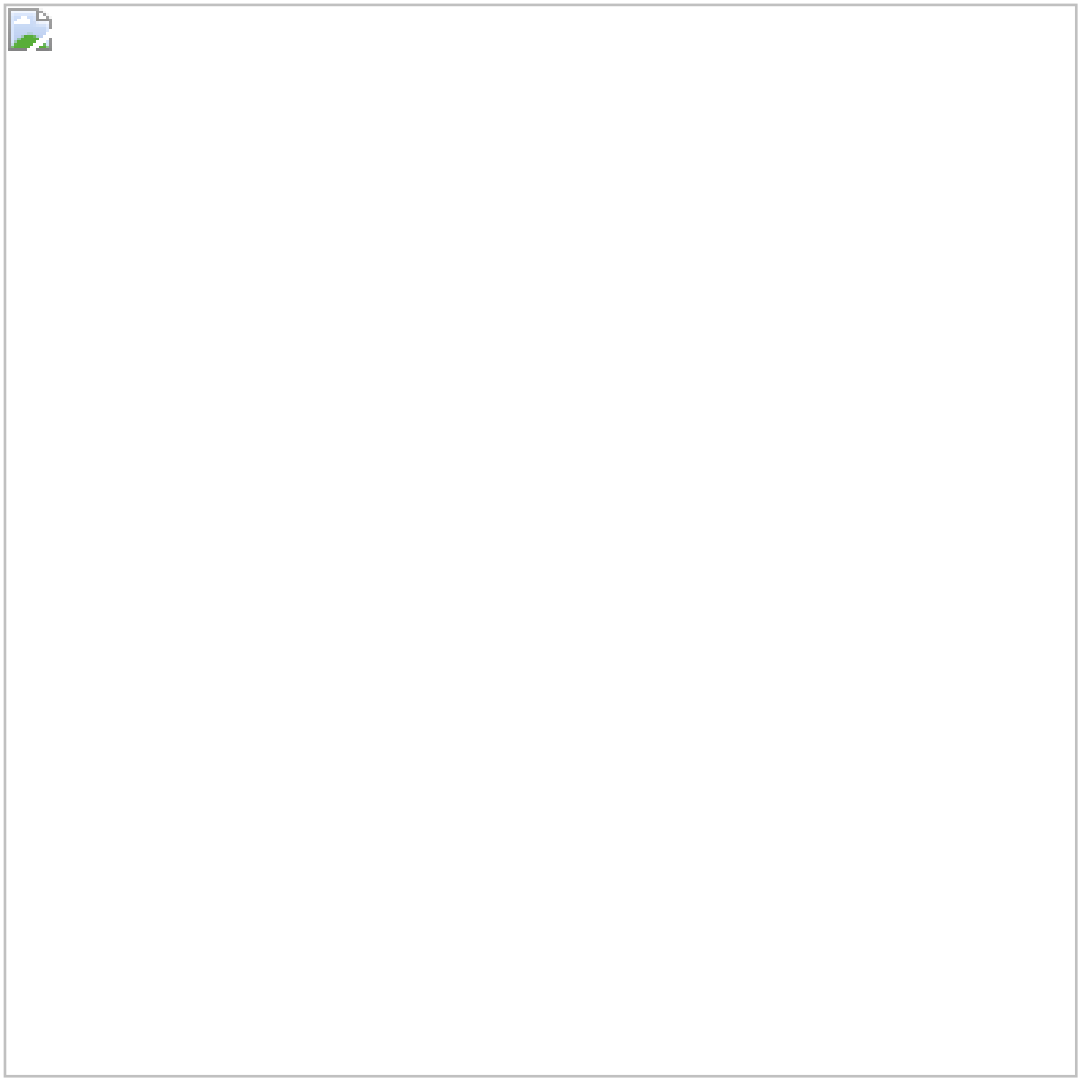

Isolation and Characterization of 11 Microsatellite Markers for Glochidion acuminatum (Phyllanthaceae)

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PRIMER NOTE

ISOLATION AND CHARACTERIZATION OF 11 MICROSATELLITE MARKERS FOR *GLOCHIDION ACUMINATUM* (PHYLLANTHACEAE)[1]

