Primer Note

Twenty novel polymorphic microsatellite primers in the critically endangered Melastoma tetramerum var. tetramerum (Melastomataceae)

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• Premise of the study: Microsatellite markers were identified for Melastoma tetramerum var. tetramerum (Melastomataceae), a critically endangered shrub endemic to the Bonin Islands, to reveal genetic characteristics in wild and restored populations.
• Methods and Results: Using next-generation sequencing, 27 microsatellite markers were identified. Twenty of these markers were polymorphic in M. tetramerum var. tetramerum, with two to nine alleles per locus and expected heterozygosity ranging from 0.10 to 0.71. Among the 20 polymorphic markers, 15 were applicable to other closely related taxa, namely M. tetramerum var. pentapetalum, M. candidum var. candidum, and M. candidum var. alessandrense.
• Conclusions: These markers can be potentially useful to investigate the genetic diversity, population genetic structure, and reproductive ecology of M. tetramerum var. tetramerum as well as of the three related taxa to provide appropriate genetic information for conservation.

Key words: Bonin Islands; conservation; Melastoma; Melastomataceae; microsatellites; next-generation sequencing.

Melastoma L. (Melastomataceae) is a genus comprising 50 species (Stevens, 2001) that are distributed from the Indo-Malesian region to the Pacific Ocean. Around the Japanese archipelago, 10 taxa (seven species and three varieties) grow wild, four of which are vulnerable, endangered, or critically endangered (Ministry of the Environment, Japan, 2015). In the Bonin Islands, which are typical oceanic islands with unique biota and which were designated as a UNESCO Natural World Heritage Site in 2011, three Melastoma taxa are of conservation concern. Melastoma tetramerum Hayata var. tetramerum is a critically endangered shrub, endemic to Chichi-jima Island in the Bonin Islands. Only one individual was known at Higashidaira (HD) until a population comprising 125 individuals was discovered in 1993 at Higashikaigan (HK), which is 2 km from HD (Shimizu, 1997). However, by 1995 the individual at HD had died (Toyoda, 2014). The HK population also declined with forest development because a forest containing fewer gaps was unsuitable for this light-demanding shrub. Several dozen wild individuals were remaining in HK in 2007 (Ministry of the Environment, Japan, 2015). Cuttings and seeds of the extinct individual in HD and 11 individuals in HK were collected for propagation at the Koishikawa Botanical Garden of the University of Tokyo before 1995 and in 2005–2008, respectively. The source seeds or cuttings of the restored plants were derived from individuals collected at the restoration sites. The garden has stored and propagated these cuttings and seeds and has restored 558 and 252 plants to HD and HK, respectively.

To determine the genetic variation and differentiation of in situ and ex situ populations and to maintain genetic diversity in future populations, we identified 20 polymorphic microsatellite loci to genotype all available individuals of M. tetramerum var. tetramerum. The broad applicability of these markers was examined in three closely related taxa, namely, M. tetramerum var. pentapetalum Toyoda, M. candidum D. Don var. candidum, and M. candidum var. alessandrense S. Kobay. Melastoma tetramerum var. pentapetalum is an endangered variety, endemic to two mountains on Haha-jima Island, which is 37 km from Chichi-jima Island. This variety has flowers with five petals, whereas M. tetramerum var. tetramerum has flowers with four petals. More than 100 individuals of M. tetramerum var. pentapetalum had been observed in 1994; however, only several dozen were found in 2007 (Shimizu, 1997; Ministry of the Environment, Japan, 2015). Melastoma candidum var. candidum is a common variety native to Okinawa, Taiwan, China, and Indochina (Ohwi, 1978). Melastoma candidum var. alessandrense is endemic to Kita-Iwo-To Island, an uninhabited island isolated 200 km from Chichi-jima Island. This variety is vulnerable, with a total of several hundred individuals known in 2007 (Ministry of the Environment, Japan, 2015).

Methods and Results

An individual of M. tetramerum var. tetramerum was collected at HK and stored at Koishikawa Botanical Garden (voucher: komaki201601; Appendix 1) for use in developing microsatellite loci. From silica gel–dried leaf tissues, total genomic DNA was extracted using the QIAGEN DNeasy Plant Mini Kit (QIA-GEN, Hilden, Germany), and a fragment DNA library was constructed using Ion

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Shear Plus Reagents (Life Technologies, Carlsbad, California, USA) and the Ion Plus Fragment Library Kit (Life Technologies) according to the manufacturer’s protocol. After dilution to 15 pM, the library was sequenced using the Ion PGM system (Life Technologies) with Ion PGM Template OT2 400 Kit (Life Technologies), Ion PGM Sequencing 400 Kit (Life Technologies), and Ion 318 Chip Kit v2 (Life Technologies) according to the manufacturer’s protocols. We obtained 546,612 reads with an average length of 184 bp.

