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Isolation and characterization of microsatellite markers from the RAD sequence of two temperate liana species: *Euonymus fortunei* (Celastraceae) and *Schizophragma hydrangeoides* (Hydrangeaceae)

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Abstract

Clonal reproduction of lianas is a common but important life history strategy. It is necessary to evaluate the clonal structure of liana species because clonal ability is potentially a major determinants distribution pattern of lianas. Therefore, we developed 10 microsatellite markers for *Euonymus fortunei* and *Schizophragma hydrangeoides* respectively from genomic sequences obtained from double-digest restriction site associated DNA (ddRAD). The sequence data of the developed markers were deposited on the public database. The expected heterozygosity (H_E) of *E. fortunei* and *S. hydrangeoides* ranged from 0.727 to 0.847 with an average of 0.766, and from 0.734 to 0.924 with an average of 0.812, respectively. All loci were under HWE except for a locus of *S. hydrangeoides* (*sh*07). These markers should contribute to the understanding of the life history of temperate liana species.

Keywords: *liana, Celastraceae, Hydrangeaceae, microsatellite, simple sequence repeat, ddRAD.*

Introduction

Lianas are woody vines that require host trees to climb up to the forest canopy. Clonal reproduction of lianas is a common but important life history strategy. Unlike most tree species, liana species often reproduce through clonal stems (i.e. stolons) on the forest floor (Putz, 1984). The clonal ability enables lianas to effectively colonize suitable environments (e.g., canopy gaps; Ledo and Schnitzer, 2014), wait in the understory for the canopy to open (the "sit and wait" strategy; Greenberg et al., 2001), expand their distribution horizontally over long distances (Yorke et al., 2013) and search for host trees to climb (Kato et al., 2011). Thus, clonal reproduction is thought to be one of the major determinants in the distribution pattern of liana species. Genetic tools are useful for evaluating the contribution of clonal reproduction to the distribution of lianas because clonally reproduced stems below the ground and/or their connection with each other in the past is often difficult to detect using observational methods. However, few studies have demonstrated the use of genetic tools on liana distribution for the evaluation of clonal structure. Furthermore, comparison of the clonal structure of co-occurring liana species is necessary for the general understanding of the distribution and life history strategies of liana species. In the present study, we developed 10 microsatellite markers for two co-occurring liana species, namely *Euonymus fortunei* and *Schizophragma hydrangeoides*, from genomic sequences of double-digest restriction site associated DNA (ddRAD; Peterson et al., 2012). These markers would help clarify the population genetic structure of *E. fortunei* and *S. hydrangeoides*, and more importantly,

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would contribute to the understanding of their life history strategies.

Methods

Sequence data and primer design

Two individuals each of both *E. fortunei* and *S. hydrangeoides* were used to construct a sequencing library. Genomic DNA was extracted from leaf samples with DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Double-digest restriction site associated DNA was generated according to the methods described by Peterson et al. (2012) with EcoRI and MseI as restriction sites and was sequenced as 2 × 300 bp paired-end reads on MiSeq (Illumina, San Diego, CA, USA). Overlapping paired-end sequencing reads were combined with bbMerge (http://jgi.doe.gov/data-and-tools/bbtools/) and were processed with dDocent pipeline (Puritz et al., 2014) to construct the reference sequences. All the reference sequences were used for the CMIB (CD-HIT-EST, MISA, ipcress and BlastCLUST) pipeline (Ueno et al. 2012) to design primers with the number of repeat units ≥6, 5, 4, 3, and 3 for di-, tri-, tetra-, penta-, and hexa-simple sequence repeats (SSRs), respectively. Primer pairs were BLASTed against the NCBI nr database with an e-value of 1e-3. We selected 150 primer pairs for SSRs with the number of repeat units ≥10 and based on the BLAST hits. Forward primers with tail sequences (Blacket et al. 2012) and reverse primers with 'pig-tail' (Brownstein et al. 1996) were synthesized by Hokkaido System Science Co., Ltd. (Hokkaido, Japan).

Sampling, PCR, and fragment analysis

Leaf samples were taken from 30 and 29 individuals of *E. fortunei* and *S. hydrangeoides*, respectively, which were collected along the roadside and stream near Ogawa Forest Reserve (OFR) located in the north of Ibaraki Prefecture, Japan. All the samples were collected from locations that were at least 50-m apart to avoid collecting clonally reproduced stems.

