Analysis of microsatellites in the vulnerable orchid Gastrodia flavilabella: the development of microsatellite markers, and cross-species amplification in Gastrodia

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Abstract

Background: Gastrodia flavilabella is a mycoheterotrophic orchid that obtains carbohydrates and nutrients from its symbiotic mycorrhizal fungi. The species is an endemic and vulnerable species enlisted in the “A Preliminary Red List of Taiwanese Vascular Plants” according to the IUCN Red List Categories and Criteria Version 3.1. G. flavilabella dwells the underground of broadleaf and coniferous forest with richness litter. Based on herbarium records, this species is distributed in central Taiwan. Twenty eight microsatellite loci were developed in G. flavilabella and were tested for cross-species amplification in additional taxa of G. confusoides, G. elata, and G. javanica. We estimated the genetic variation that is valuable for conservation management and the development of the molecular identification system for G. elata, a traditional Chinese medicine herb.

Results: Microsatellite primer sets were developed from G. flavilabella using the modified AFLP and magnetic bead enrichment method. In total, 257 microsatellite loci were obtained from a magnetic bead enrichment SSR library. Of the 28 microsatellite loci, 16 were polymorphic, in which the number of alleles ranged from 2 to 15, with the observed heterozygosity ranging from 0.02 to 1.00. In total, 15, 13, and 7 of the loci were found to be interspecifically amplifiable to G. confusoides, G. elata, and G. javanica, respectively.

Conclusions: Amplifiable and transferable microsatellite loci are potentially useful for future studies in investigating intraspecific genetic variation, reconstructing phylogeographic patterns among closely related species, and establishing the standard operating system of molecular identification in Gastrodia.

Keywords: Gastrodia; Conservation; Microsatellites; Mycoheterotrophic orchid; Population genetics; Simple sequence repeat markers

Background

Gastrodia is the largest achlorophyllous and mycoheterotrophic genus in the Orchidaceae with 50 to 60 species in the world. Recent studies recognized 19 species, including 13 endemic species distributed in Taiwan (Hsu 2008; Leou 2000; Hsu and Kuo 2010; Chung and Hsu 2006). Species diversity in Taiwan Island is one of the hot spots of Gastrodia in the world. Gastrodia elata Blume is an important Chinese medicine that provides supplement to protect neuron and cardiovascular systems (Baek et al. 1999). Ecologically, Gastrodia species are saprophyte (Leou 2000), growing underground of forest or bamboo grove with richness litter and obtaining carbohydrates and nutrients from its symbiotic mycorrhizal fungi, including Armillaria mellea and other microbial species (Cha and Igarashi 1995). Due to such a unique growth form, Gastrodia species are difficult to find except the flowering

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and flowering seasons, generally 2 to 4 weeks after budding. Most *Gastrodia* species are vulnerable to the human destruction. As a result, 7 species recognized as threatened species, including one as critically endangered, three as endangered, and another three as vulnerable, are evaluated by the IUCN Red List Categories and Criteria Version 3.1 (IUCN 2012) and listed in the “A Preliminary Red List of Taiwanese Vascular Plants” (Wang et al. 2012a).

*Gastrodia flavilabella* S.S. Ying is an endemic and vulnerable species with only few populations distributed at the edges of conifer plantation or natural broadleaf forests restricted to the central mountainous regions from 1,000 to 1,300 meters altitude (Leou 2000). This taxon is characterized by tuberous horizontal rhizomes ca. 4 to 1,000 to 1,300 meters altitude (Leou 2000). Unique life form and habitat preference lead his species to be rare and vulnerable. However, no data for the genetic diversity in this species or genus are available, which is critical for evaluating the population dynamics and conservation genetics for conservation management.

Microsatellite genotyping is the most popular molecular tool for evaluating the structure and genetic diversity of populations because of its high genetic variability (cf. Ho et al. 2014). With co-dominant inheritance, the information of microsatellite genotyping can estimate the effective population sizes in ancestral and present populations (Ge et al. 2014), Hardy-Weinberg Equilibrium (Ge et al. 2014), and levels of introgression (Liao et al. 2012). In addition, microsatellite genotyping technology was extended to molecular identification system for paternity testing and cultivar identification (Tsai et al. 2013).

In this study, we constructed a microsatellite enriched library and developed microsatellite loci for future estimating the population genetic diversity based on microsatellite genotyping. The application of the microsatellite primers developed in this study was tested in other taxa of *Gastrodia*, specifically three taxa for polymorphism test and 13 species for transferability test.

