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Primer Note

Development of SSR markers for *Psychotria homalosperma* (Rubiaceae) and cross-amplification in four other species[1]

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- *Premise of the study:* Twenty-six microsatellite (simple sequence repeat [SSR]) markers were characterized in *Psychotria homalosperma* (Rubiaceae), an endemic evergreen tree in the Bonin Islands, Japan, to investigate the genetic structure and gene flow of the species.
- *Methods and Results:* Using next-generation sequencing, we developed 26 SSR markers for *P. homalosperma* with perfect motifs from di- to pentanucleotide repeats. Of these, the Chichijima and Hahajima island populations of *P. homalosperma* had mean allele numbers of 6.50 and 6.81, respectively. The mean expected heterozygosities were 0.578 and 0.606, respectively. In addition, 10 and eight of these markers were successfully amplified for *P. boninensis* and *P. serpens*, respectively, occurring in the same or adjacent areas.
- *Conclusions:* The SSR markers developed in this study will be useful for future studies concerning the population genetics of *P. homalosperma* and will facilitate the development of a conservation strategy.

Key words: de novo sequencing; gene flow; heterostyly; microsatellites; *Psychotria homalosperma*; Rubiaceae.

Psychotria L. (Rubiaceae) has been recognized as an important model system for the study of heterostyly and its evolutionary transition on oceanic islands (Watanabe and Sugawara, 2015). *Psychotria homalosperma* A. Gray is an evergreen tree found only in the Chichijima and Hahajima island groups of the oceanic Bonin Islands in the northwest Pacific Ocean. Previous studies have reported that the species is distylous with self- and intramorphic incompatibilities (Watanabe et al., 2014). Revealing the mating system and the gene flow patterns in this species will help in understanding the evolutionary significance of heterostyly on oceanic islands. Meanwhile, the *Red List of Threatened Plants of Japan* and *Red List of Threatened Species in Tokyo* have described *P. homalosperma* as "vulnerable to extinction" (Tokyo Metropolitan Government, 2011; Ministry of the Environment, 2012). Recently, with the exception of some populations on Hahajima Island, natural populations of *P. homalosperma* did not regenerate successfully, apparently because of disturbances from human activities (Watanabe et al., 2009; Sugai et al., 2015). Therefore, genetic information (e.g., genetic diversity within populations and genetic differentiation between islands) will be important for the development of an effective conservation plan for this species.

Here, we developed 26 microsatellite (simple sequence repeat [SSR]) markers for *P. homalosperma* for use in evolutionary and conservation studies. These markers were tested on two natural populations of *P. homalosperma* because it is currently distributed only in the two island groups of the Bonin Islands. We also examined the transferability of these markers to four species of *Psychotria* (*P. boninensis* Nakai, *P. rubra* (Lour.) Poir., *P. manillensis* Bartl. ex DC., and *P. serpens* L.) that occur naturally in Japan and adjacent areas.

METHODS AND RESULTS

Total genomic DNA of *P. homalosperma* was extracted from a fresh leaf collected from Sekimon (26°40′11.3″N, 142°09′16.4″E) on Hahajima Island, using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). A voucher specimen of this sample was deposited in the Makino Herbarium (MAK) of Tokyo Metropolitan University, Japan (Appendix 1). The extracted DNA of *P. homalosperma* was pyrosequenced using a 454 GS Junior System (Roche, Basel, Switzerland). Multiplex Identifier (MID) tags were used for multiplexing of the abovementioned sample and the sample of another species in the Bonin Islands, i.e., *Gynochthodes boninensis* (Ohwi) E. Oguri & T. Sugaw. These samples were then combined. The raw data were demultiplexed and MID tags removed from the reads using Newbler (Roche). The identification of SSRs and design of primers from the above DNA sequences were performed using the QDD 2.1 program (Meglecz et al., 2010). This program is composed of

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the following steps to design PCR primers: (1) selection of sequences that contain SSRs, (2) elimination of redundant sequences, (3) primer design, and (4) contamination detection.

