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Short Communication

Development of nuclear microsatellite markers in *Stizophyllum* (Bignoniaceae) using next-generation sequencing‡

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Abstract

Here we developed the first set of nuclear microsatellite markers (nSSRs) for *Stizophyllum riparium* (Bignoniaceae), a widespread species of Neotropical liana. Thirty-two sets of primers were isolated from a genomic dataset obtained with an Illumina HiSeq Platform, and characterized for 57 individuals of *S. riparium* from three populations. Nine nSSRs were polymorphic and the number of alleles ranged from eight to 29. The unbiased expected heterozygosity per locus ranged from 0.724 to 0.952 and the polymorphic information content values ranged from 0.717 to 0.944. All nine primer pairs also amplified for two closely related species (*S. inaequilaterum* and *S. perforatum*). The new set of nuclear markers will be useful for population genetics studies of *Stizophyllum* as a whole.

Keywords: cross-amplification, genetic diversity, *Stizophyllum inaequilaterum*, *Stizophyllum perforatum*

Introduction

Stizophyllum Miers (Bignonieae, Bignoniaceae) is a small genus of Neotropical lianas, distributed from Mexico to Brazil, occurring in wet to dry forests (Lohmann and Taylor, 2014). The genus is monophyletic (Lohmann, 2006), and all species are pollinated by bees, and dispersed by wind (Gentry, 1974). Three species are currently recognized (Lohmann and Taylor, 2014): *S. inaequilaterum* Bureau & K. Schum., *S. perforatum* (Cham.) Miers and *S. riparium* (Kunth) Sandwith.

Nuclear microsatellite markers (nSSRs) are short tandem repeats of DNA (Powell *et al.*, 1996). These markers have been widely used to estimate genetic diversity parameters (Palma-Silva *et al.*, 2009; Hmeljevski *et al.*, 2017), gene flow, population genetic structure (Barbará *et al.*, 2007; Lexer *et al.*, 2016), demography (Pinheiro *et al.*, 2011), and phylogeography (Collevatti *et al.*, 2012; Tóth *et al.*, 2017). Although these markers are extremely informative for genetic studies, they were only isolated for a single species of Bignoniaceae (Figueira *et al.*, 2010). Here, we developed and characterized nSSRs markers for *Stizophyllum riparium* using genomic data and examined their transferability.

Experimental

Genomic DNA of one individual of *S. riparium* (Nogueira 170) was extracted from silica-dried leaf material using the Invisorb Plant Mini Kit (Invitek, Berlin, Germany). The genomic library was constructed following Nazareno *et al.* (2015), and sequenced on an Illumina HiSeq 2000 system (Illumina Inc., San Diego, CA, USA) at the University of São

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Table 1. Nuclear microsatellite markers developed for *Stizophyllum riparium*, including the repeat motif, forward (F) primer sequence labelled with 6-FAM or JOE fluorescent dyes and reverse (R) primer sequence, annealing temperature (T_a), allele size range and GenBank accession numbers

Locus	Repeat motif	Primer sequences (5′–3′)	T_a (°C)	Allele size range (bp)	GenBank accession no.
stiz63	$(AG)_{14}$	F: **6-FAM –** CGTGAGGAAAAGGGCTGTTT R: CTTGTGGCGTGGATATGGTC	48	92–119	KP863502
stiz104	$(AC)_{17}$	F: **JOE –** GGTTTATTCACTCATGCCATGC R: TGCGCTAAAATCTAGGATGCT	51	109–129	KP863504
stiz112	$(TC)_{16}$	F: **JOE –** TCTGCTTGAACTTGTGACTCA R: AGTACTTAAATCGAGCGAGCG	48	79–90	KP863505
stiz123	$(TC)_{16}$	F: **JOE –** GCCAAAGCCCTTTCTACA R: ACAAACATAGCACACACAAG	51	94−116	KP863506
stiz133	$(CTT)_{11}$	F: **6-FAM –** ACAGATGGTTGTGGGTTTGA R: GGTGATGAGACCCCTTTTCT	51	88–126	KP863507
stiz135	$(CTT)_9$	F: **6-FAM –** TCAATTTCTCCACACCTCGC R: CAGGAATTGGTGCATGTCATG	51	91–102	KP863508
stiz138	$(CTT)_8$	F: **6-FAM –** ACCCTCGACATGTTCTCTCC R: GTGACAGCAAGTGAAGAGCA	51	93–124	KP863509
stiz154	$(CTC)_{11}$	F: **6-FAM –** CCATTGCTGTTCATTTCTAC R: CATTCTTTTACGTTCTTGGC	48	99–117	KP863510
stiz156	$(CTC)_8$	F: **JOE –** TGCTGCTCTTCACTTCAGGA R: CTCATGTGCGGTGGTTGC	48	88–111	KP863511