**Methods**

**Sampling and DNA extractions**

Twenty individuals from each of four taxa in *Gastrodia*, including *G. flavilabella* from Nantou, *G. elata* from China, *G. javanica* (Blume) Lindl. from Lanyu Islet, and *G. confusoides* T. C. Hsu, S. W. Chung & C. M. Kuo from Taichung (Table 1) were sampled for polymorphism test. One individual of *G. flavilabella* was used to construct a microsatellite enriched library and to develop microsatellite loci. To test the transferability of these newly designed microsatellite primers, two individuals of other 13 native taxa listed in Table 1, specifically 8 endemic species, were sampled from the field. The sample location, sample size, and deposited herbarium for the voucher specimens are listed in Table 1. To avoid the contamination from the symbiotic mycorrhizal fungi, we collected the flower buds or seed pods for extracting total genomic DNA. Total DNA was

**Table 1 Sample location for each species of the *Gastrodia***

| Species Location | Species code | Sample size | Latitude | Longitude | Herbarium |
|------------------|--------------|-------------|----------|-----------|-----------|
| *Gastrodia elata* | Yunnan, China | Gel | 20 | N 27°46/07′ | E 104°15/39′ | TAIE |
| *Gastrodia javanica* | Lanyu, Taiwan | Gja | 20 | N 22°00/53′ | E 121°34/17′ | TAIE |
| *Gastrodia confusoides* | Taichung, Taiwan | Gco | 20 | N 24°14/21′ | E 120°54/81′ | TAIE |
| *Gastrodia albida* | Taipei, Taiwan | Gal | 2 | N 24°50/36′ | E 121°33/28′ | TAIE |
| *Gastrodia appendiculata* | Nantou, Taiwan | Gap | 2 | N 23°41/17′ | E 120°47/26′ | TAIE |
| *Gastrodia autumnalis* | Taoyuan, Taiwan | Gau | 2 | N 24°47/34′ | E 121°26/08′ | TAIE |
| *Gastrodia clausa* | Taipei, Taiwan | Gcl | 2 | N 25°04/57′ | E 121°37/33′ | TAIE |
| *Gastrodia fontinalis* | Taipei, Taiwan | Gfo | 2 | N 24°51/27′ | E 121°32/19′ | TAIE |
| *Gastrodia gracilis* | Chaiyi, Taiwan | Ggr | 2 | N 23°29/28′ | E 120°43/42′ | TAIE |
| *Gastrodia leoui* | Chaiyi, Taiwan | Gle | 2 | N 23°29/28′ | E 120°43/42′ | TAIE |
| *Gastrodia nantoensis* | Nantou, Taiwan | Gna | 2 | N 23°41/17′ | E 120°47/27′ | TAIE |
| *Gastrodia nipponica* | Taipei, Taiwan | Gni | 2 | N 24°51/05′ | E 121°32/11′ | TAIE |
| *Gastrodia pubilabiata* | Nantou, Taiwan | Gpu | 2 | N 23°40/23′ | E 120°47/54′ | TAIE |
| *Gastrodia shimizuana* | Pingtung, Taiwan | Gsh | 2 | N 22°12/12′ | E 120°47/16′ | TAIE |
| *Gastrodia theana* | Nantou, Taiwan | Gth | 2 | N 23°51/57′ | E 120°55/42′ | TAIE |

Note: TAIE = the herbarium of the Taiwan Endemic Species Research Institute. Sample size, location, coordinates, and voucher specimens are indicated.
Isolation of microsatellite DNA loci and identification
In order to develop the molecular markers for evaluating the genetic variation of populations and testing transferability in *Gastrodia* species, we selected one individual of *G. flabilabella* to build (AG)n, (AC)n, (TTG)n, (TCC)n, (ACG), (CCA)n, (AACT)n, and (AGAT)n enriched DNA library. Microsatellite loci were isolated following the magnetic bead enrichment method (Liao et al. 2009; Hsu et al. 2013), modified from the method proposed by Zane et al. (2002) based on AFLP, magnetic bead enrichment, and TA cloning protocol. Genomic DNA of *G. flabilabella* was digested using the restriction enzyme Msel (Promega, Madison, Wisconsin, USA) and DNA fragments from 400 to 1000 bp were isolated from agarose gels using the HiYield™ Gel PCR DNA Fragments Extraction Kit (RBC Bioscience). The purified partial genomic library was ligated to adaptors (complementary oligo A: 5′-TACCTAGGACTCAT-3′, and 5′-phosphorylated oligo B: 5′-GACGATGAGTCCTGAG-3′). The partial genomic library was enriched using 15 cycles of prehybridization polymerase chain reaction (PCR) using adaptor specific primers (5′-GATGAGTCCTGAGTAAN-3′, hereafter referred to as Msel-N). The enriched partial genomic library was denatured and hybridized to eight different biotinylated probes [Biotin-(AG)15, Biotin-(AC)15, Biotin-(TTG)10, Biotin-(TCC)10, Biotin-(ACG)10, Biotin-(CCA)10, Biotin-(AACT)8, and Biotin-(AGAT)8] from each of four taxa were selected (Table 1). PCR re- action cocktail contained 20 ng template DNA, 0.2 μM each of forward and reverse primers, 2 μL 10 × PCR re-action buffer, 2 mM dNTP mix, 2 mM MgCl2, 0.5 U Taq DNA polymerase (Promega), plus adding sterile water to total volume to 20 μL. PCR amplifications were executed by a Labnet MultiGene 96-well Gradient Thermal Cycler (Labnet, Edison, NJ, USA). PCR products were checked by 10% PAGE electrophoresis to separate the target DNA bands and which were following confirmed based on cloning and sequencing. These SSR primer pairs with confirmed target DNA bands were chosen for polymorphism evaluation.

