanghai, China) following the manufacturer protocol. DNA quality and concentration were checked by 1.5% gel electrophoresis with a NanoDrop (Thermo Scientific Inc.). Firstly, we randomly selected 25 SSR primers and tested them for amplification and specificity, from which we selected the 18 best primers and labeled the forward sequence with an FAM fluorescent dye at the 5'-end. The 25-µL PCR mixture contained 2 µL 50-100 ng genomic DNA, 2.5 µL 10X *Taq* buffer (plus Mg^{2+}), 0.25 mM of each dNTP, 0.25 µM of each primer, 0.2 U *Taq* polymerase (TaKaRa Bio, Dalian, China), and PCR water.

PCR amplification was conducted using T100™ Thermal Cycler (Bio-Rad, CA, USA) with the following cycling parameters: an initial denaturation step of 3 min at 95°C, followed by 30 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 50-55°C, an extension of 30 s at 72°C, and a final extension step for 10 min at 72°C. The quality of the PCR products was checked on 1.5% agarose gel prior to sequencing, and the sequences were analyzed using the GeneMarker® software (SoftGenetics). The polymorphic data recorded were used to estimate the intrapopulation genetic diversity of *I. sinensis* using the ATETRA 1.2 program (Van Puyvelde et al., 2010).

RESULTS

Illumina *de novo* sequencing and assembly

In order to investigate the *I. sinensis* transcriptome, RNA was extracted from several different tissues and a library was successfully created and sequenced using Illumina paired-end technology. A total of 19,522,164 reads with a length of 100 bp each were generated. The reads were assembled into 125,629 contigs that were reduced to 50,110 after a strict filtration step that removed adaptors and low-quality sequences. The average length of the high-quality contigs was 755 bp, and ranged between 201 and 14,750 bp. Further processing was conducted on the 50,110 contigs using the EMBOSS getorf tool (Rice et al., 2000) to generate 50,063 unigenes.

Functional annotation and classification

A GO-based functional annotation of the ESTs was performed through BLASTp using

the National Center for Biotechnology Information database. The BLAST results revealed that of the 50,063 unigenes generated, 9979 (19.9%) were homologous to known proteins and 40,084 (80.1%) were homologous to expressed, hypothetical, or unknown proteins. Further analyses conducted using the GoPipe program revealed that 5294 (10.6%) of the unigenes significantly matched 3011 GO functional terms (Figure 1). These unigenes were grouped into three main categories of functional classification: cellular components, biological processes, and molecular functions. A single unigene could have been linked to one or several cellular components, active in one or more biological processes, and involved in one or more molecular functions; therefore, some unigenes were simultaneously annotated into two or three categories. Figure 1 shows that assignments to biological processes constituted the majority (17,039, 37.3%), followed by molecular functions (14,641, 32.1%) and cellular components (13,944, 30.6%).

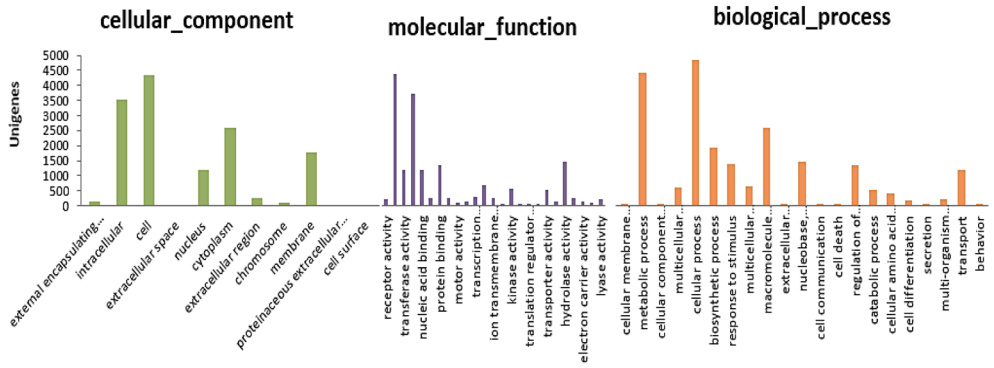

Figure 1. Gene Ontology (GO) classification of 3011 unigenes.

A search of the KEGG database revealed that of the 50,063 unigenes identified, 2363 (4.73%) were involved in KEGG pathways and were assigned to five main categories (Figure 2). The categories were metabolism, general information processing, environmental information processing, cellular processes, and organismal systems. These classifications were further reduced to 25 KEGG pathways, most of which were in line with the biological processes already observed in the GO annotations, including some unigenes being annotated into two or more classifications.