DNA was extracted from leaf samples (10 mg) using extraction buffers from DNeasy kit (QIAGEN, Hilden, Germany). We started the initial polymerase chain reaction (PCR) for the two individuals of *E. fortunei* and *S. hydrangeoides* in a 10-μL reaction mixture containing primer mix, 1 X Multiplex PCR master mix (QIAGEN, Hilden, Germany), and 5–10 ng of template DNA. The primer mix contained both the forward and reverse primers and one of the fluorescently-labeled TAIL primers (Blacket et al. 2012). PCR was performed using the following thermal profiles: 15 min at 95°C, followed by 35 cycles of 30 s at 94°C, 90 s at 60°C, 60 s at 72°C, and a final extension step at 60°C for 30 min. The PCR products were analyzed using a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) with GeneScan 600 LIZ size standard (ThermoFisher Scientific, Waltham, MA, USA). Electropherograms from each marker were checked with caution for patterns of peaks using Geneious R9 (Kearse et al., 2012). For the rest of the samples, PCR amplification and genotyping were performed as described above.

Characterization of SSR markers

For each locus, the following genetic diversity indices were calculated using GenAlEx version 6.5 (Peakall and Smouse, 2012): the number of alleles (*Na*), the effective number of alleles (*Ne*), observed heterozygosity (H_O) and expected heterozygosity (H_E).

Results and Discussion

Amplified products were successfully obtained from *E. fortunei* using 10 primers in the multiplex PCR, and these products exhibited polymorphism (Table 1). The *Na* for the 10 polymorphic loci ranged from four to 11, with an average of eight. The H_O and H_E ranged from 0.567 to 0.900, with an average of 0.766, and from 0.727 to 0.847, with an average of 0.766, respectively. There was no significant linkage disequilibrium ($P > 0.05$) for all the locus pairs after Bonferroni correction using FSTAT 2.9.3 (Goudet, 1995). No significant deviations from Hardy–Weinberg equilibrium (HWE; $P > 0.05$) were detected for any of the loci. The sequences data of the developed markers were deposited in DNA Data Bank of Japan (DDBJ) with accessions numbers LC312174 to LC312193.

Table 1
Characteristics of 10 microsatellite loci for *Euonymus fortunei* (N = 30)

Locus ID	Forward primer sequence	Reverse primer sequence	SSR motif sequence	T_a (°C)	PCR product size range	*Na*	*Ne*	H_O	H_E	DDBJ Accession number
ef01	CGGAGAGCCGAGAGGTG TCCTGTTCAAATCACATC CTCCG	GTTTCTTCCGAGCGAA TGTAAGGACACGC	(AG)13	60	273-303	11	4.688	0.9	0.787	LC312174 LC312184
ef02	GCCTCCCTCGCGCCATGT CGATCAACCCACCGGAA CAG	GTTTCTTTCAACGAGC TCAGGATGTTCC	(TTA)14	60	246-285	9	4.787	0.8	0.791	LC312175 LC312185
ef03	CGGAGAGCCGAGAGGTG CGATGGCGAAATCGGAA GAAGCAG	GTTTCTTATCATCTGCA GTGTGTCGGGTGC	(TC)11	60	192-212	8	4.053	0.621	0.753	LC312176 LC312186
ef04	CGGAGAGCCGAGAGGTG CATAATTTGCCTAGGTCC TTTCTTG	GTTTCTTATTCAGTCTC AGCGTTCCGGCTC	(CT)16	60	272-296	9	6.207	0.9	0.839	LC312177 LC312187
ef05	CAGGACCAGGCTACCGT GACACCAAGTGATCAAC CTGCATTC	GTTTCTTGGAGCCTTT CCACTTCTGCTCTC	(AT)14	60	153-193	9	4.215	0.6	0.763	LC312178 LC312188
ef06	CAGGACCAGGCTACCGT GAGGTCAAACCATGCCA GAACTTGC	GTTTCTTGCCGCTTCTT TGTCCTGAACTCG	(TC)14	60	185-199	7	3.659	0.767	0.727	LC312179 LC312189
ef07	CAGGACCAGGCTACCGT GACCACTCAATACCCTCC AAGCCC	GTTTCTTCGATTTCCCA ACTCCAGAGTCTCC	(GA)11	60	290-298	5	3.982	0.8	0.749	LC312180 LC312190
ef08	CAGGACCAGGCTACCGT GACAGAGCTGCAAACAT ATTTGGGAGC	GTTTCTTGTGAAATGG CAGTGGTATGGATGC	(TG)14	60	211-227	8	4.036	0.867	0.752	LC312181 LC312191
ef09	CGGAGAGCCGAGAGGTG TCGCATCCTCATCACAAC TCCCAC	GTTTCTTAGGCGGATC AGTGTAGTCCTTGG	(CT)12	60	183-199	10	6.522	0.833	0.847	LC312182 LC312192
ef10	CGGAGAGCCGAGAGGTG ACCTAGGCAGACCTCGA GAACTC	GTTTCTTAGCCTCAAA TCTCCAAGAATCTCCC	(CT)12	60	170-176	4	3.203	0.567	0.688	LC312183 LC312193

