Twenty Novel Polymorphic Microsatellite Primers in the Critically Endangered Melastoma tetramerum var. tetramerum (Melastomataceae)

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PRIMER NOTE

TWENTY NOVEL POLYMORPHIC MICROSATELLITE PRIMERS IN THE CRITICALLY ENDANGERED *MELASTOMA TETRAMERUM* VAR. *TETRAMERUM* (MELASTOMATACEAE)[1]

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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 (QIAGEN, Hilden, Germany), and a fragment DNA library was constructed using Ion

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

Locus[a,b]	Primer sequences (5′–3′)	Repeat motif	Allele size (bp)[c]	Multiplex PCR set[d]	Fluorescent tag[e]	GenBank accession no.
Mte002*	F: GCACCTCCACACATTGCTC R: ACGCACGTCCTGTTAGGG	$(GA)_9$	258	—	6-FAM	KX394447
Mte003	F: ATTTGCATGGCCAGTTGCG R: TGACAATCAGTTCCAACACGTC	$(AG)_{10}$	171–173	C	6-FAM	KX394448
Mte005	F: CTCCTTCCGATCGTCGTTATG R: CGATGGTGTCTAACTAAGCTTCC	$(AG)_{10}$	212–230	C	VIC	KX394449
Mte007	F: CATCTCTCTCGGATCCAATTCC R: GGTCACCGCGATAAACGAC	$(GA)_{12}$	177–181	B	NED	KX394450
Mte008	F: CCCGTCTACAGCAAGAGTCC R: GAGTATCAGAGTTGTTAGCTGATCG	$(TA)_9$	166–168	A	6-FAM	KX394451
Mte012	F: CAAAGCCCAACATCGGGAC R: ACTAATGGAGTACGAATAGCAACG	$(TG)_{12}$	163–178	A	NED	KX058005
Mte014	F: ACCTTGGCATCTTCACAAAGG R: TGGGCAACACTGGGATCTG	$(GAA)_{15}$	209–237	D	NED	KX394452
Mte015*	F: ACCTCGGAAGTGTCCATGAG R: GAAGTGCTGAAGCGTCTCG	$(CT)_{13}$	193	—	VIC	KX394453
Mte017	F: ACTCGCCTTATTTGAGTATCCG R: AAGTGGCCATTCATCCACG	$(CT)_9$	203–209	E	6-FAM	KX394454
Mte018	F: CGGAGAAGGACGAATGTGC R: TTGAAGGGAGGTGGCAGAC	$(CAA)_9$	184–186	C	NED	KX058008
Mte019	F: AGTTTGGACCCATCCCATTTG R: AGGGTAGTGAAACAGCTAAGG	$(AG)_{10}$	167–170	B	6-FAM	KX058009
Mte022*	F: CACCCGAAGCACGAATCAC R: TCGGGAAAGACCCAGTTCG	$(AAT)_9$	203	—	NED	KX237522
Mte023	F: CCCTTCATCCCAAGCAACG R: GGACCGTCGATTGAGTCCG	$(CT)_{11}$	225–227	B	6-FAM	KX058010
Mte025*	F: TTTCCCGCCAACTTCATCG R: GCTCGAAATCTTCCAGGCG	$(AG)_9$	215	—	6-FAM	KX237523
Mte026*	F: TCACACCCATGGCACTCTG R: AACTGCATCACCAGGCAAG	$(AG)_8$	191	—	NED	KX237524
Mte027*	F: GGAGAAAGAAAGGATATCATCTGTCG R: ACGTCTATTTGGGCCTCGG	$(CT)_{10}$	245	—	NED	KX237525
Mte029	F: CATCGTCCCACATGCTGTC R: GCTTTGATCCCAATCCGCC	$(CTT)_8$	264–279	C	6-FAM	KX058011
Mte030	F: ACGGCTTTGCAGTTAAGGTC R: GCATCAGATCCCACAGGAG	$(AG)_9$	240–241	E	NED	KX058012
Mte032	F: GGACACTTGCATCACCCTTC R: GTCGAGGTAAATCTCAATCGCC	$(GT)_9$	229–233	D	VIC	KX058013
Mte033	F: GTTGAAACGGGATTGTTTAGCG R: AACGGTACCACGGACATCG	$(CTT)_{10}$	223–235	B	VIC	KX058014
Mte034	F: CGATCATGCAAAGGATATCTGC R: AGCCAGGCAGCCAATTTAC	$(TTC)_8$	225–228	A	6-FAM	KX058015
Mte035	F: CCCAGTGGATGGAGTTTGC R: TCCTTAATTGGGTTTAGGGACAAC	$(AG)_{13}$	262–264	E	6-FAM	KX058016
Mte038*	F: TCCCACATTTCCGATTTCAAC R: GAAGGGAGGCATTCATGGG	$(GA)_8$	187	—	VIC	KX237526
Mte039	F: CTTCCCTGAGCTGCAATCC R: AGACAGGCTACAAGGCTCC	$(CT)_{12}$	238–240	E	VIC	KX058017
Mte040	F: CCCAGGAATCCCAAATCCAG R: CTGATTCGCTTGCCGACAG	$(GAA)_8$	185–188	D	6-FAM	KX058018
Mte041	F: TCGGAGCAGCCATAGAACG R: TGCCTCGACCTCCTGAAATC	$(TCG)_{10}$	272–299	D	NED	KX058019
Mte042	F: GTTTGAAGCTTATGGCCAAGAC R: GCAGGAAAGTGTTGAGAAGACC	$(AG)_8$	170–174	A	VIC	KX058020

