Characterization and transferability of microsatellites for *Gentiana lawrencei* var. *farreri* (Gentianaceae)

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PREMISE OF THE STUDY: Microsatellite markers were developed for a medicinal herb, *Gentiana lawrencei* var. *farreri* (Gentianaceae), for the future assessment of population genetic structure and potential hybridization events with related taxa.

METHODS AND RESULTS: Using the 454 FLX+ sequencing platform, we obtained 81,717 clean reads with an average length of 291 bp. A total of 3031 primer pairs were designed, and 96 were selected for validation. A set of 20 fluorescently labeled primer pairs was further selected and screened for polymorphisms in three *G. lawrencei* var. *farreri* populations and one *G. veitchiorum* population. Among the four populations, the average number of alleles per locus was 15.2. Finally, a set of 17 unlinked loci were determined to be in Hardy–Weinberg equilibrium after two linked loci were removed.

CONCLUSIONS: The identified simple sequence repeat markers will be useful for genetic diversity and evolution studies in *G. lawrencei* var. *farreri* and related taxa.

KEY WORDS: *Gentiana*; Gentianaceae; medicinal herb; microsatellite primers; transferability.

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*Gentiana lawrencei* Burkill var. *farreri* (Balf. f.) T. N. Ho (Gentianaceae) is a perennial wildflower that is endemic to the Qinghai–Tibetan Plateau (QTP) and used in traditional Chinese and Tibetan medicine (Ho and Liu, 2001). *Gentiana veitchiorum* Hemsl., which is a perennial wildflower as well, is closely related with *G. lawrencei* var. *farreri* in phylogeny (Ho et al., 1996; Ho and Liu, 2001; Favre et al., 2010). The two species’ plastomes have very similar structures and low sequence variation (Fu et al., 2016; Fu, unpublished data). The morphological distinctions between the two species are primarily in leaf shape and flower color, with *G. lawrencei* var. *farreri* having linear stem leaves and a pale blue corolla and *G. veitchiorum* having narrowly elliptic stem leaves and an intense blue corolla. The two species are common in the QTP alpine meadow and sympatric in the central QTP (Ho and Liu, 2001). According to our field observations and previous studies, both species are outcrossing (Hou et al., 2009). The flowering and fruiting periods of *G. lawrencei* var. *farreri* and *G. veitchiorum* are from August to October and July to October, respectively (Ho and Liu, 2001). Therefore, hybridization and gene flow may occur between them. If simple sequence repeat (SSR) markers developed in *G. lawrencei* var. *farreri* could be applied in *G. veitchiorum*, they could be used as molecular markers for studies of genetic structure, hybridization, species divergence, and gene flow of these two closely related species. Because phylogenetic relationships and species divergences of numerous *Gentiana* L. species are still unclear (Ho and Liu, 2001; Favre et al., 2010), developed polymorphic SSRs would also aid in studies of population genetics and evolution within *Gentiana*.

METHODS AND RESULTS

In this study, we tested the amplification and evaluated polymorphisms using four populations: three populations of *G. lawrencei* var. *farreri* and one population of *G. veitchiorum* (Appendix 1). Total genomic DNA was extracted from dried leaves using a modified cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987). Genomic DNA (600 μg) was fragmented with nitrogen at 45 pounds per square inch (psi) for 2 min; 500–800-bp fragments were used for further study. The fragments were ligated to adapters using T4 ligase and amplified by PCR with corresponding adapter primers. Genome libraries were constructed using eight biotin-labeled probes (pGA, pAC, pAAT, pAAC, pAAG, pATGT, pGATA, and pAAAT) and a selective hybridization with streptavidin-coated beads (Invitrogen, Grand Island, New York, USA) (Armour et al., 1994; Kandpal et al., 1994; Glenn and Schable, 2005). Library quality inspection and sequencing of clones was carried out following the descriptions in Yang et al. (2012). Subsequently, entire libraries were equally pooled and sequenced on a Roche 454 GS FLX+ sequencer (454 Life Sciences/Roche, Penzberg, Germany) using titanium reagents at Shanghai Personal
Biotechnology Co., Ltd. (Shanghai, China). Processing and analysis of the sequencing data were performed with GS-FLX+ software version 2.9 (454 Life Sciences/Roche). Using a series of normalization, correction, and quality-filtering, the sequencing data were processed to remove low-quality and adapter sequences using EMBOSS (Rice et al., 2000). A total of 87,097 reads were generated from the pooled *G. lawrencei* var. *farreri* library, and 81,717 read sequences were used for further analysis after adapter removal. The average read sequence length was 291 bp, with a maximum length of 791 bp. Clean reads were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (accession number SRP101615).