Microsatellite loci were located and primers were designed using MSATCOMMANDER version 0.8.2 (Faircloth, 2008) under the following conditions: more than eight microsatellite motif repeats, 40–60% of GC content in the PCR product.

**Table 1. Characteristics of the 27 microsatellite loci amplified in Melastoma tetramerum var. tetramerum.**

| Locus | Primer sequences (5′–3′) | Repeat motif | Allele size (bp) | Multiplex PCR set* | Fluorescent tag† | GenBank accession no. |
|-------|--------------------------|--------------|------------------|--------------------|------------------|----------------------|
| Mte002* | F: GCACCTCCACACATGCTC     | (GA)$_b$    | 258              | —                  | 6-FAM            | KX394447             |
|       | R: ACGCACGTCCTGTTAGGG     |             |                  |                    |                  |                      |
| Mte003 | F: ATTTGGATGGGCAATGTCG    | (AG)$_{10}$ | 171–173          | C                  | 6-FAM            | KX394448             |
|       | R: TGGACAATGAGTCCCAAGAGCTC|             |                  |                    |                  |                      |
| Mte005 | F: CTCTCTCGAGTCCTGTTAG    | (AG)$_{10}$ | 212–230          |                    | VIC              | KX394449             |
|       | R: CGATGTGTTCTAATGTTCTCC  |             |                  |                    |                  |                      |
| Mte007 | F: CATCTCTCGATGCAATATTCC  | (GA)$_{12}$ | 177–181          | B                  | NED              | KX394450             |
|       | R: GTGACCGGATAAAAGCAC     |             |                  |                    |                  |                      |
| Mte008 | F: CUCUCCATGACAGAAATGCC  | (TA)$_b$    | 166–168          | C                  | 6-FAM            | KX394451             |
|       | R: GAGATACAGAGTTGGATAGCTG |             |                  |                    |                  |                      |
| Mte012 | F: CAAAGGCGGCAAATGCGG     |             |                  |                    |                  |                      |
|       | R: ACTAACTGAGTGAATATGACGG|             |                  |                    |                  |                      |
| Mte014 | F: ACCTTTGCGATCTTCAAGAG  | (GAA)$_{15}$| 209–237          | D                  | NED              | KX394452             |
|       | R: TGCCGAGACTGGGAGTCTG    |             |                  |                    |                  |                      |
| Mte015* | F: ACCTTGGAAGTGTCCTAGAG  | (CT)$_b$    | 193              | —                  | VIC              | KX394453             |
|       | R: GAGATGCAGAGTGCTCTG     |             |                  |                    |                  |                      |
| Mte017 | F: ACTGCTCTTATGTGATCTGG  | (CT)$_{12}$ | 203–209          | E                  | 6-FAM            | KX394454             |
|       | R: AAGTGTCGATCCTTACAG     |             |                  |                    |                  |                      |
| Mte018 | F: CGGAGAGGAGCAGATGTCG    | (CA)$_b$    | 184–186          | C                  | NED              | KX058008             |
|       | R: TTGAGAGGGAGTGCCAGAC    |             |                  |                    |                  |                      |
| Mte019 | F: AGTTGGAGCCATTCCATTGG  | (AG)$_{10}$ | 167–170          | B                  | 6-FAM            | KX058009             |
|       | R: AGGTTAGTGAAAACGTCAGG   |             |                  |                    |                  |                      |
| Mte022* | F: CGCCGAAGCGAATGCAAG     | (AA)$_{10}$ | 203              | —                  | NED              | KX237522             |
|       | R: TCAGGGAAAAGCAGCTTGC    |             |                  |                    |                  |                      |
| Mte023 | F: CCCTTCATCCAACAGAAGC   | (CT)$_{11}$ | 225–227          | B                  | 6-FAM            | KX058010             |