The de novo sequencing produced 148,586 reads with an average length of 422 bp. These reads were registered in the DNA Data Bank of Japan (DDBJ) Sequence Read Archive (DRA004086). SSR loci were identified as having bordered sequences with more than five repeats for di- to hexanucleotide motifs, and the length of one sequence was more than 80 bp. According to these criteria, a total of 5544 reads contained SSR loci. To eliminate redundancy, all sequences containing SSRs were subjected to an "all-against-all" BLAST with an *E*-value of 1E−40. Subsequently, 2384 reads were selected from whole sequences containing SSRs. PCR primers were designed using Primer3 (Rozen and Skaletsky, 2000) implemented in the QDD program. Finally, a total of 1475 SSR primer pairs were designed by Primer3.

Amplification and polymorphism tests were performed for 48 selected primer pairs with consideration for the SSRs (single motifs of di-, tri-, tetra-, and pentanucleotides with 8–13 repeats) and the type of design ("A" in QDD 2.1). Four universal primers with different fluorescent tags, as designed by Blacket et al. (2012), were prepared. The 5′ end of each forward

Table 1. Twenty-six SSR markers for *Psychotria homalosperma*.[a]

Locus	Primer sequences (5′–3′)	Fluorescent label[b]	PIG-tail[c]	Repeat motif	Allele size range (bp)	GenBank accession no.
Ph0095	F: TTAAGCGGCCATAAATTAAGAAGA R: TGGTATGAGGTATGATGTCTGGAA	GCCTTGCCAGCCCGC	GTT	$(CT)_8$	137–145	LC093233
Ph0172	F: GTGCCTGGATCGATATCATTACTT R: CCGGTCTCTTCGTATACCTCTAAA	CGGAGAGCCGAGAGGTG	GTTT	$(CT)_8$	114–122	LC093234
Ph0248	F: TTCAGTGACTCAACTCGTGATTTC R: TCTGAGTACCTGGAATTGTGATTG	CAGGACCAGGCTACCGTG	GTT	$(AT)_8$	213–223	LC093235
Ph0288	F: TTCAGGACAAGCCAATAAACTACC R: AAGTTTACGATGAGGTTCCTACCA	CGGAGAGCCGAGAGGTG	GTTT	$(TA)_{11}$	80–130	LC093236
Ph0353	F: AGGAGATTGCATTATTAACAACCG R: CTTATGTCCCATTTCAACAGTGTG	CAGGACCAGGCTACCGTG	GTTT	$(AT)_{10}$	173–217	LC093237
Ph0401	F: CCCTAGGTAAATCAAAGCAAGAAG R: TCTTCTTATAGCGATTGAGCATGA	GCCTCCCTCGCGCCA	GTT	$(ACA)_{10}$	136–151	LC093238
Ph0432	F: AATTCAACCGCTCTTCAATATCAA R: TCTGGTACACTGATCTGCTTTCTG	GCCTTGCCAGCCCGC	GTT	$(AG)_8$	123–125	LC093239