T_a: annealing temperature, *Na*: Number of alleles per locus, *Ne*: Number of effective alleles per locus, H_O: observed heterozygosity, *HE*: expected heterozygosity

Similar results were obtained for *S. hydrangeoides*, where amplified products were successfully obtained using ten primers in the multiplex PCR, and these products exhibited polymorphisms (Table 2). The *Na* for the 10 polymorphic loci ranged from seven to 19, with an average of 10.2. The H_O ranged from 0.586 to 0.897, with an average of 0.738, and the H_E ranged from 0.734 to 0.924, with an average of 0.812. There was no significant linkage disequilibrium ($P > 0.05$) for any of the locus pairs after Bonferroni correction using FSTAT 2.9.3 (Goudet,

Table 2
Characteristics of 10 microsatellite loci for *Schizophragma hydrangeoides* (N = 29)

[1]

Locus ID	Forward primer sequence	Reverse primer sequence	SSR motif sequence	T_a (°C)	PCR product size range	*Na*	*Ne*	H_O	H_E	DDBJ Accession number
sh01	GCCTCCCTCGCGCCA TGAGAACCTGAAGAGT AGTCCTCGC	GTTTCTTGGTTGAA ATGGTGGCGGAATT GC	(AG)14	60	132-156	12	7.097	0.793	0.859	LC312154 LC312164
sh02	CAGGACCAGGCTACC GTGTCTTCTTGCAAG TGTAACAGAAGCTC	GTTTCTTGTTGTTG AGAGCTGCTGTGGT GC	(GAA)13	60	153-186	10	3.763	0.655	0.734	LC312155 LC312165
sh03	CGGAGAGCCGAGAG GTGAGCTCCTCAACC AATTCAAGGTTCC	GTTTCTTACATCCG CGTTGGCTCTCATG G	(CT)11cacaa(AC)8	60	257-297	19	13.141	0.897	0.924	LC312156 LC312165
sh04	CAGGACCAGGCTACC GTGTGCAAGTATGAC ACAGCAAACAAAG	GTTTCTTGCGAAAT GTGGAGAGACCCT GG	(AT)12	60	255-267	7	5.820	0.724	0.828	LC312157 LC312167
sh05	CAGGACCAGGCTACC GTGCCGCCTTCACAT GCACACAACAC	GTTTCTTACAAGAT GCTTCAGAGGAGC AGC	(TC)13	60	136-162	12	6.300	0.759	0.841	LC312158 LC312168
sh06	CGGAGAGCCGAGAG GTGAAGTTATTTCCT TTGACCCTCACAC	GTTTCTTCCTCAGG AACATCGCTGCATC G	(TA)13	60	221-245	7	5.644	0.759	0.823	LC312159 LC312169
sh07	GCCTTGCCAGCCCGC ACAGGTATAAGCTCC ACTATAGTTGC	GTTTCTTGGGTACA ATCACCGGTATCAG CG	(TA)13	60	159-179	9	5.021	0.586	0.801	LC312160 LC312170
sh08	CAGGACCAGGCTACC GTGCCATCACGGGTA CACGTGCAAAG	GTTTCTTATTCGATC CTGACGCCGGAACT C	(TA)12	60	218-254	11	4.976	0.793	0.799	LC312161 LC312171
sh09	CAGGACCAGGCTACC GTGAGTGGGAATAGC TGGACCCTTAC	GTTTCTTGAGACCC TCTGTGTTGCCTAC C	(TA)13	60	193-205	7	3.976	0.586	0.749	LC312162 LC312172
sh10	CGGAGAGCCGAGAG GTGTTCCGATCCGTT CCATCCGAATG	GTTTCTTCTCCGTC GTCAACAACTCCGG	(CT)12	60	241-263	10	5.780	0.828	0.827	LC312163 LC312173

T_a: annealing temperature, *Na*: Number of alleles per locus, *Ne*: Number of effective alleles per locus, H_O: observed heterozygosity, H_E: expected heterozygosity

1995). Significant deviations from HWE ($P < 0.05$) were detected for a locus *sh07*. The sequences data of the developed markers were deposited in DDBJ with accessions numbers LC312154 to LC312173.

The microsatellite markers described here will contribute to the evaluation and comparison of genetic structure and genetic diversity within and among the populations of *E. fortunei* and *S. hydrangeoides*. These findings would aid in the understanding of the life history strategies of temperate liana species.

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