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- *Premise of the study:* Microsatellite markers were developed for *Glochidion acuminatum* (Phyllanthaceae) to investigate pollen dispersal distances and thereby to assess the effectiveness of specialized *Epicephala* moths as pollinators.
- *Methods and Results:* Using next-generation sequencing, 11 polymorphic microsatellite primer pairs were developed for *G. acuminatum*. The primer pairs were tested on 49 individuals from two populations in Japan. The number of alleles per locus ranged from two to 13, and the expected heterozygosity ranged from 0.12 to 0.85. Probability of identity for all loci combined was lower than 1.27×10^{7}.
- *Conclusions:* The microsatellite markers developed in this study will be useful for evaluating the benefit of specialized *Epicephala* moth pollination to *Glochidion* plants.

Key words: active pollination; *Epicephala*; *Glochidion acuminatum*; obligate pollination mutualism; Phyllantheae; pollen dispersal distance.

The monoecious tree genus *Glochidion* J. R. Forst. & G. Forst. (Phyllanthaceae, Phyllantheae) consists of more than 300 species that occur widely in the tropical to subtropical parts of Asia, Australia, and the Pacific. All *Glochidion* species studied to date are pollinated exclusively by species-specific, seed-parasitic moths of the genus *Epicephala* Meyrick (Gracillariidae) (Kato et al., 2003; Kawakita and Kato, 2009; Hembry et al., 2013). Female *Epicephala* moths actively collect pollen from the anthers of *Glochidion* and subsequently deposit pollen on the stigmas of female flowers to ensure that fruits are produced for their seed-feeding larvae. The female moths then oviposit into ovules, and the hatched larvae consume a subset of the seeds in each fruit, leaving the rest intact. Despite the apparently large cost of seed damage, specialization to *Epicephala* moth pollination has evolved several times independently in Phyllantheae (Kawakita and Kato, 2009). This suggests that the benefit plants gain from *Epicephala* pollination exceeds the cost of seed loss by pollinator larvae. Thus, it is necessary to quantify the benefit of *Epicephala* pollination by investigating the outcrossing rates and pollen dispersal distances in *Glochidion* plants.

Here, we report 11 nuclear microsatellite loci for *G. acuminatum* Müll. Arg. that were developed using next-generation sequencing (NGS). These markers will facilitate the assessment of outcrossing rates and pollen dispersal distances in *Glochidion* plants pollinated by specialized *Epicephala* moths.

METHODS AND RESULTS

A fresh leaf sample of a single *G. acuminatum* individual was collected for library preparation from a natural population at Nagakumo-toge, Amami-Oshima Island, Japan (28°25′34.3″N, 129°34′34.6″E), and genomic DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) protocol (Okuyama and Kawakita, 2012). Restriction-site associated genomic libraries were prepared twice for two batches of NGS runs. Initially, 200 ng of DNA were digested with the *Nde*I restriction enzyme (New England Biolabs, Beverly, Massachusetts, USA) and ligated with adapters (5′-C*C*A*TCTCATCCCTGCGTGTCTCCGACTCAGGCTCTTCCG*A*T*C-3′ and 5′-/5Phos/-T*A*G*ATCGGAAGAGCATCACCGACTGCCCATAGAG*A*G*G-3′; * signifies a phosphorothioate bond; /5Phos/ signifies a phosphorylation) using T4 DNA Ligase (Enzymatics, Beverly, Massachusetts, USA). Because initial amplification of this ligation product produced an excess of short-sized fragments not suitable for isolating microsatellites, the ligation product was size-selected to 350–450 bp using the E-Gel SizeSelect system (Life Technologies, Gaithersburg, Maryland, USA) for the second batch of library preparation. Ligation products of both batches were amplified with adapter-specific primers (F: 5′-CCTCTCTATGGGCAGTCGGTGAT-3′ and R: 5′-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3′) using Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Scientific, Waltham, Massachusetts, USA) under the following PCR conditions: initial denaturation at 94°C for 30 s, followed by 28 cycles of 98°C for 10 s, 65°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 5 min. After PCR, fragments of 350–450 bp in length were selected using the E-Gel SizeSelect for both batches, and PCR products were purified with the AMPure XP purification kit (Beckman Coulter, Brea, California, USA). The resulting libraries were quantified using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, California, USA) and were diluted to 26 pM for template preparation. Fragments of the library were mixed with capture beads and amplified by emulsion polymerase chain reaction (emPCR) using the Ion PGM Template OT2 400 Kit (Life Technologies). After emPCR, beads were collected, and beads capturing the DNA library were enriched using Ion PGM Enrichment Beads (Life Technologies). Sequencing was