To investigate genetic polymorphisms, 20 individuals from each of four taxa were selected (Table 1). PCR reaction cocktail contained 20 ng template DNA, 0.2 μM each of forward and reverse primers, 2 μL 10 × PCR reaction buffer, 2 mM dNTP mix, 2 mM MgCl2, 0.5 U Taq DNA polymerase (Promega), plus adding sterile water to total volume to 20 μL. PCR amplifications were executed by a Labnet MultiGene 96-well Gradient Thermal Cycler (Labnet). The PCR protocol was piloted at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 48–65°C for 30 s, 72°C for 30 s, and a final extension of 72°C for 10 minutes with the Labnet MultiGene 96-well Gradient Thermal Cycler (Labnet). The PCR products were separated by electrophoresis on a 10% polyacrylamide gel (acylamide: bisacrylamide 29: 1, 80 V for 14–16 hours) and determined the allele size by a 25 or 50 bp DNA Step Ladder (Promega). The bands of ampli- cons were then imaged under UV light using the Flo Gel FGIS-3 fluorescent gel image system (Top BIO Co., Taipei, Taiwan), and the sizes of bands were estimated using Quantity One software version 4.62 (Bio-Rad Laboratories, Hercules, California, USA).

Genetic variation analysis
Several genetic variation parameters were calculated using GenAIEx version 6.5 (Peakall and Smouse 2012), including the number of alleles (*Na*), the number of effective alleles (*Ne*), the observed and expected heterozygosity (*Ho* and *He*), Shannon’s information index (*H*), fixation index (*F*), by general setting on 2, 3, and 5 of match, mismatch, and indel for alignment parameters and 20 for minimum alignment score to report repeat. The pair of specific primers for each microsatellite locus detected by Tandem Repeats Finder was designed using FastPCR software version 6.4.18 (Kalander et al. 2011) based on the setting of parameters at a PCR product size ranging from 100 to 400 bp, an optimum annealing temperature of 55°C, and a GC content ranging from 35% to 70%.

DNA amplification and genotyping
To optimize PCR at various annealing temperatures, we evaluated each primer pair using a gradient PCR procedure. All primer pairs were tested for PCR amplification on DNA extracted from each species, i.e., two individuals of each 17 taxa. The protocol was executed at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 48–65°C for 30 s, 72°C for 30 s, and a final extension of 72°C for 10 minutes with the Labnet MultiGene 96-well Gradient Thermal Cycler (Labnet, Edison, NJ, USA). PCR products were checked by 10% PAGE electrophoresis to separate the target DNA bands and which were following confirmed based on cloning and sequencing. These SSR primer pairs with confirmed target DNA bands were chosen for polymorphism evaluation.

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Hardy–Weinberg equilibrium ($H_{WE}$) was evaluated using Arlequin software version 3.5.1.2 (Excoffier and Lischer 2010).

Results and discussion
Enrichment microsatellite library and sequencing results
We sequenced 1047 positive plasmids from eight microsatellite enrich libraries and confirmed 257 microsatellite loci from SSR enrich library (Table 2). Among the derived repeats of microsatellite loci, the di-, tri-, tetra-, penta-, and hexanucleotide motif was existed in 106 (41.25%), 120 (46.69%), 9 (3.05%), 11 (4.28%) and 9 (1.67%) loci, respectively (Table 2). Di- (41.25%) and trinucleotide repeats (46.69%) comprised the largest group of repeat motifs and accounted for more than four-fifths of the total SSR content, while the rest amounted to less than 12.06%. Generally, di- and trinucleotide repeats overstepped other types of repeats in all the species and mostly contributed to the major fraction of SSRs (Wei et al. 2014). Among the repeat motifs within G. flabilabella, di- and trinucleotide repeats were the commonest motifs, representing for 87.94%, similar to Sesamum indicum (Wei et al. 2014), Arabidopsis thaliana, Sorgihum bicolor (Sonah et al. 2011), and Brassica napus (Cheng et al. 2009).

