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 $1.27 \times 10^{-7}$.

**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; Phyllanthaceae; pollen dispersal distance.

The monoecious tree genus *Glochidion* J. R. Forst. & G. Forst. (Phyllanthaceae, Phyllanthae) 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 *Glochidion* plants pollinated by *Epicephala* moth pollination exceeds the 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 was digested with the *Nde*I restriction enzyme (New England Biolabs, Beverly, Massachusetts, USA) and ligated with adapters (5′-CCTCTCTATGGGCAGTCGGTGAT-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). Ligation products of both batches were amplified with adapter-specific primers (F: 5′-CCCTGCGTGTCTCCCGACTCGGCGTCC-3′ and R: 5′-CCATCCTCCCTGGGCTTTCCGAATTCG-3′). The resulting libraries were quantified using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, California, USA) and diluted to 26 µM 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 performed on the Ion PGM Infinity System.
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 ≥ 26 dinucleotide, ≥ 24 trinucleotide, or ≥ 28 tetranucleotide repeats using MSATCOM-MANDER (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 (either labeled with FAM, NED, PET, or VIC; Table 1). The PCR amplification profiles include an initial denaturation at 95 °C, 90 s at 57 °C, and 30 min at 72 °C, followed by 30 cycles of 30 s at 95 °C, 90 s at 57 °C, and 3 min at 72 °C, and a final extension for 30 min at 60 °C. The PCR product size was analyzed 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.

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

| Locus | Primer sequences (5′–3′) | Repeat motif | Allele size range (bp) | T_a (°C) | Fluorescent label | GenBank accession no. |
|-------|-------------------------|--------------|------------------------|----------|------------------|----------------------|
| Gacu1 | F: ATCCGGTTTAGTTGGAATGC  | (CT)         | 92–100                 | 57       | NED              | AB934119             |
|       | R: ATCTAACACACACGTGAAATATGCTA |             |                        |          |                  |                      |
| Gacu2 | F: CCACACACCGCCTATCAA    | (CT)         | 143–154                | 57       | VIC              | AB934120             |
|       | R: TGGACCGAGAAGGACACATGC  |             |                        |          |                  |                      |
| Gacu3 | F: TGCAATATAGTAGACAGACATACC| (CT)        | 115–125                | 57       | VIC              | AB934121             |
|       | R: GCAACAGGATTTTACATCC   |             |                        |          |                  |                      |
| Gacu4 | F: TGGTTTGCTGTCATTCTTATA | (AT)        | 186–201                | 57       | PET              | AB934122             |
|       | R: TGGGCAACAATACGTTTTA    |             |                        |          |                  |                      |
| Gacu5 | F: CTTCACACACACACACGCT    | (CT)_10      | 103–130                | 57       | FAM              | AB934123             |
|       | R: GGCACCTGCGGAGAGGAAATAA |             |                        |          |                  |                      |
| Gacu6 | F: TGGACCTGCAAGTAAAGAGCAA | (AT)_10     | 180–189                | 57       | PET              | AB934124             |
|       | R: TTTGAAATTGTTGCGAGCATT  |             |                        |          |                  |                      |
| Gacu7 | F: CCAAATTTGCTGCTCTTTGGAG| (AT)        | 202–222                | 57       | NED              | AB934125             |
|       | R: CCATCAATAGATAGGCGTTCTT |             |                        |          |                  |                      |
| Gacu8 | F: ACAACACCGCGCTATTTACA  | (CT)_11      | 175–196                | 57       | NED              | AB934126             |
|       | R: ATGCCGCTTTAGCTGAG      |             |                        |          |                  |                      |
| Gacu9 | F: TTTTGTTCACCATATAGACATC | (ATC)_13    | 173–176                | 57       | VIC              | AB934127             |
|       | R: GCATGAGCTGAAATAAAAAACCA|             |                        |          |                  |                      |
| Gacu10| F: CGGACCAAATCTCCAAAAATC  | (AG)        | 148–154                | 57       | PET              | AB934128             |
|       | R: TACCTGGTTTGGTTGTTTACAGG|             |                        |          |                  |                      |
| Gacu11| F: TCTTTTTCATTTTTCTTGGCATTTG | (AC)_11  | 114–124                | 57       | PET              | AB934129             |
|       | R: GGATAGTAGAGGAGGTTGGA   |             |                        |          |                  |                      |

Note: T_a = annealing temperature.

* Sequences of the fluorescent labels: FAM = GCCTCCCTCGCGCCA; NED = CAGGACGAGCTACCGTG; PET = CGGAGAGCAGAGAGGTG; VIC = GCCTTGGCCACCGCC.

A total of 5720 and 11789 reads with ≥ 4 trinucleotide, ≥ 4 tetranucleotide, or ≥ 6 dinucleotide repeats were selected from the two runs, consisting of 4240 and 9072 reads with ≥ 4 trinucleotide, ≥ 4 tetranucleotide, or ≥ 6 dinucleotide repeats, respectively. Sequences of the fluorescent labels: FAM = GCCTCCCTCGCGCCA; NED = CAGGACGAGCTACCGTG; PET = CGGAGAGCAGAGAGGTG; VIC = GCCTTGGCCACCGCC.

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

| Population | Locus | H_o | H_e | P_ID | A | H_o | H_e | P_ID |
|------------|-------|-----|-----|------|---|-----|-----|------|
| Nagakumo-toge (N = 33) | 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| 1   | 0.46 | 0.69 | 0.13 | 7 | 0.53 | 0.85 | 0.04 |

Note: A = number of alleles; H_o = expected heterozygosity; H_e = observed heterozygosity; N = sample size; P_ID = probability of identity.

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.
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