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TABLE 1. Characteristics of 11 microsatellite loci developed for *Glochidion acuminatum*. Values are based on 49 individuals sampled from two populations in Amami-Oshima, Japan.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	T_a (°C)	Fluorescent label[a]	GenBank accession no.
Gacu1	F: ATCCGGTTTAGTTGGAATGC	$(AT)_9$	92–100	57	NED	AB934119
	R: ATCTAACCACACGTGAAATATGCTA					
Gacu2	F: CCACAACACGCTGATTCAA	$(AT)_9$	143–154	57	VIC	AB934120
	R: TTGACACGAACGAACATGC					
Gacu3	F: TCGAACTATAGTGAGCACAGATACC	$(CT)_9$	115–125	57	VIC	AB934121
	R: GCAAGCAGGAGTTTCATCC					
Gacu4	F: TGGTGTGGTGTCAAATCTCTAATA	$(AT)_9$	186–201	57	PET	AB934122
	R: TGGGCACAAATCACGTTTTA					
Gacu5	F: CTTCCAACCAAAACAACGTCT	$(CT)_{10}$	103–130	57	FAM	AB934123
	R: GGAACTGGGGAGACCAATAA					
Gacu6	F: TGAGCTGCAGTAAAGAGCAAA	$(AT)_{10}$	180–189	57	PET	AB934124
	R: TTTGAAATTGTGTTCGAGCATT					
Gacu7	F: CCAAATTGTGTGTCCTGGAG	$(AT)_9$	202–222	57	NED	AB934125
	R: CCATCAATGATATGAGCGTCTT					
Gacu8	F: ACAAACGGTGCCGTATTACA	$(CT)_{11}$	175–196	57	NED	AB934126
	R: ATTGGAGGGCTTATGCTGAG					
Gacu9	F: TTTTGTTCCATCATATAGGCATC	$(ATC)_5$	173–176	57	VIC	AB934127
	R: GCATGAGCTTGAAATAAAAACAA					
Gacu10	F: CGAGCAAAATCTCCAAAAATC	$(AG)_6$	148–154	57	PET	AB934128
	R: TACCGTGGTTGTGTTTCAGG					
Gacu11	F: TCTTTTCATTTTCTTCGCATTG	$(AC)_{11}$	114–124	57	PET	AB934129
	R: GAGATGAGTGAGGAGGGTTGA					

Note: T_a = annealing temperature.

[a] Sequences of the fluorescent labels: FAM = GCCTCCCTCGCGCCA; NED = CAGGACCAGGCTACCGTG; PET = CGGAGAGCCGAGAGGTG; VIC = GCCTTGCCAGCCCGC.

performed on an IonPGM (Life Technologies) using 800 flows on a 318 sequencing chip, obtaining 990,270 and 2,060,314 reads for the first and second batches, respectively.