Ph0517	F: AAAGAATAAACAGCAAGTTAGGCT R: TAGAATACAAACATGGGATGACGA	CAGGACCAGGCTACCGTG	GTT	$(AT)_8$	140–166	LC093240
Ph0539	F: GTCGGTTGTCATCGTCATTAAATA R: CGGGAAGTCTGTCAAGACAAGTAT	GCCTTGCCAGCCCGC	GTTT	$(ACAA)_8$	195–219	LC093241
Ph0587	F: AGTTGCTTAGAAATGCAAAGATGT R: TAGTAGTGAATCGCAAAGGCAA	CAGGACCAGGCTACCGTG	GTT	$(CT)_8$	108–128	LC093242
Ph0606	F: GATGGTCATATGTTGATTGTGGAG R: TTTCGCACGAAAGAAGAAACAT	CAGGACCAGGCTACCGTG	G	$(GAGGA)_8$	142–173	LC093243
Ph0639	F: TGCAGTCTCGTCGCTTTAATAGAT R: CCTCTTAGATTGTCATTGTTATCGG	GCCTTGCCAGCCCGC	GTTT	$(ATT)_8$	103–127	LC093244
Ph0711	F: GGTTTAATATTGGCTGGACCTTCT R: AGAAATGATTTAACACCCACTTCG	CGGAGAGCCGAGAGGTG	GTTT	$(AT)_{12}$	141–159	LC093245
Ph0770	F: ATTGCCTCTGTCTATCTTTGGTTG R: GCATTTATCTTGTGGCTCAAATGT	GCCTTGCCAGCCCGC	GTTT	$(AT)_8$	109–149	LC093246
Ph0789	F: ATCCACGTGTACCCATAATTTGTT R: TAGAGTGGTAGGACTTGGGAATTG	GCCTTGCCAGCCCGC	GTT	$(AAT)_{10}$	271–287	LC093247
Ph0855	F: TCTCGGCTAGTACTGATGATAGGAA R: GAAAGGTAGGAGGTAACTATGCCC	GCCTCCCTCGCGCCA	GTTT	$(AT)_9$	167–177	LC093248
Ph0878	F: TTTGTAACTTGACTAGAATTCGGC R: AGTATTCAACTCGAATGTTAAATGAA	GCCTTGCCAGCCCGC	GTTT	$(TA)_8$	163–219	LC093249
Ph0954	F: CTGCTGAGCCCATAATAAATTACA R: ACATTCCCATCATAGCATTAGGAC	CAGGACCAGGCTACCGTG	GTTT	$(AAT)_{10}$	154–169	LC093250
Ph1051	F: ACATTTGTTACCTACGGCTACGAT R: TATACAACTCATGCTCCATTGTCC	GCCTCCCTCGCGCCA	GTT	$(GAT)_{13}$	194–224	LC093251
Ph1073	F: TTTGGTGTACTAGAAGGAATTGGG R: GCTCTTCACCTCGCATCTCTT	CGGAGAGCCGAGAGGTG	GTTT	$(TG)_8$	341–371	LC093252
Ph1122	F: ACACTCCGGAAACTTCCCAC R: CCAGTCCTCGAGAAGAAGTGATTA	GCCTCCCTCGCGCCA	GTTT	$(AAG)_9$	122–149	LC093253
Ph1126	F: CTTAGACGGATGATCATGAATGG R: AACAGTAGGATGAAAGGAGTGGAA	CAGGACCAGGCTACCGTG	GTTT	$(AT)_{12}$	213–257	LC093254
Ph1163	F: AAACTTAATGTCACCTCTAATGCCA R: ATACCCTATCAATTTCTCCACGCT	CGGAGAGCCGAGAGGTG	GTTT	$(TG)_9$	167–183	LC093255
Ph1284	F: GATCAGAAATCAGCCAAGCAT R: CTGTTTAGGCTTCTAACTCAAAGTTGC	GCCTCCCTCGCGCCA	GTTT	$(AG)_8$	99–103	LC093256
Ph1346	F: TGATCTATGGATTTGACGTAACCA R: CAATACAGAAGAGGTGGAGGAAAG	GCCTCCCTCGCGCCA	GTTT	$(CTT)_8$	227–249	LC093257
Ph1387	F: TGATTCTCAATTGCATGTATTGGT R: ATGACTTGGGTTTAAGTTGGAAGA	CGGAGAGCCGAGAGGTG	GTTT	$(TA)_9$	131–135	LC093258