Development of microsatellite markers
Totally, we designed 144 microsatellite primer pairs based on the flanking sequences from 257 microsatellite loci. All primer pairs were screened using a gradient PCR protocol with a Labnet MultiGene” 96-well Gradient Thermal Cycler (Labnet) to find the best annealing temperature. Finally, 28 primer pairs showed desired DNA bands and were selected for future diversity evaluation. The characteristics of 28 microsatellite loci are listed in Table 3. Of the 28 loci, 26 are complete microsatellite loci, including 13 carrying a dinucleotide motif, 11 with a trinucleotide motif, 1 with a pentanucleotide motif, and 1 with a hexanucleotide motif, and 2 remaining loci are carried a compound motif. The sequences of 28 loci reported in this paper are available from GenBank (accession numbers: LK934509–LK934536) (Table 3).

Genotyping and population genetics analysis
To inspect the level of genetic polymorphism at each locus, 20 individuals were collected in the field from the remaining wild population of G. flabilabella (Table 1). All the 28 new microsatellite loci identified in G. flabilabella were successfully amplified. Of the 28 loci, 12 microsatellite loci were monomorphic and 16 were polymorphic (Table 4). Genetic variation indices for 16 polymorphic loci, including the number of alleles ($Na$), the number of effective alleles ($Ne$), the observed and expected heterozygosity ($Ho$ and $He$), Shannon's information index ($H$) and fixation index ($F_{IS}$), were estimated. $Ne$ represents here an estimate of the number of equally frequent alleles in a model population following the formula of $Ne = 1/ (1-He)$. As shown in Table 4, $Na$ ranged from 2 to 15, $Ne$ varied from 1.08 to 8.85, $Ho$ ranged from 0 to 1.00 and mean was 0.163, and $He$ varied from 0.08 to 0.89 and mean was 0.444. The Shannon’s information index ($H$) and fixation index ($F_{IS}$) ranged from 0.17 to 2.41 and from -1.00 to 1.00, and the mean was 0.882 and 0.697, respectively. Significant deviations from Hardy–Weinberg equilibrium ($H_{WE}$) were detected at all loci (Table 4).

To test the transferability and genetic diversity, 20 individuals from each of three taxa, including G. elata, G. javanica, and G. confusoides, were tested. Of the 28 loci, 13, 7, and 17 markers worked in G. elata, G. javanica, and G. confusoides, respectively. Of the 13, 7, and 17 microsatellite loci, 9, 5, and 12 were monomorphic and 4, 2, and 5 were polymorphic (Table 4). In addition, three loci, including CT6-90, CT6-99, and CT-AG-157, are monomorphic within each of four species, but polymorphic between species. As shown in Table 4, the ranges for the $Na$, $Ne$, $Ho$ and $He$ were varied from 1 to 7, 1.00 to 4.37, 0.00 to 1.00, and 0.33 to 0.77 in G. elata, 1 to 2, 1.00 to 2.00, 0.11 to 1.00, and 0.10 to 0.50 in G. javanica, and 1 to 7, 1.00 to 4.35, 0.00 to 1.00, and 0.06 to 0.77 in G. confusoides. The Shannon’s information index ($H$) and fixation index ($F_{IS}$) ranged from 0.69 to 1.64 and from -1.00 to 1.00, and the mean was 1.113 and 0.147 in G. elata, from 0.21 to 0.69 and from -1.00 to -0.056, and the mean was 0.450 and -0.528 in G. javanica, and from 0.13 to 1.64 and from -1.00 to -0.056, and the mean was 0.670 and -0.266 in G. javanica.

| No. of repeat units | Di- | Tri- | Tetra- | Penta- | Hexa- | Mix | Total |
|---------------------|-----|------|--------|--------|-------|-----|-------|
| 4                   | 1   | 13   | 8      | 1      | 0     | 0   | 23    |
| 5                   | 0   | 5    | 0      | 1      | 0     | 0   | 6     |
| 6                   | 2   | 4    | 0      | 0      | 1     | 0   | 7     |
| 7                   | 5   | 4    | 0      | 0      | 0     | 0   | 9     |
| 8                   | 4   | 3    | 0      | 0      | 0     | 0   | 7     |
| 9                   | 3   | 4    | 0      | 0      | 0     | 0   | 7     |
| 10                  | 6   | 4    | 0      | 0      | 2     | 0   | 12    |
| 11                  | 2   | 4    | 0      | 0      | 0     | 0   | 6     |
| 12                  | 3   | 3    | 0      | 0      | 2     | 0   | 8     |
| ≥13                 | 80  | 76   | 1      | 9      | 4     | 2   | 172   |
| Total               | 106 | 120  | 9      | 11     | 9     | 2   | 257   |