The MicroSatellite identification tool (MISA; Thiel et al., 2003) was used to identify reads that contained SSRs. The minimum motif repeat was defined as six for di-, and five for tri-, tetra-, penta-, and hexanucleotides. A total of 6381 SSRs were identified. All SSRs with flanking sequence lengths ≥20 bp were assigned into clusters based on 98% similarity of flanking sequences using UCLUST version 1.2.22q (Edgar, 2010). We determined SSR lengths in each cluster with Perl and defined the SSR length polymorphism value as *n* when there were *n* kinds of SSR lengths in a cluster. Among the 4831 SSR-containing sequences, a total of 2168 SSRs with flanking sequence lengths ≥20 bp were detected and assigned into 1023 clusters. Of these, 207 clusters (20.33%) had an SSR length polymorphism value ≥2; subsequently, a total of 3031 primer pairs were designed in Primer3 (Rozen and Skaletsky, 1999).

To amplify microsatellite regions, PCR amplifications were carried out in a 15-μL reaction volume containing approximately 15 ng of template DNA, 1× PCR buffer (with MgCl₂), 0.2 μM of each primer, 0.4 μM of each dNTP, and 1 unit of *Taq* DNA polymerase (TaKaRa Biotechnology Co., Dalian, Liaoning, China). The PCR cycling profile included an initial step at 95°C for 5 min; followed by 35 cycles of 95°C for 45 s, 50–55°C for 30 s, and 72°C for 30 s; and final extension at 72°C for 7 min. We analyzed the PCR products