EST-SSR marker development and characterization

MiSa was employed to further analyze the 50,063 unigenes generated; using the default parameters, 1982 SSR motifs were discovered. We then analyzed the frequency of different repeat motif types (Table 1). The repeat motifs were not equally represented; trinucleotides had the highest percentage (61.3%), dinucleotides accounted for 25.3%, and tetranucleotides accounted for 10.9%. Compound repeats (3.7%) and penta-hexanucleotides (2.5%) were the least frequent, and the most common di-, tri-, and tetranucleotide motifs were TA/TA (18.2%), GGA/TCC (8.1%), and CTTC/GAAG (8.7%), respectively (Figure 3).

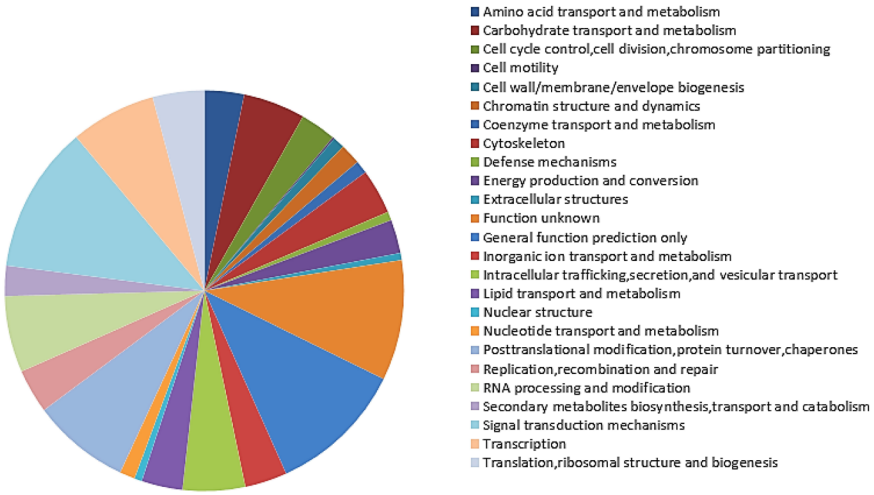

Figure 2. Kyoto Encyclopedia of Genes and Genome (KEGG) assignment of 2363 unigenes based on cellular processes, genetic information processing, environmental information processing, metabolism, and organismal systems.

Table 1. Distribution of expressed sequence tag-simple sequence repeat motifs based on the number of repeat units.

Repeats	Di-	Tri-	Tetra-	Penta-Hexa	Total	Percentage
5	0	676	157	14	847	44.4
6	265	237	26	5	533	27.9
7	130	241	11	2	384	20.1
8	33	16	8	20	77	4.0
9	11	1	0	2	14	0.7
10	13	0	0	4	17	0.9
11	25	0	2	0	27	1.4
≥12	6	0	4	0	10	0.5
Total	483	1171	208	47	1909	100.0
Percentage	25.3	61.3	10.9	2.5	100.0	

Di- = dinucleotides; Tri- = trinucleotides; Tetra- = tetranucleotides; Penta-Hexa = pentanucleotides and hexanucleotides.

Validation of EST-SSR primers

SSR primers were designed using the Primer 3.0 program based on the flanking regions of the SSR-containing sequences. A total of 1438 SSR primers (72.6%) were designed. Twenty-five primer pairs were randomly selected and tested for amplification and specificity using genomic DNA extracted from four accessions of *I. sinensis*. Eighteen (72%) markers were successfully amplified, produced clear bands after gel electrophoresis, and generated products of the expected size at various annealing temperatures.

We genotyped 24 accessions in order to authenticate the markers and their suitability for further molecular studies of *I. sinensis*. All 18 markers exhibited a high level of polymorphism and a significant amount of diversity was observed. Heterozygosity values ranged from 0.08 to 0.91, and Shannon-Wiener diversity index values ranged from 0.17 to 2.55. The locus sequences have been submitted to GenBank (Table 2).