Table 2 Summary of different SSR repeat motif types related to variation of repeat unit numbers in 257 Gastrodia flabilabella SSR loci selected by the length of repeat motif more than 20 bps
| Locus    | Repeat motif | Primer sequence (5’-3’) | Allele size (bps) | Ta (°C) | Genbank accession no. |
|----------|--------------|--------------------------|-------------------|---------|-----------------------|
| CT3-32   | (GGA)\(_9\)  | F: TAACGGGGGAATGGGGAGGCG R: TTGGATCTCTCCCTGTTACG | 137–146 | 52 - 54 - 54 - | LK934509 |
| CT6-4    | (GA)\(_{10}\) | F: CAGAATAGGGCCACACCTC R: GTGAGCTACTGCTGGCATGCC | 110–151 | 55 - - - | LK934510 |
| CT6-35   | (TG)\(_{64}\) | F: GTCTGTCCATTGTATATG R: GCAGTAATGCTTGTGATTTG | 250–252 | 55 - 52 - 50 | LK934511 |
| CT6-65   | (TGT)\(_{36}\) | F: CACCGAGCTTTTGTCTAATG R: GCAATAAATAGTACAGCAGC | 247–262 | 55 52 - 51 | LK934512 |
| CT6-90   | (TTG)\(_7\)  | F: CAAACAGCAAGATGCATAG R: ACATCTCCTCCCTGGATGTTC | 132 | 55 55 52 55 | LK934513 |
| CT6-99   | (CAA)\(_7\)  | F: GGCATTATCCCTGGATATGG R: GGGCTTTCATTTGATCATGC | 138 | 55 50 - 55 | LK934514 |
| CT6-120  | (CACAG)\(_{38}\) | F: TAGCACCCATAAAGGAAGGCC R: GTCGGAGATCAAAATGGAAATG | 316 | 55 - - - | LK934515 |
| CT6-142  | (AAC)\(_7\)  | F: GTCATGCACACTTCCTCCG R: CATGATATATCCCTCCAC | 128–131 | 55 55 - 55 | LK934516 |
| CT-ACT-74| (AG)\(_{20}\) | F: GAGGTCAATCTAAGATTTTC R: TGGCTTACAACTTTCTCCCTTC | 122–156 | 54 - - | LK934517 |
| CT-ACT-88| (TGA)\(_9\)  | F: TATGGGATTTGGAGTTTGAG R: CTCATCTTTGATACACTTC | 101 | 54 - 51 | LK934518 |
| CT-ACT-136| (CT)\(_{12}\) | F: ATTAGGGTGATCCGACACC R: TGGCGAAGGCTGAGAACGCT | 140–142 | 54 55 55 54 | LK934519 |
| CT-AG-35 | (GA)\(_{12}\) | F: TCTTCCGGACACTTCATAC R: TTCAGAGGATCTGCTCCATCG | 133–137 | 52 55 55 55 | LK934520 |
| CT-AG-45 | (CTT)\(_{12}\) | F: CAGAAAGCAGCACAATGCTAC R: TCTGAATTTTGGGAGTGAGCC | 115–121 | 50 54 - 52 | LK934521 |
| CT-AG-55 | (TGCCCTC)\(_5\) | F: GTCGGGAGATTACACTTACG R: AAGGAAGGCTGAGGATAG | 108–110 | 50 50 - 55 | LK934522 |
| CT-AG-85 | (TG)\(_{5}\) (AG)\(_{28}\) | F: CCCATATGTCTCTTGTCATC R: GCCTCCACATCTCCTCCTTC | 208–248 | 54 - - - | LK934523 |
| CT-AG-88 | (AG)\(_{15}\) | F: ACAACCTACAAGATCTAAG R: CTTTATTTGTGGGTACCGG | 152 | 55 54 - 55 | LK934524 |
| CT-AG-114| (TG)\(_{13}\) | F: AGTGATAGTGAACACCCCTC R: TAGATCTCTAGCTCCTACAC | 104 | 50 - - | LK934525 |
| CT-AG-127| (TC)\(_3\)  | F: AACCTCCTGCGCTTCTTCTTG R: TGGTTTTGGGGCCAGAGCTG | 117–123 | 54 - - - | LK934526 |
| CT-AG-140| (AG)\(_{15}\) | F: AGCTCTGCCTCTCAAGCTTGG R: GAAGGATCAAGCTGGGAGG | 120–126 | 54 55 55 55 | LK934527 |
| CT-AG-144| (AG)\(_{18}\) | F: GGGATGTTCAATCAACTAAG R: TAACTGATAGCTGCCTCCAC | 113–115 | 52 55 55 55 | LK934528 |
| CT-AG-145| (TC)\(_{14}\) (ACTC)\(_3\) | F: ATCTCCTAGTATCCTAACCCTG R: AATGAGCTCCTTGAGCTTC | 140 | 54 - 55 | LK934529 |
javanica. Significant deviations from Hardy–Weinberg equilibrium ($H_{WE}$) were detected at 4 of 4, 1 of 2, and 4 of 5 polymorphic loci (Table 4).

