New microsatellite markers recognize differences in tandem repeats among four related *Gastrodia* species (Orchidaceae)

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*Gastrodia* is the most species-rich genus among mycoheterotrophic plants, and is thus an essential taxon to understand the mechanism of species diversification in mycoheterotrophs. In this study, we developed microsatellite markers with high transferability for four *Gastrodia* species to examine genetic differentiation and similarity among species, populations and individuals. The 12 microsatellite markers developed from a *G. fontinalis* library showed high transferability for the ramets that identified *G. nipponica*, *G. kuroshimensis* and *G. takeshimensis*. In addition to the high transferability of these markers, we observed low allele variation within a sampled population of each species and allele differences among the four species. The 12 markers described here will be useful for investigating the genetic differences among and within the *Gastrodia* species, which evolved by a limitation of gene flow.

**Key words:** cross amplification, genetic variation, inbreeding, Ion PGM, mycoheterotrophy

*Gastrodia* is the most species-rich genus among mycoheterotrophic fungi, which obtain carbon from their associated fungi without photosynthesis. Numerous recent studies have re-examined the diversity of *Gastrodia* species in various Asian countries (e.g., Hsu and Kuo, 2010; Suetsugu, 2013, 2014, 2016a, 2016b; Huang et al., 2015; Hsu et al., 2016; Tsukaya and Hidayat, 2016; Suetsugu et al., 2018a, 2018b; Ma et al., 2019), and, as a result, the genus has been updated to include approximately 100 accepted species. Therefore, it is a crucial taxon to identify species diversification in mycoheterotrophic plants.

Multiple speciation mechanisms and modes of reproductive isolation are known in plants (Rieseberg and Willis, 2007). In the case of *Gastrodia*, limitation of gene flow between individuals may play an essential role in speciation. Many *Gastrodia* species have an automatic selfing strategy [e.g., *G. nipponicaoides* Suetsugu, *G. okinawaensis* Suetsugu (Suetsugu, 2017) and *G. damingshanensis* A.Q. Hu & T.C. Hsu (Hu et al., 2014)]. In addition to these automatic selfing chasmogamous species, four putative completely cleistogamous species are also described. In *G. clausa* (Hsu et al., 2012), *G. takeshimensis* Suetsugu (Suetsugu, 2013), *G. flexistyloides* Suetsugu (Suetsugu, 2014) and *G. kuroshimensis* Suetsugu (Suetsugu, 2016a, 2016b), chasmogamous flowers have never been observed. Complete cleistogamy is an ultimate form of reproductive isolation from other individuals, and dominance of autogamy is likely to be an essential factor in the evolution of complete cleistogamy. *Gastrodia nipponica*, a sister species of completely cleistogamous *G. takeshimensis*, showed high inbreeding coefficient values, suggesting a high frequency of self-fertilization, in a preliminary microsatellite analysis (Kishikawa et al., 2019). However, the recently discovered *G. ×
**G. nipponica** is considered a natural hybrid between *G. nipponica* and *G. uraiensis* (Suetsugu et al., 2018a), which suggests the existence of outcrossing in *G. nipponica*.

To clarify the current and historical status of gene flow in such *Gastrodia* species, microsatellite analysis is a compelling option. Microsatellite analysis should be able to identify the switching point of self-fertilization and outcrossing in *Gastrodia* species. Therefore, we developed microsatellite markers with high transferability for four *Gastrodia* species to examine genetic differentiation and similarity among species, populations and individuals.

Genomic DNA was extracted from a fresh sample of *G. fontinalis* (Table 1) using the DNeasy Plant Mini Kit (Qiagen). *Gastrodia fontinalis* is a chasmogamous sister species of the putative completely cleistogamous *G. kuromishimensis*. A DNA fragment library was constructed using the Ion Xpress Plus Fragment Library Kit (Thermo Fisher Scientific), amplified using the Ion PGM Template OT2 400 Kit (Thermo Fisher Scientific), and then sequenced using the Ion PGM Sequencing 400 Kit (Thermo Fisher Scientific) and an Ion 318 Chip v2 (Thermo Fisher Scientific). After filtering for identical reads, 683,018 sequences were screened for potential microsatellite loci using MSATCOMMANDER (Faircloth, 2008). Primers were designed for all sequences containing more than ten dinucleotide or eight trinucleotide tandem repeats using Primer3 software (Rozen and Skaletsky, 2000) with the default settings. A total of 51 primer pairs were obtained for screening. Twelve primer pairs (Table 2) showing clear peak patterns were selected after an amplification trial using eight *G. fontinalis* ramets from a population on Takeshima Island, Kagoshima Prefecture, Japan.

