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

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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 distylos 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 Sekinson (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...
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 submitted to 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 primer (pig-tail sequences attached to the reverse primer) and the 3' end of each reverse primer (PS-tail sequences attached to the forward primer) are shown in Table 1.

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

| Locus | Primer sequences (5′–3′) | Fluorescent labela | PIG-tailb | Repeat motif | Allele size range (bp) | GenBank accession no. |
|-------|--------------------------|-------------------|-----------|--------------|------------------------|-----------------------|
| Ph0095 | F: TTAAGCCGGCCATATAATTTAGAGA | GCTTGGACGCGCCG | GTT | (CT),4 | 137–145 | LC093233 |
|        | R: TATTATGGGAGGATGAGTTGGA | CCTTTCATTTCGTTAATCTA | GCTTGGACGCGCCG | GTT | (CT),4 | 114–122 |
| Ph0172 | F: GTGGCTTGATATGATGTTGGA | GCTTGGACGCGCCG | GTT | (CT),4 | 137–145 | LC093233 |
|        | R: TTTTGAGGAGGATGAGTTGGA | CCTTTCATTTCGTTAATCTA | GCTTGGACGCGCCG | GTT | (CT),4 | 114–122 |
| Ph0248 | F: TTGGAGGGGAGGATGAGTTGGA | GCTTGGACGCGCCG | GTT | (CT),4 | 137–145 | LC093233 |
|        | R: TTTTGAGGAGGATGAGTTGGA | CCTTTCATTTCGTTAATCTA | GCTTGGACGCGCCG | GTT | (CT),4 | 114–122 |
| Ph0288 | F: TTTTGAGGAGGATGAGTTGGA | GCTTGGACGCGCCG | GTT | (CT),4 | 137–145 | LC093233 |
|        | R: TTTTGAGGAGGATGAGTTGGA | CCTTTCATTTCGTTAATCTA | GCTTGGACGCGCCG | GTT | (CT),4 | 114–122 |

**Notes:**

- a Fluorescent label sequences attached to the forward primer (Blacket et al., 2012).
- b PIG-tail sequences attached to the reverse primer (Brownstein et al., 1996).
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  | A1 | A2 | A3 | A4 | Chichijima Island (N = 24) | Hahajima Island (N = 24) |
|--------|----|----|----|----|---------------------------|---------------------------|
|        |    |    |    |    | A1 | A2 | A3 | A4 | A1 | A2 | A3 | A4 |
| Ph0095 | 4  | 4  | 1.25 | 0.139 | 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 | 0.487 | 0.909 | 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  | 0.549 | 0.425 | 0.118 | 13 | 0.727 | 0.902 | 0.194 |
| Ph0539 | 8  | 6  | 0.375 | 0.421 | 0.109 | 6  | 0.708 | 0.720 | 0.194 |
| 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 |
| Ph0757 | 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 | 0.687 | 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  | 3  | 0.833 | 0.749 | -0.112 | 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; A1 = total number of alleles per locus; Fis = fixation index; Ho = expected heterozygosity; Ho = observed heterozygosity; N = number of genotyped individuals.

*None of the loci deviated significantly from Hardy–Weinberg equilibrium.*
Table 3. Transferability of the 26 SSR markers for the four species of Psychotria in Japan.*

| 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                    | No                |
| 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).

*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.

LITERATURE CITED

Blacket, M. J., C. Robin, R. T. Good, S. F. Lee, and A. D. Miller. 2012. Universal primers for fluorescent labelling of PCR fragments—an efficient and cost-effective approach to genotyping by fluorescence. Molecular Ecology Resources 12: 456–463.

Brownstein, M. J., J. D. Carpenter, and J. R. Smith. 1996. Modulation of non-templated nucleotide addition by Taq DNA polymerase: Primer modifications that facilitate genotyping. BioTechniques 20: 1004–1006, 1008–1010.

Goudet, J. 2002. FSTAT v2.9.3.2. Website http://www2.unil.ch/popgen/softwares/fstat.htm [accessed 14 April 2016].

Miegiez, E., C. CosteDOat, V. Dubut, A. Gilles, T. MalauSa, N. Pich, and J.-F. Martin. 2010. QDD: A user-friendly program to select microsatellite markers and design primers from large sequencing projects. Bioinformatics 26: 403–404.

Ministry of the Environment, Government of Japan. 2012. The 4th Version of the Red List of Threatened Plants of Japan. Website http://www.env.go.jp/pl/bk/stsp/Final/2012/pl/2012_redlist.pdf [accessed 27 October 2015] [in Japanese].

Nakamura, K., T. Denda, O. Kameshima, U. Uehara, and M. Yokota. 2003. Chromosome numbers of Ophiophriza and Psychotria (Rubiaceae) in the Ryukyus. Biological Magazine Okinawa 41: 15–24 [in Japanese with English abstract].

Peakall, R., and P. E. Smouse. 2006. GenAIEx 6: Genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes 6: 228–295.

Rozen, S., and H. J. Skaletsky. 2000. Primer3 on the WWW for general users and for biologist programmers. In S. Misener and S. A. Krawetz [eds.], Methods in molecular biology, vol. 132: Bioinformatics methods and protocols, 365–386. Humana Press, Totowa, New Jersey, USA.

Sugai, K., K. WatANabe, A. Muki, H. Kato, and T. SugAWaRa. 2015. Conservation of endangered species Psychotria homalosperma endemic to the Bonin (Ogasawara) Islands (2). Annual Report of Ogasawara Research 38: 65–73 [in Japanese].

Tokyo Metropolitan Government. 2011. 2011 Red List of Threatened Species in Tokyo: Islands version. Tokyo Metropolitan Government, Tokyo, Japan [in Japanese].

U.S. Fish and Wildlife Service. 2015. Endangered Species. Website http://www.fws.gov/endangered/ref=toobar [accessed 2 November 2015].

WatANabe, K., H. Kato, and T. SugAWaRa. 2009. Conservation of endangered species Psychotria homalosperma endemic to the Bonin (Ogasawara) Islands. Annual Report of Ogasawara Research 32: 11–26 [in Japanese].

WatANabe, K., H. Kato, and T. SugAWaRa. 2014. Distylly and incompatibility in Psychotria homalosperma (Rubiaceae), an endemic plant of the oceanic Bonin (Ogasawara) Islands. Flora 209: 641–648.

WatANabe, K., and T. SugAWaRa. 2015. Is heterostyly rare on oceanic islands? AoB Plants 7: plv087.

Yamazaki, T. 1993. Psychotria L. In K. Iwatsuki, T. Yamazaki, D. E. Boufford, and H. Obha [eds.], Flora of Japan Illa, 225–227. Kodansha Ltd., Tokyo, Japan.

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        |
| P. boninensis Nakai     | Sekimon, Hahajima Island, Bonin Islands, Tokyo, Japan | 26°40'11.3"N | 142°09'16.4"E | MAK436004        |
| 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        |