For orchids, only few researches were used simple sequence repeats to evaluate the genetic diversity. The genetic diversity, including the means of the observed ($H_o$) and expected heterozygosity ($H_e$) (Table 4), of G. flabilabella was low compared with that of other Orchidaceae species, such as Dendrobium huoshanense (0.512 and 0.569) (Wang et al. 2012b), Dendrobium officinale (0.720 and 0.740) (Xie et al. 2010), Dendrobium officinale (0.514 for $H_o$) (Lu et al. 2012), and Dendrobium no-bile (0.350 and 0.608) (Lu et al. 2014). Unfortunately, no data for any Gastrodia taxa or mycoheterotrophic orchids are available for the comparison of genetic variability. However, the low observed and expected heterozygosity values implied that rare and mycoheterotrophic taxa tend to possess low levels of genetic diversity due to stochastic losses of genetic polymorphisms resulting from genetic drift (cf. Ge et al. 2014). In addition, significant deviations from Hardy–Weinberg equilibrium ($H_{WE}$) were detected at all loci in the remained population, and these deviations were credited to the heterozygote deficiency likely due to the unique interactions between orchids and pollinators (Boberg et al. 2014). Besides, the habitat preferences (Mallet et al. 2014) strengthened the isolation among populations.

### Table 3 Summary of general information for the 28 microsatellite loci isolated from Gastrodia flabilabella (Continued)

| Locus     | Repeat | Forward Primer | Reverse Primer | Annealing Temp | Accession | Concordance |
|-----------|--------|----------------|----------------|----------------|-----------|-------------|
| CT-AG-157 | (TG)14 | F: TGCAGTAATAGCATTGCGAG | R: AGGCTGGCACCTGTACTTTTC | 56 | 55 | - | 55 | LK934530 |
| CT-AGAT-19 | (TC)10 | F: TACATTGATTAGGATGCCCTC | R: ACATTGTTGCTCCTCCTCACAC | 55 | 50 | - | 50 | LK934531 |
| CT-AGAT-26 | (TG)88 | F: GATGATCTATGTGGTCTG | R: TGGCATATAGCATTGCGAG | 295 | 55 | - | - | LK934532 |
| CT-AGAT-131 | (CCA)7 | F: TCCAATCCTAGTAGCTCTG | R: GTTGAATTITAGTGAGAGG | 139 | 55 | 50 | LK934533 |
| CT-CCA-71 | (TGG)14 | F: ACATGAGTAGGAGCATCCTC | R: TTTCATCTCCCCACAGCTGC | 150–156 | 50 | - | 50 | LK934534 |
| CT-CCA-108 | (CCA)127 | F: CATGGTGGGACATAAAACTG | R: GTGGTTGTAGTCACTGACCTC | 489–516 | 47 | - | - | LK934535 |
| CT-CCA-137 | (CCA)16 | F: AATCTGACAGCCCTTCCAG | R: TGGAGGAGGCTGCTAGAGG | 150 | 55 | 55 | LK934536 |

Note: $F$ = the forward primer; $R$ = the reverse primer; $T_a$ = optimized annealing temperature.

### Conclusions

For conservation purposes, 28 new microsatellite loci, including 12 monomorphic and 16 polymorphic loci, were isolated from G. flabilabella. The genetic diversity indices assessed using these 16 polymorphic microsatellite loci for the remained populations of this endemic and vulnerable species revealed that these markers are potentially useful for future studies, especially those focusing on evaluating the genetic variation and identifying distinct evolutionary units within populations for conservation management. Genetic diversity was characterized for three other related species using these 28 microsatellite markers. Furthermore, successful amplification in 13 other Gastrodia taxa indicated the transferability of these primer pairs. The interspecies transferability made these microsatellite loci useful for...
Table 4 Genetic diversity characteristics of the 28 microsatellite loci tested on four *Gastrodia* taxa