To test the genetic variation of the 12 selected microsatellite loci, the following samples of four *Gastrodia* species (Table 1) were collected: 50 ramets from a *G. fontinalis* population on Takeshima Island, 28 ramets from a *G. nipponica* population in Munakata City, Fukuoka Prefecture, 13 ramets from a *G. kuromishimensis* population on Kuroshima Island, Kagoshima Prefecture, and 27 ramets from a *G. takashimensis* population on Takeshima Island. PCR amplification was performed in 5-μl reactions using the QIAGEN Multiplex PCR Kit (QIAGEN) and a protocol for fluorescent dye-labeled primers (Blacket et al., 2012). Each reaction contained the following components: 10 ng of genomic DNA, 2.5 μl of Multiplex PCR Master Mix, 0.01 μM forward primer, 0.2 μM reverse primer, and 0.1 μM fluorescently labeled primer. Amplifications used the following setting: 95 °C for 15 min; 33 cycles at 94 °C for 30 s, 57 °C for 1.5 min and 72 °C for 1 min; and an extension at 60 °C for 30 min. Product sizes were determined using an ABI PRISM 3130 Genetic Analyzer and GeneMapper software (Applied Biosystems). For each species, we calculated observed heterozygosity (*H*<sub>O</sub>) and expected heterozygosity (*H*<sub>E</sub>) using GenALEx 6.5 (Peakall and Smouse, 2006, 2012). Calculation of inbreeding coefficients (*F*<sub>IS</sub>) and testing of deviation from Hardy–Weinberg equilibrium for polymorphic loci were performed by FSTAT version 2.9.3 (Goudet, 1995). For the evaluation of divergence among species, *F*<sub>ST</sub> (Weir and Cockerham, 1984) and *F*<sub>ST</sub>' (Meirmans and Hedrick, 2011) were calculated by FSTAT version 2.9.3 (Goudet, 1995) and GenAlEx 6.5 (Peakall and Smouse, 2006, 2012), respectively. We also calculated allele size difference between pairs of related species to clarify the accumulation of mutations of each locus. The allele size difference was defined as the absolute value of the difference in allele size between the two species, and it should be noted that the change in allele size is not necessarily in one direction.

In *G. fontinalis*, we found that eight of 12 loci were polymorphic. The ranges of *H*<sub>O</sub> and *H*<sub>E</sub> in the polymorphic loci were 0.02–0.08 (mean = 0.04) and 0.16–0.34 (mean = 0.26), respectively (Table 3). The range of *F*<sub>IS</sub> was 0.67–1.00 (mean = 0.85), and all eight polymorphic loci had significant deviations from Hardy–Weinberg equilibrium (*P* < 0.05, after Bonferroni correction). In *G. nipponica*, three of 12 loci showed polymorphism. The ranges of *H*<sub>O</sub> and *H*<sub>E</sub> in the polymorphic loci were 0.04–0.21 (mean = 0.13) and 0.04–0.50 (mean = 0.26), respectively (Table 3). The range of *F*<sub>IS</sub> was 0.00–0.58 (mean = 0.34), and two polymorphic loci had significant deviations from Hardy–Weinberg equilibrium (*P* < 0.05, after Bonferroni correction). Loss of allele variation in all loci was observed in the two putative cleistogamous species, *G. kuromishimensis* and *G. takashimensis*. High *F*<sub>IS</sub> values

### Table 1. Four Gastrodia species and their reported localities

| Species/Breeding system | Distribution records (reference) | Note |
|-------------------------|----------------------------------|------|
| *G. fontinalis* / chasmogamous | Takeshima Island (Suetsugu, 2014), Kuroshima Island (Suetsugu, 2016b) | Putative sister of *G. kuromishimensis* |
| *G. nipponica* / chasmogamous | Southern Japan and Taiwan (Hsu and Kuo, 2010) | Putative sister of *G. takashimensis* |
| *G. kuromishimensis* / cleistogamous | Kuroshima Island, Akusekijima Island and Yakushima Island (Suetsugu, 2016a) | |
| *G. takashimensis* / cleistogamous | Takeshima Island (Suetsugu, 2013), Yakushima Island, Kuroshima Island, Nakanoshima Island and Tanegashima Island (Suetsugu, 2017) | |
Microsatellites of four Gastrodia spp. in chasmogamous species and loss of allele variation in cleistogamous species correspond with a previous study using different microsatellite markers (Kishikawa et al., 2019).