Paulo (Brazil). We used a Perl script to clean raw reads and exclude reads with low Phred scores and with more than three uncalled bases (see Nazareno *et al.*, 2015).

The nuclear genome was separated from the chloroplast genome using *de novo* assembly, based on sequence coverage. Contigs with coverage lower than 10x corresponded to the nDNA, since organelles have high-copy numbers and the DNA derived from organelles is oversampled in the total genomic sample sequenced (Nazareno *et al.*, 2015). The remaining *de novo* contigs were assembled into larger contigs in Sequencher 5.3.2 (Gene Codes Inc., Ann Arbor, MI, USA) based on at least 20 bps overlap and 98% similarity. The UNIX 'grep' function was used to find reads and assemble them. We used a Perl script to look for motifs commonly found in plant DNA (Kalia *et al.*, 2011) using a threshold of 10 repeats for dinucleotide, eight repeats for trinucleotide and four repeats for tetra-, penta- and hexanucleotides.

Primer pairs for a total of 202 nSSRs (online Supplementary Appendix S1) were designed using Primer3web 4.0.0 (Rozen and Skaletsky, 1999), with the following parameters: primer size 18–23 bp, annealing temperatures 50–62°C, with GC content 40–80%. A set of 32 nSSR primers for nSSR with the most common nucleotide repeats was synthesized (i.e. AG, AC, TC and AAG; see Morgante and Olivieri, 1993; Kalia *et al.*, 2011). PCR amplifications were performed in a final volume of 10 µl that contained 15 ng of genomic DNA, 10 mM of each primer with forward primers labelled with 6-FAM or JOE fluorescent dyes (Macrogen, Seoul, South Korea, Table 1), 25 mM $MgCl_2$ (Promega Corporation, Madison, WI, USA) and 5 µl 1x of KAPA2G® Fast RedyMix (Kapa Biosystems, Wilmington, MA, USA). The cycling conditions were as follows: an initial denaturation step of 3 min at 94°C, followed by 30 cycles of 30 s at 94°C for denaturation, 30 s at the specific annealing temperature for each primer pair (Table 1), 72°C for 60 s and final extension of 5 min at 72°C. To test the utility of the primers, PCR products were detected using a 1.0% agarose gel electrophoresis with a 100-bp range DNA ladder (Promega Corporation).

The synthesized primers were used to check for polymorphisms in 57 individuals from three populations representing the limits of the species distribution (online Supplementary Appendix S2). DNA was extracted from silica-dried leaf material or herbarium specimens using the Invisorb Plant Mini Kit. The amplicons with fluorescent labels were resolved to genotype on an automated DNA sequencer, ABI 3500 XL (Applied Biosystems, Foster City, CA, USA) with GeneScan 500 Rox Size Standard (Applied Biosystems).

All polymorphic nSSRs were tested for cross-amplification in six individuals of *S. inaequilaterum* and *S. perforatum* (online Supplementary Appendix S2). The PCR conditions

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Table 2. Characterization of the nine nuclear microsatellite markers in three *Stizophyllum riparium*[a] populations (sample size, $n = 57$)