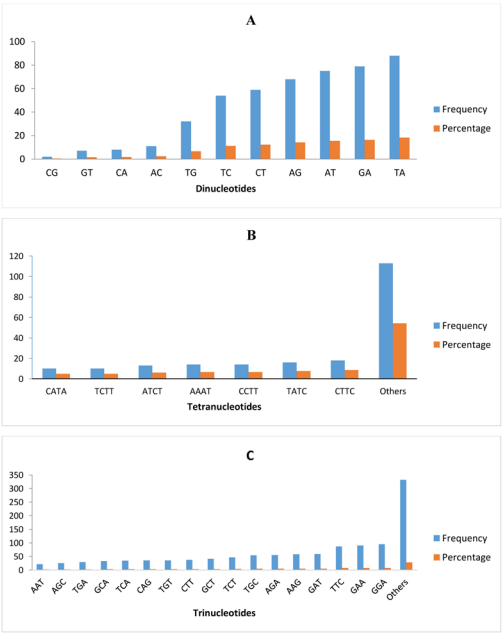

Figure 3. Frequency distribution (number and percentage) of the main expressed sequence tag-simple sequence repeats based on motif type. **A.** Dinucleotides. **B.** Tetranucleotides. **C.** Trinucleotides.

Table 2. Characterization of 18 polymorphic expressed sequence tag-simple sequence repeat markers in *Isoëtes sinensis*.

Locus	Repeat motif	Primer 5'-3'	GenBank accession No.	Tm (°C)	Allele size range (bp)	H_i	H'
SJSSR4	$(GCA)_5$ N $(CAG)_5$	F:GCAGCAGATTCAGATGCAGA R: GTTCCTGATGCTGGTGGTCT	KT989976	53	145-198	0.737	1.5301
SJSSR5	$(CA)_6$	F: CACACCCAACATACATACGCA R: ATGGGGTGAAACAACAGGAG	KT989977	53	144-172	0.8367	1.9181
SJSSR6	$(ACTC)_6$	F: AGAAAAATGATCGCCTGTGG R: TGCACTATGGAAAGCTTGTGA	KT989978	55	129-141	0.8307	1.9513
SJSSR9	$(TGC)_6$	F: GCAAAGTCCACCAAGCAGAT R: AATGCCCTGGCAGTTAACAC	KT989979	55	225-229	0.2176	0.3744
SJSSR11	$(GCT)_6$	F: CAGCATCAATGAGCAAAGGA R: GCTTGGGGCAAGTAGCTATG	KT989980	53	218-233	0.7688	1.5746
SJSSR12	$(GAT)_5$	F: GTGGTTGATTTGGGGTCATC R: CCCTCTTTGCCAACAGTGAT	KT989981	53	205-240	0.7673	1.7722
SJSSR14	$(CT)_{10}$	F: GGCCAGAGAACAGGAGAAAG R: CCAAGTGGAAATTATGTCGCT	KT989982	55	255-281	0.8774	2.281
SJSSR45	$(TC)_6$	F: AAGGCCAACACAAAAACTGG R: CGCCCACTAATCAGGACACT	KT989983	52	243-255	0.7315	1.4196
SJSSR46	$(AAC)_7$	F: TCGAAAATTCGAAGATCGCT R: ATAATTTGGAGGTTGCGACG	KT989984	52	226-252	0.7679	1.5833
SJSSR50	$(TCA)_5$	F: ACCATAGTGGGGTGAAGGAA R: CCTGAGTCTCATGTGCAAGC	KT989985	54	195	0.0799	0.1732
SJSSR53	$(TCT)_5$	F: AGGGATTGCTAGCGCTGTTA R: GGCAAAACAAAAGCATCCAT	KT989986	54	195-286	0.9106	2.5589
SJSSR15	$(TGT)_6$	F: CTCTTGCCTCTGCTCTTGCT R: TGATAGAGGGTGGGAAATCG	KT989987	55	241-275	0.8127	1.7587
SJSSR24	$(TCA)_6$	F: GTTTGGGCATTTTTCCCTCT R: TGAAAAGCCTATGGTTGGGT	KT989988	53	131-241	0.7869	2.0675
SJSSR32	$(TTCC)_5$	F: AAATGCCACCAATCCATCAT R: AAATCAGGTCCGATGGTCTG	KT989989	52	228-243	0.852	1.9835
SJSSR39	$(GCA)_6$	F: CAGTGACATGCCCGTTCATA R: GTGGAGTTGGAGGCAACAAT	KT989990	53	228	0.2778	0.4506
SJSSR8	$(GAC)_7$	F: CTTGGTAGCGAACATGAGCA R: ATGATGGCATCTTCCTCAGC	KT989991	53	193-206	0.818	1.9951
SJSSR22	$(AAC)_5$	F: TACGCAGAGCCCCTTCTCTA R: GCCCTCGGTATTGAATCTGT	KT989992	55	197-224	0.5256	1.1476
SJSSR26	$(GTT)_5$	F: ATAACAAAAAGGGCGCTTGA R: CTTAATTTCTGCAGGAGCCG	KT989993	52	116-274	0.631	1.1042

Tm = melting temperature; H_i = heterozygosity index; H' = observed Shannon-Wiener information index.