The microsatellite markers developed from G. fontinalis showed high transferability for G. nipponica, G. kuroshimensis and G. takeshimensis. All markers were successfully amplified for all samples of the four analyzed species. This result can be explained by a high degree of sequence similarity in the primer annealing sites among the four Gastrodia species and suggests a very low frequency of sequence polymorphism in the sequences neighboring the microsatellite repeats. The high transferability of the present markers should permit not only microsatellite analysis of each species but also integrated genetic variation comparison within these Gastrodia species.

Table 2. Characteristics of 12 microsatellite primers developed from Gastrodia fontinalis

| Locus | Primer sequences (5'-3') | Repeat motif | $T_a$ (°C) | Fluorescent label | DDBJ/EMBL/GenBank accession no. |
|-------|--------------------------|--------------|------------|------------------|----------------------------------|
| Gfont013 | TTCGAGTGTGGCCAGATGGG | (CA)$_{21}$ | 57 | FAM | LC485254 |
| Gfont017 | ACCATGAGTGGATCCCTGGTG | (TA)$_{10}$ | 57 | VIC | LC485255 |
| Gfont021 | ATCCAAGGCAACAATAAGG | (GT)$_{10}$ | 57 | NED | LC485256 |
| Gfont022 | ATTCATGCAACCCAGAGGC | (GA)$_{12}$ | 57 | FAM | LC485257 |
| Gfont027 | GCCATTAGCGTGGGAGATGC | (GA)$_{12}$ | 57 | NED | LC485258 |
| Gfont028 | AACACACACTTTCTCAAAGG | (TG)$_{14}$ | 57 | FAM | LC485259 |
| Gfont034 | TGTCAAGATAAGGAACTGATG | (GAT)$_{18}$ | 57 | FAM | LC485260 |
| Gfont035 | GACGCTACCCGATACACC | (CTT)$_{8}$ | 57 | PET | LC485261 |
| Gfont038 | CAAACGTCCTGGCCTAGAAC | (GAA)$_{12}$ | 57 | PET | LC485262 |
| Gfont043 | CGCTAGAAAGTGGCCTCAAC | (ATT)$_{8}$ | 57 | VIC | LC485263 |
| Gfont048 | GCAGTCATCAATTCGAGG | (AGC)$_{10}$ | 57 | NED | LC485264 |
| Gfont049 | TCATACATTCCACAGTGGGC | (GAA)$_{13}$ | 57 | FAM | LC485265 |
| \(T_a\) = annealing temperature.

Sequence of the fluorescent labels: FAM = 5'-GCCTCCCTCGCAGCA-3', VIC = 5'-GCCTTGCCAGCCCGGC-3', NED = 5'-CAGGACCAGGCTACTGTG-3', PET = 5'-CGGAGGAGCCAGAGGG-3'.

Species-specific alleles were observed in many loci (Table 3), and the size difference of the major alleles between two species was notable (Table 4). All two-species pairs have different major alleles in at least five loci, and the average size difference of major alleles between two species ranged from 1.0 bp (G. nipponica and G. takeshimensis) to 10.9 bp (G. fontinalis and G. nipponica). However, high transferability of microsatellite markers and allele difference are not always compatible. It is common for amplification to fail in several loci in cross-amplification tests (e.g., G. flavilabella and its related species, Tsai et al., 2014), and even if amplified well, similar-sized alleles are observed among taxa (e.g., Livistona rigid and its related species, Kaneko et al., 2011; Stachyurus macrocarpus var. macrocarpus and var. prunifolius, Kaneko et al., 2009). Therefore, microsatellite markers that show taxon-specific alleles are useful for taxon identification, and the genotype data of such markers are valuable for
The low genetic variation and high genetic divergence among *Gastrodia* species can be explained by severely limited gene flow as a result of the species' selfing mechanism. These genetic and ecological characteristics may be related to factors of evolution into complete cleistogamy and speciation of this diverged mycoheterotrophic taxon. The diversity of *Gastrodia* plants has just been reconfirmed, and genetic analysis using our microsatellite markers and SNP markers such as RAD-seq will be useful for further studies about the ecology and evolution of the genus *Gastrodia*.

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