|       | R: GGACCTGGATGCTGAGCC     |             |                  |                    |                  |                      |
| Mte025* | F: TTCCCAGCAACCTCTAGTG   | (AG)$_{10}$ | 215              | —                  | 6-FAM            | KX237523             |
|       | R: GTCGAAATCCCTCAAGCGG    |             |                  |                    |                  |                      |
| Mte026* | F: TCGCATCCATGGGACTGCTG  | (AG)$_{12}$ | 191              | —                  | NED              | KX237524             |
|       | R: AAGTGCATCCAGGAGAC      |             |                  |                    |                  |                      |
| Mte027* | F: GGAGAAAGAAGAGATATCCTAGTCTG | (CT)$_{10}$ | 245              | —                  | NED              | KX237525             |
|       | R: ACCTCTATTTGGGCTCAG     |             |                  |                    |                  |                      |
| Mte029 | F: CATCGTCCACATGCTGTC     | (CTT)$_b$   | 264–279          | C                  | 6-FAM            | KX058011             |
|       | R: GCTTTGATCCAAATCGGCC    |             |                  |                    |                  |                      |
| Mte030 | F: ACGCTCTGAGTTAAGGTC     | (AG)$_b$    | 240–241          | E                  | NED              | KX058012             |
|       | R: GCACTACGATCCCAAGGG     |             |                  |                    |                  |                      |
| Mte032 | F: GCAGACTTGGATCCACCTTC  | (GT)$_b$    | 229–233          | D                  | VIC              | KX058013             |
|       | R: GTGAGGAGTAATCTCTAGGCC  |             |                  |                    |                  |                      |
| Mte033 | F: GTGAAACCGGATGTITTTAGG  | (CTT)$_{10}$| 223–235          | B                  | VIC              | KX058014             |
|       | R: AACGTTCCACGGCAATCG     |             |                  |                    |                  |                      |
| Mte034 | F: CGATCTGACAAGAGATCTG    | (TTC)$_b$   | 225–228          | A                  | 6-FAM            | KX058015             |
|       | R: AGAGCCGCTCAAGGCTTCC    |             |                  |                    |                  |                      |
| Mte035 | F: CCGAGTGATGGGATTTGGC   | (AG)$_{13}$ | 262–264          | E                  | 6-FAM            | KX058016             |
|       | R: TCTCGAAAATGCTTGAACGAC  |             |                  |                    |                  |                      |
| Mte038* | F: TCCACATTTCCAGTTTACAC  | (GA)$_b$    | 187              | —                  | VIC              | KX237526             |
|       | R: GAAGGGAGCCTATAGGCG     |             |                  |                    |                  |                      |
| Mte039 | F: CTCCTCAGTGAGTCAATCC   | (CT)$_{12}$ | 238–240          | E                  | VIC              | KX058017             |
|       | R: AGACGGGCTCAAAGGCTC     |             |                  |                    |                  |                      |
| Mte040 | F: CCGAGGATCCCAATCCAG     | (GAA)$_{10}$| 185–188          | D                  | 6-FAM            | KX058018             |
|       | R: CTGATGTGTTGCTGACAG     |             |                  |                    |                  |                      |
| Mte041 | F: TGGAGCAGGCATGAAAGC    | (TC)$_{10}$ | 272–299          | D                  | NED              | KX058019             |
|       | R: GGCTGAGCCTGTCGAAACTC   |             |                  |                    |                  |                      |
| Mte042 | F: GTTTGAGCTTATGGCCAGAGC  | (AG)$_b$    | 170–174          | A                  | VIC              | KX058020             |
|       | R: GCAGAGAAGCTTGAGAAGAGC  |             |                  |                    |                  |                      |