The reads were screened for potential microsatellite loci with ≥6 dinucleotide, ≥4 trinucleotide, or ≥4 tetranucleotide repeats using MSATCOMMANDER (Faircloth, 2008). Totals of 3520 and 7389 reads satisfying the above criteria were found from the two runs, consisting of 4840 and 1813 dinucleotide, 1278 and 1694 trinucleotide, and 315 and 396 tetranucleotide repeats, respectively. Reads containing at least 50 bp to the upstream of the repeat region were selected, and primer pairs were designed using Primer3 (Untergasser et al., 2007). Primer pairs were successfully designed for 100 loci, including one tetranucleotide, 17 trinucleotide, and 82 dinucleotide repeats. To facilitate multiplex PCR, the forward primer of each locus was synthesized with one of four tag sequences (Blacket et al., 2012). Initial amplification trials were conducted with three to seven *G. acuminatum* individuals from the Nagakumo-toge population using standard PCR procedures, and amplified products were sequenced using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) and an ABI 3130 Genetic Analyzer (Applied Biosystems) to verify whether the target loci were correctly amplified. The 34 loci that were amplified successfully were then tested for polymorphism using eight individuals from the Nagakumo-toge population. For this test, PCR amplifications were performed using the Type-it Microsatellite PCR Kit (QIAGEN, Hilden, Germany) in a final volume of 5 µL, containing 25 ng of DNA, 2.5 µL of Type-it Microsatellite Master Mix, 0.1 µM of forward primer, 0.2 µM of reverse primer, and 0.1 µM of fluorescently tagged primers complementary to the above tag sequences (either labeled with FAM, NED, PET, or VIC; Table 1). The PCR amplification profiles include an initial denaturation at 95°C for 5 min, followed by 32 cycles of 30 s at 95°C, 90 s at 57°C, and 30 min at 72°C, and a final extension for 30 min at 60°C. The PCR product size was analyzed using an ABI 3130 Genetic Analyzer and GeneMapper version 4.0 (Applied Biosystems). Of the 34 loci tested, 11 were polymorphic and were thus amplified in 33 and 16 individuals from two populations in Amami-Oshima Island (Nagakumo-toge, as described above, and Sumiyo, 28°13′48.9″N, 129°21′46.5″E). One specimen from each population has been vouchered at KYO (voucher numbers: *K. Mochizuki 00001* and *00002*).

The number of alleles per locus (A), observed heterozygosity (H_o), and expected heterozygosity (H_e) were calculated using GenAlEx version 6.5 (Peakall and Smouse, 2012) for each locus for each population. Probability of identity (P_{ID}) for each locus and for all loci combined was obtained using GenAlEx to assess the utility of these loci for paternity analysis. Overall, A, H_o, H_e, and P_{ID} per locus per population ranged from two to 13, from 0 to 0.92, from 0.12 to 0.85, and from 0.04 to 0.77, respectively (Table 2). The P_{ID} for all 11 loci combined was 8.44×10^9 and 1.27×10^7 in Nagakumo-toge and Sumiyo, respectively.

CONCLUSIONS

The 11 microsatellite markers developed here for *G. acuminatum* were characterized by high levels of polymorphism and low probability of identity, indicating that they can be used for paternity analysis in natural populations. These markers will be useful for investigating outcrossing rates and pollen dispersal distances to evaluate the benefit of *Epicephala* moth pollination to *Glochidion* plants.

TABLE 2. Properties of the 11 newly developed microsatellite markers for two *Glochidion acuminatum* populations in Japan.

	Nagakumo-toge (N = 33)				Sumiyo (N = 16)			
Locus	A	H_o	H_e	P_{ID}	A	H_o	H_e	P_{ID}
Gacu1	5	0.57	0.70	0.14	3	0.80	0.66	0.19
Gacu2	4	0.22	0.35	0.45	3	0.15	0.15	0.74
Gacu3	5	0.65	0.65	0.18	6	0.50	0.64	0.16
Gacu4	6	0.68	0.67	0.17	3	0.54	0.49	0.32
Gacu5	13	0.88	0.84	0.04	5	0.92	0.70	0.14
Gacu6	9	0.48	0.76	0.09	8	0.56	0.77	0.09
Gacu7	8	0.80	0.79	0.07	4	0.50	0.69	0.16
Gacu8	6	0.32	0.32	0.47	3	0.20	0.18	0.67
Gacu9	2	0.52	0.49	0.38	2	0.33	0.46	0.39
Gacu10	2	0.05	0.21	0.65	2	0.00	0.12	0.77
Gacu11	5	0.46	0.69	0.13	7	0.53	0.85	0.04

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; N = sample size; P_{ID} = probability of identity.

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