| Locus     | *Gastrodia flavilabella* | *Gastrodia elata* | *Gastrodia javanica* | *Gastrodia confusoides* |
|-----------|--------------------------|-------------------|----------------------|-------------------------|
|           | Na Ne Ho He H F<sub>IS</sub> | Na Ne Ho He H F<sub>IS</sub> | Na Ne Ho He H F<sub>IS</sub> | Na Ne Ho He H F<sub>IS</sub> |
| CT3-32    | 4 1.23 0.00 0.19* 0.43 1.000 | — — — — — — — | — — — — — — — | — — — — — — — |
| CT6-4     | 15 8.85 0.10 0.89* 2.41 0.887 | — — — — — — — | — — — — — — — | — — — — — — — |
| CT6-35    | 2 1.08 0.00 0.08* 0.17 1.000 | — — — — — — — | — — — — — — — | — — — — — — — |
| CT6-65    | 5 2.94 0.32 0.66* 1.23 0.515 | 1 1.00 — — — — | — — — — — — — | — — — — — — — |
| CT6-90    | 1 1.00 — — — — | 1 1.00 — — — — | 1 1.00 — — — — | 1 1.00 — — — — |
| CT6-99    | 1 1.00 — — — — | 1 1.00 — — — — | 1 1.00 — — — — | 1 1.00 — — — — |
| CT6-120   | 1 1.00 — — — — | — — — — — — — | — — — — — — — | — — — — — — — |
| CT-6-142  | 2 1.04 0.00 0.04* 0.10 1.000 | 1 1.00 — — — — | — — — — — — — | — — — — — — — |
| CT-ACT-74 | 10 4.03 0.16 0.75* 1.80 0.783 | — — — — — — — | — — — — — — — | — — — — — — — |
| CT-ACT-88 | 1 1.00 — — — — | — — — — — — — | — — — — — — — | — — — — — — — |
| CT-ACT-136| 2 2.00 1.00 0.50* 0.69 -1.000 | 1 1.00 — — — — | — — — — — — — | — — — — — — — |
| CT-AG-35  | 3 2.56 0.00 0.61* 1.00 1.000 | 7 4.37 0.05 0.77* 1.64 0.935 | 2 1.11 0.11 0.10 0.21 -0.056 | 2 1.11 0.00 0.10* 0.20 1.000 |
| CT-AG-45  | 3 1.09 0.00 0.08* 0.20 1.000 | 1 1.00 — — — — | — — — — — — — | — — — — — — — |
| CT-AG-55  | 2 1.95 0.00 0.49* 0.68 1.000 | 1 1.00 — — — — | — — — — — — — | — — — — — — — |
| CT-AG-85  | 8 3.97 0.02 0.75* 1.62 0.972 | 5 3.86 1.00 0.74* 1.43 -0.349 | — — — — — — — | — — — — — — — |
| CT-AG-88  | 1 1.00 — — — — | — — — — — — — | — — — — — — — | — — — — — — — |
| CT-AG-114 | 1 1.00 — — — — | — — — — — — — | — — — — — — — | — — — — — — — |
| CT-AG-127 | 3 1.14 0.00 0.12* 0.28 1.000 | — — — — — — — | — — — — — — — | — — — — — — — |
| CT-AG-140 | 4 2.34 0.00 0.57* 0.97 1.000 | 4 1.49 0.00 0.33* 0.69 1.000 | 1 1.00 — — — — | 1 1.00 — — — — |
| CT-AG-147 | 2 1.17 0.00 0.15* 0.28 1.000 | 1 1.00 — — — — | — — — — — — — | — — — — — — — |
| CT-AG-145 | 1 1.00 — — — — | — — — — — — — | — — — — — — — | — — — — — — — |
| CT-AG-157 | 1 1.00 — — — — | 1 1.00 — — — — | 1 1.00 — — — — | 1 1.00 — — — — |
| CT-AGAT-19| 1 1.00 — — — — | 2 2.00 1.00 0.50* 0.69 -1.000 | — — — — — — — | — — — — — — — |
| CT-AGAT-26| 1 1.00 — — — — | — — — — — — — | — — — — — — — | — — — — — — — |
| CT-AGAT-131| 1 1.00 — — — — | — — — — — — — | — — — — — — — | — — — — — — — |
| CT-CCA-137| 1 1.00 — — — — | — — — — — — — | — — — — — — — | — — — — — — — |
| Mean      | 3.07 1.896 0.163 0.444 0.882 0.697 | 2.077 1.480 0.513 0.585 1.113 0.147 | 1.286 1.159 0.555 0.300 0.450 -0.528 | 1.588 1.324 0.612 0.386 0.67 -0.266 |

The number of different alleles (Na), number of effective alleles (Ne), observed heterozygosity (Ho), expected heterozygosity (He), Shannon’s information index (H), and fixation index (F<sub>IS</sub>) are reported.

*Significant deviation from Hardy-Weinberg equilibrium: P < 0.05.
future research aiming to reconstruct the phylogeographic patterns and the process of speciation among closely related species. Additionally, the transferable microsatellite loci will be potentially useful for future studies that focus on establishing the standard operating system of molecular identification for *Gastrodia elata*, a traditional Chinese medicine.