[a] Annealing temperature for all loci was 57°C.
[b] Fluorescent label sequences attached to the forward primer (Blacket et al., 2012).
[c] PIG-tail sequences attached to the reverse primer (Brownstein et al., 1996).

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primer was attached to the same sequence as a tail. Additionally, all reverse primers were given a PIG-tail (5′-GTTT-3′, 5′-GTT-3′, or 5′-G-3′) at the 5′ end of the sequence to reduce stuttering due to the inconsistent addition of adenine by *Taq* DNA polymerase (Brownstein et al., 1996). PCR amplification was performed using the Type-it Microsatellite PCR Kit (QIAGEN). Multiplex PCRs were performed for each of the four primer pair sets. The thermal cycler program was as follows: 95°C for 5 min followed by 32 cycles of 95°C for 30 s, 57°C for 1.5 min, and 72°C for 30 s, as well as a final extension step of 60°C for 30 min. PCR products were mixed with a GeneScan 600 LIZ Size Standard (Life Technologies, Waltham, Massachusetts, USA) and loaded onto an ABI3130 Genetic Analyzer (Life Technologies). Fragment lengths were examined using GeneMapper 4.0 (Life Technologies).

We then tested two populations of *P. homalosperma* from Chichijima and Hahajima islands to evaluate their allelic polymorphism. A total of 48 individuals were tested: 24 from Higashidaira (27°04′35.7″N, 142°13′14.9″E) on Chichijima Island and 24 from Sekimon (26°40′26.3″N, 142°09′21.6″E) on Hahajima Island. In addition, transferability among four other *Psychotria* species occurring in Japan (*P. boninensis* [N = 8] from Chichijima Island of the Bonin Islands; *P. rubra* [N = 8], *P. manillensis* [N = 8], and *P. serpens* [N = 8] from the Ryukyu Islands) was tested using the same PCR conditions described above. Voucher specimens of the representative individuals were deposited in MAK (Appendix 1). To characterize each locus, the number of alleles per locus (A), observed heterozygosity (H_o), expected heterozygosity (H_e), and fixation index (F_{IS}) were calculated using GenAlEx 6.501 (Peakall and Smouse, 2006). The Hardy–Weinberg equilibrium (HWE) at each locus of each population and the linkage disequilibrium (LD) between loci of each population were tested with FSTAT 2.9.3.2 (Goudet, 2002).

Of the 48 tested SSR markers, 26 primer pairs were successfully amplified and showed polymorphism among 48 individuals of *P. homalosperma* (Table 1). The mean A was 6.50 (1–19) in the Chichijima population and 6.81 (2–18) in the Hahajima population (Table 2). For the Chichijima population, the mean H_o and H_e were 0.547 (0.083–1.000) and 0.578 (0.080–0.905), respectively. For the Hahajima population, the corresponding values were 0.581 (0.043–0.917) and 0.606 (0.122–0.910), respectively (Table 2). None of the loci deviated significantly from HWE. No significant LD between markers was observed in either of the populations. Of the 26 SSR markers tested, 10 were successfully amplified for *P. boninensis* and eight for *P. serpens* (Table 3), while none could be amplified for *P. rubra* or *P. manillensis* (Table 3).

CONCLUSIONS

Twenty-six novel SSR markers were developed for *P. homalosperma* using a next-generation sequencing approach. These markers are likely to be useful for evaluating the genetic structure and gene flow of *P. homalosperma*, which will subsequently facilitate the development of a conservation strategy for this species. The developed markers are unlikely to be useful for the study of the other tested *Psychotria* species in Japan, most likely because *P. homalosperma* is assigned to a section (sect. *Pelagomapouria* Fosb.) that is different from those of the other tested species (Yamazaki, 1993); moreover, *P. rubra* and *P. manillensis* are polyploid (the former is tetraploid [$2n = 42$] and the latter octoploid [$2n = 84$]) (Nakamura et al., 2003). However, future studies should examine the applicability of these markers to critically endangered sect. *Pelagomapouria* species found in the Hawaiian Islands (U.S. Fish and Wildlife Service, 2015).

Table 2. Characteristics of 26 SSR markers in the two populations of *Psychotria homalosperma*.

Locus	A_T	Chichijima Island (N = 24)				Hahajima Island (N = 24)			
		A	H_o	H_e	F_{IS}[a]	A	H_o	H_e	F_{IS}[a]
Ph0095	4	4	0.125	0.193	0.351	3	0.417	0.434	0.040
Ph0172	3	3	0.083	0.081	−0.032	2	0.542	0.478	−0.132
Ph0248	6	3	0.625	0.598	−0.045	6	0.583	0.668	0.126
Ph0288	15	12	0.375	0.785	0.522	12	0.833	0.831	−0.003
Ph0353	20	15	0.792	0.870	0.090	16	0.875	0.873	−0.002
Ph0401	6	3	0.625	0.612	−0.021	6	0.750	0.760	0.013
Ph0432	2	2	0.083	0.080	−0.043	2	0.208	0.187	−0.116
Ph0517	13	7	0.609	0.690	0.118	13	0.727	0.902	0.194
Ph0539	8	6	0.375	0.421	0.109	6	0.708	0.720	0.016
Ph0587	6	4	0.708	0.666	−0.064	4	0.875	0.662	−0.321
Ph0606	7	5	0.208	0.194	−0.071	6	0.583	0.644	0.094
Ph0639	7	4	0.375	0.353	−0.061	5	0.083	0.639	0.870
Ph0711	10	8	0.833	0.773	−0.077	9	0.625	0.819	0.237
Ph0770	15	11	0.542	0.656	0.175	7	0.375	0.360	−0.041
Ph0789	10	8	0.833	0.787	−0.058	7	0.750	0.748	−0.002
Ph0855	6	5	0.750	0.709	−0.058	5	0.542	0.475	−0.141
Ph0878	25	19	0.667	0.905	0.264	12	0.833	0.826	−0.008
Ph0954	5	4	0.625	0.678	0.078	5	0.708	0.704	−0.006
Ph1051	11	9	1.000	0.841	−0.189	7	0.750	0.688	−0.091
Ph1073	3	1	—	—	—	3	0.417	0.338	−0.234
Ph1122	9	4	0.833	0.749	−0.112	9	0.708	0.824	0.140
Ph1126	22	12	0.917	0.850	−0.079	18	0.875	0.910	0.038
Ph1163	8	7	0.833	0.779	−0.070	6	0.917	0.793	−0.157
Ph1284	3	3	0.250	0.223	−0.121	3	0.167	0.155	−0.073
Ph1346	7	7	0.417	0.426	0.022	3	0.208	0.190	−0.096
Ph1387	3	3	0.200	0.541	0.630	2	0.043	0.122	0.643
Average	9.00	6.50	0.547	0.578	0.050	6.81	0.581	0.606	0.038