*Monomorphic loci are marked with an asterisk.

Annealing temperature for all loci was 57°C.

Allele size indicates the size of PCR products identified in 30 individuals from the wild population on Chichi-jima Island and restored or collected individuals originating from the extinct population on Chichi-jima Island.

Loci with identical letters were amplified in the same PCR.

Amplified with M13 primers with fluorescent dye at each 5′ end: 6-FAM = 5′-CACGACGTTGTAAAACGAC-3′, VIC = 5′-TGTTGAATTGTTGAGCGG-3′, NED = 5′-CTAFAAGGCCACCGGTGTTG-3′.
products, annealing temperature of 57–60°C, and 150–400 bp of product sizes. Any primers with self-annealing sequences or hairpin structures were removed using Primer3 (Rozen and Skaletsky, 1999). Any primer pairs were removed if melting temperatures of forward and reverse primers differed more than 1°C. After filtering the candidate microsatellite regions, 42 microsatellite regions were retained for primer testing. M13 sequences, which enable fluorescent labeling of PCR products, were ligated to the 5’ end of forward primers (Table 1).

The primers were tested using eight individuals of M. tetramerum var. tetramerum, consisting of one restored individual at HD (voucher: 04110; Appendix 1) and seven wild individuals at HK (voucher: komaki201601; Appendix 1). PCR was performed in a final reaction volume of 5 µL, which included 4–240 ng of template DNA, 0.01 µM of each forward primer, 0.2 µM of each reverse primer, 1 µM of each M13 primer with fluorescent labels, and 2.5 µL of QIAGEN Multiplex PCR MasterMix (QIAGEN). The PCR conditions were as follows: initial denaturation at 95°C for 15 min; followed by 33 denaturation cycles at 94°C for 30 s, annealing at 57°C for 90 s, extension at 72°C for 90 s; and a final extension at 72°C for 10 min. One microliter of the PCR product was electrophoresed using a 3130xl Genetic Analyzer (Applied Biosystems, Foster City, California, USA) with 10 µL of HiDi Formamide (Applied Biosystems) and 0.15 µL of GeneScan 500 LIZ Size Standard (Applied Biosystems).

Out of 42 microsatellite loci tested, 27 loci were amplified and characterized in all eight individuals of M. tetramerum var. tetramerum, and 20 were found to be polymorphic in M. tetramerum var. tetramerum (Table 1). The applicability of these 20 loci was examined in the four Melastoma taxa: 30 individuals of M. tetramerum var. tetramerum, including 11 (10 restored and one stocked) individuals from HD and 19 (eight wild and 11 stocked) individuals from HK; 12 individuals of M. tetramerum var. pentapetalum from two populations on Hahajima Island; 12 individuals of M. candidum var. candidum from one population in Aha, Okinawa; and three individuals of M. candidum var. alessandrense from one population on Kita-Iwo-To Island (Appendix 1). The genotypes were identified using GeneMapper version 4.1 (Applied Biosystems). The number of alleles and observed and expected heterozygosities were calculated using GENALEX version 6.5 (Peakall and Smouse, 2012). Linkage disequilibrium (LD) and deviation from Hardy–Weinberg equilibrium (HWE) were tested using GENEPOP version 4.2 (Rousset, 2008). Out of the 20 loci, 15 were amplified in all four taxa tested, whereas the remaining five only amplified in the two varieties of M. tetramerum (Table 2). The total number of alleles per locus observed across the four taxa ranged from two to 11 (Table 2). In M. tetramerum var. tetramerum, the observed and expected heterozygosities were in the ranges of 0.10–0.73 and 0.10–0.71, respectively (Table 2). A total of six loci (Mte005, Mte007, Mte019, Mte023, Mte035, and Mte041) significantly deviated from HWE (P < 0.05) in M. tetramerum var. tetramerum. The extremely small population size of M. tetramerum var. tetramerum could have strongly affected its genetic variation and caused HWE deviation by genetic drift. A significantly high LD was found between Mte019 and Mte023, Mte007 and Mte019, Mte007 and Mte014, and Mte007 and Mte012 (P < 0.05) in every taxon.

CONCLUSIONS

Twenty novel polymorphic loci identified in the critically endangered M. tetramerum var. tetramerum will be used for the assessment of the genetic diversity, effective population size, inbreeding coefficient, and population genetic structure of the extant and extinct populations of this variety and three related taxa, which will enable evaluation and improvement of the ongoing conservation management programs.

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**APPENDIX 1. Voucher information for *Melastoma* taxa examined in this study.**

| Taxon                                           | Voucher specimen accession no. (Herbarium)a | Collection locality                                      | Geographic coordinates       |
|-------------------------------------------------|--------------------------------------------|---------------------------------------------------------|-----------------------------|
| *M. tetramerum* Hayata var. *tetramerum*         | komak201601 (TI)                           | Higashikaigan (HK), Chichi-jima Island, Bonin Islands  | 27°24'08.1"N, 142°13'35.9"E |
| *M. tetramerum* var. *tetramerum*               | 04110 (TI)                                | Higashidaara (HD), Chichi-jima Island, Bonin Islands   | 27°04'30.8"N, 142°13'24.8"E |
| *M. tetramerum* var. *pentapetalum* Toyoda      | komak201602 (TI)                           | Mt. Sakaigatake, Haha-jima Island, Bonin Islands       | 26°40'26.2"N, 142°09'13.8"E  |
| *M. candidum* D. Don var. *candidum*             | KYO 00019995 (KYO)                         | Aha, Kunigami, Okinawa                                 | 26°42'30.46"N, 128°16'6.89"E |
| *M. candidum* var. *alessandrense* S. Kobay.    | komak201603 (TI)                           | Kita-Iwo-To Island, Bonin Islands                      | 25°26'16.22"N, 141°16'56.91"E |

*aVouchers are deposited at the herbarium of the University of Tokyo (TI) and the herbarium of Kyoto University (KYO).