[a] Monomorphic loci are marked with an asterisk.

[b] Annealing temperature for all loci was 57°C.

[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.

[d] Loci with identical letters were amplified in the same PCR.

[e] Amplified with M13 primers with fluorescent dye at each 5′ end: 6-FAM = 5′-CACGACGTTGTAAAACGAC-3′, VIC = 5′-TGTGGAATTGTGAGCGG-3′, NED = 5′-CTATAGGGCACGCGTGGT-3′.

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

TABLE 2. Genetic properties of 20 polymorphic microsatellite loci in *Melastoma tetramerum* var. *tetramerum* and their applicability to three related taxa.[a]

		M. tetramerum						*M. candidum*					
		var. *tetramerum* (n = 30)			var. *pentapetalum* (n = 12)			var. *candidum* (n = 12)			var. *alessandrense* (n = 3)		
Locus	Total A	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e
Mte003	3	2	0.333	0.420	1	0.000	0.000	1	0.000	0.000	2	0.333	0.278
Mte005	7	6	0.467	0.708*	2	0.167	0.153	4	0.167	0.625	1	0.000	0.000
Mte007	5	3	0.367	0.653*	1	0.000	0.000	—	—	—	—	—	—
Mte008	3	2	0.567	0.486	1	0.000	0.000	—	—	—	—	—	—
Mte012	4	3	0.333	0.352	1	0.000	0.000	—	—	—	—	—	—
Mte014	11	9	0.733	0.676	1	0.000	0.000	2	0.417	0.469	1	0.000	0.000
Mte017	4	2	0.300	0.339	1	0.000	0.000	2	0.417	0.469	1	0.000	0.000
Mte018	4	2	0.233	0.299	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000
Mte019	2	2	0.167	0.495*	1	0.000	0.000	—	—	—	—	—	—
Mte023	4	2	0.267	0.480*	1	0.000	0.000	3	0.000	0.542	1	0.000	0.000
Mte029	7	3	0.367	0.485	1	0.000	0.000	3	0.667	0.625	1	0.000	0.000
Mte030	6	2	0.100	0.095	1	0.000	0.000	5	0.583	0.507	1	0.000	0.000
Mte032	4	3	0.633	0.523	1	0.000	0.000	—	—	—	—	—	—
Mte033	7	3	0.533	0.509	1	0.000	0.000	3	0.167	0.434	2	1.000	0.500
Mte034	4	2	0.100	0.095	1	0.000	0.000	2	0.083	0.219	1	0.000	0.000
Mte035	5	3	0.233	0.415*	2	0.000	0.153	4	0.333	0.573	1	0.000	0.000
Mte039	4	2	0.133	0.124	2	0.000	0.153	4	0.500	0.549	1	0.000	0.000
Mte040	4	2	0.167	0.153	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000
Mte041	8	5	0.400	0.607*	2	0.167	0.153	2	0.000	0.153	1	0.000	0.000
Mte042	4	3	0.367	0.363	1	0.000	0.000	3	0.667	0.559	2	1.000	0.500

Note: A = number of alleles per locus; H_e = expected heterozygosity; H_o = observed heterozygosity; n = number of individuals sampled.

[a] Voucher and locality information are provided in Appendix 1.

* Significant deviation from Hardy–Weinberg equilibrium ($P < 0.05$).

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 3130*xl* 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*	komaki201601 (TI)	Higashikaigan (HK), Chichi-jima Island, Bonin Islands	27°24′08.1″N, 142°13′35.9″E
M. tetramerum var. *tetramerum*	04110 (TI)	Higashidaira (HD), Chichi-jima Island, Bonin Islands	27°04′30.8″N, 142°13′24.8″E
M. tetramerum var. *pentapetalum* Toyoda	komaki201602 (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.	komaki201603 (TI)	Kita-Iwo-To Island, Bonin Islands	25°26′16.22″N, 141°16′56.91″E

[a]Vouchers are deposited at the herbarium of the University of Tokyo (TI) and the herbarium of Kyoto University (KYO).