Note: A = number of alleles per locus; A_T = total number of alleles per locus; F_{IS} = fixation index; H_e = expected heterozygosity; H_o = observed heterozygosity; N = number of genotyped individuals.

[a] None of the loci deviated significantly from Hardy–Weinberg equilibrium.

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Table 3. Transferability of the 26 SSR markers for the four species of *Psychotria* in Japan.[a]

Locus	*P. boninensis* (*N* = 8)	*P. rubra* (*N* = 8)	*P. manillensis* (*N* = 8)	*P. serpens* (*N* = 8)
Ph0095	No	No	No	No
Ph0172	No	No	No	2
Ph0248	1	No	No	2
Ph0288	No	No	No	No
Ph0353	2	No	No	2
Ph0401	No	No	No	No
Ph0432	1	No	No	1
Ph0517	No	No	No	No
Ph0539	1	No	No	2
Ph0587	No	No	No	No
Ph0606	1	No	No	1
Ph0639	No	No	No	No
Ph0711	No	No	No	No
Ph0770	1	No	No	2
Ph0789	No	No	No	2
Ph0855	No	No	No	No
Ph0878	2	No	No	No
Ph0954	No	No	No	No
Ph1051	2	No	No	No
Ph1073	No	No	No	No
Ph1122	No	No	No	No
Ph1126	2	No	No	No
Ph1163	No	No	No	No
Ph1284	No	No	No	No
Ph1346	2	No	No	No
Ph1387	No	No	No	No

Note: No = amplification failed or nonspecific (three or more polymorphic bands detected).

[a]The number of alleles is given for loci for which amplification was successful. Calculation of the descriptive genetic parameters was not performed because of small sample sizes.

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Appendix 1. Voucher and locality information of five species used in the development and evaluation of SSR markers for *Psychotria homalosperma*. Voucher specimens were deposited at Makino Herbarium, Tokyo Metropolitan University (MAK), Tokyo, Japan.

Taxon	Locality	Latitude	Longitude	Voucher no.
P. homalosperma A. Gray	Higashidaira, Chichijima Island, Bonin Islands, Tokyo, Japan	27°04′35.7″N	142°13′14.9″E	MAK436002
	Sekimon, Hahajima Island, Bonin Islands, Tokyo, Japan	26°40′11.3″N	142°09′16.4″E	MAK436004
P. boninensis Nakai	Yoakeyama, Chichijima Island, Bonin Islands, Tokyo, Japan	27°05′16.9″N	142°12′58.8″E	MAK436340
P. rubra (Lour.) Poir.	Mt. Nago-dake, Nago, Okinawa, Japan	26°35′14.5″N	128°00′40.7″E	MAK435900
P. manillensis Bartl. ex DC.	Sueyoshi-Park, Naha, Okinawa, Japan	26°13′38.0″N	127°42′54.5″E	MAK435896
P. serpens L.	Okinawa College, Henoko, Nago, Okinawa, Japan	26°31′37.0″N	128°01′45.4″E	MAK435906

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