	SWA ($n = 17$)					SAM ($n = 22$)					EAM ($n = 18$)					ALL ($n = 57$)				
Locus	*A*	A_e	H_o	H_e	HWE(χ^2)[a]		*A*	A_e	H_o	H_e	HWE(χ^2)[a]		*A*	A_e	H_o	H_e	HWE(χ^2)[a]		*A*	A_e	H_o	H_e	PIC
Stiz63	10	2.949	0.375	0.544	115.149	***	17	13.634	0.727	0.948	198.367	***	13	6.680	1.000	0.875	147.600	***	29	13.370	0.719	0.933	0.925
Stiz104	5	3.125	0.688	0.808	34.477	***	15	10.376	1.000	0.926	176.000	***	8	3.522	0.611	0.737	63.292	***	17	9.242	0.796	0.900	0.892
Stiz112	4	2.338	0.750	0.782	20.006	**	6	4.324	0.900	0.788	19.797	ns	3	2.174	0.800	0.559	6.667	ns	8	4.812	0.804	0.800	0.793
Stiz123	14	10.889	0.563	0.883	98.000	*	15	9.025	1.000	0.913	181.785	***	11	6.857	1.000	0.891	69.600	ns	22	16.667	0.956	0.951	0.940
Stiz133	9	4.983	1.000	0.902	93.395	***	18	13.164	0.905	0.947	241.733	***	19	14.450	1.000	0.959	209.667	*	27	17.742	0.836	0.952	0.944
Stiz135	3	1.446	0.944	0.916	19.437	***	10	3.639	0.364	0.742	106.394	***	1	1.000	0.000	0.000	–		11	3.536	0.170	0.724	0.717
Stiz138	9	5.885	0.882	0.636	66.000	**	14	8.018	1.000	0.897	161.000	***	10	6.964	0.941	0.882	44.373	ns	22	8.986	0.852	0.897	0.889
Stiz154	7	4.091	0.889	0.806	53.422	***	12	8.417	1.000	0.902	72.646	ns	7	3.400	0.412	0.727	50.034	***	15	8.379	0.796	0.889	0.881
Stiz156	11	8.127	0.688	0.829	94.151	***	8	4.819	1.000	0.813	75.692	***	9	4.500	0.889	0.800	69.658	***	15	8.191	0.889	0.886	0.878
Mean	8	4.870	0.753	0.790	–	–	12.8	8.380	0.877	0.875	–	–	9	5.505	0.739	0.714	–	–	18	10.103	0.758	0.881	0.873

A, number of alleles; A_e, effective number alleles; H_o, observed heterozygosity; H_e, unbiased expected heterozygosity; *n*, sample size for each population; PIC, polymorphic information content.
Statistical deviation from HWE is indicated as $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and ns, not significant.
[a]Voucher and locality information are provided in online Supplementary Appendix S2.

and the visualization of the amplicons were as previously described. When bands of the expected size were visualized, amplifications were considered successful.

The nSSR profiles were analysed using GeneMarker (Holland and Parson, 2011). For each nSSR, the number of alleles (*A*), the effective number of alleles (A_e), the observed heterozygosity, unbiased expected heterozygosity (H_e) and Hardy–Weinberg equilibrium (HWE) were estimated using GenAlEx 6.41 (Peakall and Smouse, 2006). Polymorphic information content (PIC) was estimated using the formula $PIC = 1 - \Sigma(Pi)^2$. Gametic disequilibrium was tested for each population using FSTAT (Goudet, 1995). In order to avoid false-positives, *P*-values (= 0.05) were adjusted by applying a sequential Bonferroni correction for multiple comparisons (Rice, 1989). To check the presence of null-alleles, we used Micro-Checker 2.2.3 (Van Oosterhout *et al.*, 2004).

Results and discussion

Nine primer pairs amplified successfully (Table 1), with *A* ranging from eight to 29 and H_e varying from 0.724 to 0.952 for all populations (Table 2). No significant deviation of HWE in terms of heterozygosity deficiency was detected for any loci (Table 2). PIC values ranged from 0.717 to 0.944 and no pairs of nSSR loci were found to be in significant genotypic disequilibrium after Bonferroni correction ($P < 0.0001$). Null alleles were detected (see online Supplementary Appendix S3). All nSSR markers successfully amplified for *S. inaequilaterum* and *S. perforatum* (online Supplementary Appendix S4).

The nine polymorphic markers developed in this study showed high rates of polymorphism, and are suitable for population genetics studies. Highly polymorphic markers are fundamental tools to ensure accurate estimation of the genetic diversity parameters and the genetic structure of populations (Pinheiro *et al.*, 2011). Most genetic studies within tribe Bignonieae to date were conducted at macroevolutionary scales (e.g. Fonseca and Lohmann, 2015), with microevolutionary studies being still scarce in Bignoniaceae. The set of nSSR markers developed here will be useful for future population genetics studies of *Stizophyllum* species, including cpSSR markers (Beyer *et al.*, 2017).

Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S1479262119000108

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