| Locus | Primer sequences (5′–3′) | Repeat motif | Fluorescent dye | *Tₐ* (°C) | Allele size range (bp) | Total no. of alleles | GenBank accession no. |
|-------|--------------------------|--------------|----------------|-----------|------------------------|---------------------|----------------------|
| Law4  | F: TGCAAGGTCTACCTCTCTT  | (GA)₂₈       | FAM            | 55        | 226–296                | 21                  | MG008318             |
|       | R: TAACTCTTGCTGAAATTCTGA |              |                |           |                        |                     |                      |
| Law5  | F: TTTCAGGCTGCAATTCTCTA | (CAA)₂₈      | FAM            | 55        | 199–265                | 16                  | MG008319             |
|       | R: ATGGCTGCAAACAAAGATT  |              |                |           |                        |                     |                      |
| Law12 | F: AGTGTCGCAAACACACACT  | (ACA)₂₂      | FAM            | 55        | 85–202                 | 3                   | MG008320             |
|       | R: AGCTGATTTTTGTTGTTATG |              |                |           |                        |                     |                      |
| Law19 | F: ATACATGAGTCTCGGCAAGG  | (CAA)₁₉      | FAM            | 55        | 130–202                | 8                   | MG008321             |
|       | R: AGCTCGGATTCTTTTCTCTT |              |                |           |                        |                     |                      |
| Law24 | F: TGATGACCTCTCTCCAGA   | (CA)₁₇       | FAM            | 55        | 145–192                | 15                  | MG008322             |
|       | R: GGGTGTGTTGCTGGAAGTTT |              |                |           |                        |                     |                      |
| Law25 | F: CGAGGTCGATCCGAGAG    | (AG)₁₇       | FAM            | 55        | 178–230                | 6                   | MG008323             |
|       | R: AAAGCGTTTTGTGTTGTT |              |                |           |                        |                     |                      |
| Law32 | F: CGACGAGCCGACTCTCACAT | (CA)₁₆      | FAM            | 55        | 164–275                | 21                  | MG008324             |
|       | R: CGTCGAGCTCACGCTTCTCT |              |                |           |                        |                     |                      |
| Law34 | F: ATATTTTCCGCAATTAGG   | (AG)₁₅       | FAM            | 55        | 120–184                | 8                   | MG008325             |
|       | R: AACTGAAAGGCCGAAGG    |              |                |           |                        |                     |                      |
| Law37 | F: CCCGGTTTCTCCCTCTCTC | (TCT)₁₃      | FAM            | 55        | 122–161                | 12                  | MG008326             |
|       | R: GCCCTACCACTCTTCTTTAC |              |                |           |                        |                     |                      |
| Law41 | F: AGAATCCGGTTGTTGCGCA  | (AG)₁₅       | FAM            | 55        | 281–325                | 17                  | MG008327             |
|       | R: CAAGGACCCGAGTTGCTCG  |              |                |           |                        |                     |                      |
| Law43 | F: TGGATTAGGTGACCTTGG   | (ACA)₁₅      | HEX            | 58        | 104–251                | 23                  | MG008328             |
|       | R: TGCCGATTGTTGTCAGG    |              |                |           |                        |                     |                      |
| Law45 | F: CCAGTGGTTTGATCCCTTTAGGC | (AC)₁₅  | HEX            | 58        | 167–221                | 21                  | MG008329             |
|       | R: TGATGCTGCTGCCAGAGG   |              |                |           |                        |                     |                      |
| Law54 | F: GCAGGCAGATGCAGATACA  | (AG)₁₄       | HEX            | 58        | 122–226                | 30                  | MG008330             |
|       | R: CAGAACGACTCAGCTGTGTGTT |              |                |           |                        |                     |                      |
| Law57 | F: GCTGTTTTCTCTTTATTGGTGG | (TTG)₁₃ | HEX            | 55        | 189–366                | 22                  | MG008331             |
|       | R: ATACTGTTGGCGCATTCCGG |              |                |           |                        |                     |                      |
| Law70 | F: CCTATGCCCCCCGAGGTG   | (TTT)₁₂      | HEX            | 55        | 159–198                | 13                  | MG008332             |
|       | R: CAGAAAGGGTACGCCTGAA |              |                |           |                        |                     |                      |
| Law71 | F: CCTTGGCCTACGTTCTTTTTC | (TTT)₁₂  | HEX            | 55        | 163–256                | 19                  | MG008333             |
|       | R: TCATGCTTGACTTTCCTCC  |              |                |           |                        |                     |                      |
| Law77 | F: TCAATTGGTCTAGATTTTTGAGGG | (CTT)₁₂ | HEX            | 55        | 141–201                | 11                  | MG008334             |
|       | R: CACAGCTACACATCTTCTCTCT |              |                |           |                        |                     |                      |
| Law87 | F: CAGGTTCAGGAGGTTGCCTG | (TTT)₁₁      | HEX            | 55        | 136–196                | 14                  | MG008335             |
|       | R: ATCTTTGCGCTACCGAGGTC |              |                |           |                        |                     |                      |
| Law88 | F: CAGAGGTCCGAAAACACCGAGG | (GTT)₁₁  | HEX            | 55        | 321–357                | 9                   | MG008336             |
|       | R: CATGGCGACATTCTCTCTTAA |              |                |           |                        |                     |                      |
| Law95 | F: CGTTCGAGCTTACCTTGCA  | (AC)₁₀       | HEX            | 55        | 183–221                | 14                  | MG008337             |
|       | R: CGAAGATCTCCGCTAAACA |              |                |           |                        |                     |                      |