Abbreviations
Na: The number of alleles; Ne: The number of effective alleles; Ho: The observed heterozygosity; He: The expected heterozygosity; H: Shannon’s information index; FIS: The fixation index; HWE: The Hardy–Weinberg equilibrium.

Competing interests
The authors declare that they have no competing interests.

Table 5 Result of cross-species transferability in 13 *Gastrodia* taxa using the 28 microsatellite primers developed from *Gastrodia flavilabella*

| Locus    | Gal (N = 2) | Gap (N = 2) | Gau (N = 2) | Gcl (N = 2) | Gfo (N = 2) | Ggr (N = 2) | Gle (N = 2) | Gna (N = 2) | Gni (N = 2) | Gpu (N = 2) | Gsh (N = 2) | Gth (N = 2) | Gur (N = 2) | Total Species |
|----------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|--------------|
| CT3-32   | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | 3            |
| CT6-4    | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | 0            |
| CT6-35   | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | 0            |
| CT6-65   | 1           | 1           | 1           | 1           | =           | 1           | 1           | 1           | 1           | =           | 1           | 1           | 1           | 10           |
| CT6-90   | =           | 1           | 1           | 1           | =           | 1           | 1           | 1           | =           | 1           | 1           | 1           | 1           | 10           |
| CT6-99   | =           | 1           | 1           | 1           | 1           | 1           | 1           | 1           | 1           | 1           | 1           | 1           | 1           | 12           |
| CT6-120  | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | 1            |
| CT6-162  | 1           | 1           | 1           | 1           | =           | 1           | 1           | 1           | 1           | =           | 1           | 1           | 1           | 13           |
| CT-ACT-74| =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | 4            |
| CT-ACT-88| 1           | =           | =           | =           | =           | =           | =           | =           | =           | =           | 1           | 1           | =           | 5            |
| CT-ACT-136| 1           | 1           | 1           | 1           | 1           | 1           | 1           | 1           | 1           | 1           | 1           | 1           | 1           | 13           |
| CT-AG-35 | 1           | 1           | 1           | 1           | =           | 2           | 1           | 1           | 1           | =           | 1           | 1           | 1           | 11           |
| CT-AG-45 | 1           | =           | 1           | =           | =           | 1           | 1           | 1           | 1           | =           | 1           | 1           | =           | 8            |
| CT-AG-55 | 1           | =           | 1           | 1           | 1           | 1           | 1           | 1           | 1           | 1           | 1           | 1           | =           | 12           |
| CT-AG-85 | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | 0            |
| CT-AG-88 | 1           | 1           | 1           | 2           | 1           | 1           | 1           | 1           | 1           | 1           | 1           | 1           | 1           | 13           |
| CT-AG-114| =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | 0            |
| CT-AG-127| =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | 1            |
| CT-AG-140| 1           | 1           | =           | 1           | 1           | 1           | 1           | 1           | 1           | 1           | 1           | 1           | 1           | 12           |
| CT-AG-144| 1           | 1           | 1           | 1           | 1           | 1           | 1           | 1           | 1           | 1           | 1           | 1           | 1           | 13           |
| CT-AG-145| 1           | 1           | 1           | 1           | 1           | 1           | 1           | 1           | 1           | 1           | 1           | 1           | 1           | 12           |
| CT-AG-157| 1           | =           | 1           | 1           | 1           | 1           | 1           | 1           | 1           | =           | 1           | 1           | 1           | 11           |
| CT-AGAT-19| 1          | =           | 1           | =           | 1           | =           | =           | 1           | =           | 1           | =           | 5            | =           | =           |
| CT-AGAT-26| =          | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | 2            |
| CT-AGAT-131| =         | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | 4            |
| CT-CCA-71| =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | 4            |
| CT-CCA-108| =         | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | =           | 1            |
| CT-CCA-137| 1           | =           | 1           | =           | 1           | 1           | 1           | 1           | 1           | 1           | =           | 1           | 1           | 10           |
| No. of loci | 14         | 11          | 17          | 17          | 16          | 12          | 12          | 15          | 17          | 15          | 17          | 15          | 11           |

For loci that were successfully amplified, the number of alleles is given.

Authors’ contributions
T-WH, T-YC and Y-CC supervised the project. C-CT, S-KY, T-WH, and Y-CC collected plant sample in the field. C-CT, P-YW, C-CK, M-CH, and Y-CC mined the SSR primers. P-YW, M-CH, T-YC, and analyzed the data. T-WH, T-YC, and Y-CC wrote the manuscript. All authors read and approved the final manuscript.

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