DISCUSSION

Isoëtes sinensis is listed as critically endangered by the IUCN and as possibly extinct in some regions of China, e.g., Anhui and Jiangsu Provinces (China Plant Specialist Group, 2004). Despite a number of studies being conducted on remnant populations of this species that had the primary objective of saving the remaining individuals and re-establishing thriving populations (Kang et al., 2005; Chen et al., 2004, 2009, 2012), natural populations of this species continue to decline. EST-SSR markers have numerous applications in botany and have been used in conservation genetics, marker-assisted selection, and comparative genomics (Varshney et al., 2007). In this study, we used molecular tools (EST-SSRs) to facilitate further molecular studies of *I. sinensis*. The number of unigenes generated in this study is comparable to that obtained by other studies that have used Illumina sequencing technology (Chen et al., 2015; Zhou et al., 2016).

Our analyses assigned the SSR motifs discovered into three GO categories: i) biological functions, e.g., enzymatic reactions, plant hormonal regulations, and plant responses to stimuli; ii) cellular functions, e.g., genes that putatively regulate cellular functions including cell activity, nucleus activity, and cytoplasm regulation; and iii) molecular functions, e.g., protein binding, motor activity, and nucleic acid activity. Within the biological processes category, cellular process (3785, 22.21%) and metabolic process (3461, 20.31%) were highly represented. Binding (3681, 25.14%) and catalytic activity (3113, 21.26%) were the largest groups in the molecular function category, while in the cellular component category, cell (4353, 31.22%) and intracellular (3514, 25.2%) contained the majority of the unigenes. KEGG is a knowledge base for mapping molecular datasets in "-omics" studies, including transcriptomics (Kanehisa et al., 2010), and 2363 unigenes were assigned KEGG pathways, indicating that they are involved in metabolic activity. Of the 25 KEGG pathways, signal transduction mechanism (2913) and general function prediction (2696) were the most highly represented, followed by metabolism (2297) and genetic information processing (1023).

A total of 1982 EST-SSR motifs were mined for this species. Trinucleotides were the most frequent (61.3%), whereas dinucleotides are the most abundant in another aquatic species, *Nelumbo nucifera* (Zhang et al., 2014). The most frequent types of repeat among the di- and trinucleotides were TA\TA and GGA\TTC, respectively. The EST-SSRs revealed relatively high intrapopulation genetic diversity in *I. sinensis* (0.68) compared to that recorded in previous studies using AFLP markers [expected heterozygosity (H_E) = 0.118, genetic diversity (H_s) = 0.147, and Shannon's index (H') = 0.192] (Kang et al., 2005), allozymes (0.32) (Chen et al., 2009), and SSR markers (H_E = 0.361 for a Jiande population and H_E = 0.390 for a Xiuning population) (Chen et al., 2012). The high level of genetic diversity observed in the small population sizes of *I. sinensis* may be because reductions in these populations have occurred relatively recently (Kang et al., 2005). In addition, the habitats of the few remaining populations have been disturbed, resulting in overly fragmented and disparate subpopulations; consequently, this results in increased genetic diversity due to isolation. The population sample sizes used in this study were relatively small; therefore, it is difficult to draw a firm conclusion concerning genetic variation in *I. sinensis*. We recommend that extensive field surveys and the collection of an adequate number of samples should be undertaken in order to investigate the genetic diversity of this species and assist in its conservation.

In summary, 18 informative markers were authenticated from 25 that were randomly selected from 1438 markers. Further polymorphic genic SSR markers for *I. sinensis* await

development. EST-SSRs have a higher transferability rate than genomic SSRs, and a lower mutation rate (Varshney et al., 2005); therefore, the novel polymorphic SSR markers developed in this study can be used in evolutionary biology, phylogeography, and comparative genomic studies of species in the genus *Isoëtes*.

Conflicts of interest

The authors declare no conflict of interest.

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