Note: *Tₐ* = annealing temperature.
TABLE 2. Results of initial primer screening of 20 microsatellite loci developed for Gentiana lawrencei var. farreri in three populations of G. lawrencei var. farreri and one population of G. veitchiorum:

| Locus | HY (N = 18) | GZ (N = 18) | XGLL (N = 18) | SP (N = 18) |
|-------|-------------|-------------|---------------|-------------|
|       | A | H_o | H_e | A | H_o | H_e | A | H_o | H_e | A | H_o | H_e |
| Law4  | 8 | 1.000 | 0.770 | 11 | 1.000 | 0.833 | 9 | 1.000 | 0.821 | 16 | 1.000 | 0.929 |
| Law5  | 6 | 1.000 | 0.726\textsuperscript{a} | 14 | 1.000 | 0.913 | 5 | 0.900 | 0.800 | 9 | 1.000 | 0.863 |
| Law12 | 3 | 1.000 | 0.586\textsuperscript{a} | 2 | 1.000 | 0.517\textsuperscript{b} | 2 | 0.857 | 0.508 | 3 | 0.882 | 0.533\textsuperscript{b} |
| Law19 | 5 | 1.000 | 0.692 | 2 | 1.000 | 0.514\textsuperscript{b} | 2 | 1.000 | 0.514\textsuperscript{b} | 4 | 1.000 | 0.646\textsuperscript{b} |
| Law24 | 6 | 1.000 | 0.631\textsuperscript{b} | 10 | 0.941 | 0.868 | 5 | 0.857 | 0.670 | 8 | 1.000 | 0.738 \textsuperscript{b} |
| Law25 | 3 | 1.000 | 0.605\textsuperscript{b} | 4 | 1.000 | 0.656\textsuperscript{b} | 3 | 1.000 | 0.541\textsuperscript{b} | 4 | 1.000 | 0.624\textsuperscript{b} |
| Law32 | 7 | 1.000 | 0.754 | 15 | 1.000 | 0.914 | 7 | 1.000 | 0.793 | 6 | 1.000 | 0.800 |
| Law34 | 6 | 0.444 | 0.394 | 2 | 0.667 | 0.457 | 3 | 0.389 | 0.332 | 4 | 0.222 | 0.211 |
| Law37 | 6 | 1.000 | 0.686 | 5 | 1.000 | 0.667 | 7 | 1.000 | 0.709 | 8 | 1.000 | 0.701\textsuperscript{b} |
| Law41 | 15\textsuperscript{c} | 1.000 | 0.937 | 10 | 1.000 | 0.843 | 4\textsuperscript{c} | 1.000 | 0.733 | 8\textsuperscript{c} | 1.000 | 0.882 |
| Law43 | 13 | 1.000 | 0.913 | 10 | 1.000 | 0.839 | 14 | 1.000 | 0.908 | 13 | 1.000 | 0.884\textsuperscript{b} |
| Law45 | 8 | 1.000 | 0.911 | 11 | 1.000 | 0.903 | 9 | 0.923 | 0.880 | 17 | 1.000 | 0.938 |
| Law54 | 15 | 1.000 | 0.948 | 10 | 0.938 | 0.893 | 10 | 1.000 | 0.843 | 15 | 1.000 | 0.952 |
| Law57 | 6 | 1.000 | 0.692 | 9 | 1.000 | 0.890 | 7 | 1.000 | 0.779 | 9 | 1.000 | 0.869 |
| Law70 | 11 | 1.000 | 0.903 | 8 | 1.000 | 0.794 | 8 | 1.000 | 0.790 | 8 | 1.000 | 0.816 |
| Law71 | 12 | 1.000 | 0.892 | 6 | 1.000 | 0.617\textsuperscript{b} | 9 | 1.000 | 0.907 | 11 | 1.000 | 0.883 |
| Law77 | 4 | 1.000 | 0.577\textsuperscript{b} | 6 | 1.000 | 0.712 | 2 | 1.000 | 0.571 | 9 | 0.938 | 0.851 |
| Law87 | 9 | 1.000 | 0.781 | 7 | 1.000 | 0.762\textsuperscript{b} | 7 | 1.000 | 0.804 | 6 | 1.000 | 0.726 |
| Law88 | 4 | 1.000 | 0.650\textsuperscript{b} | 8 | 1.000 | 0.840 | 3 | 1.000 | 0.714 | 3\textsuperscript{c} | 0.333 | 0.733 |
| Law95 | 12 | 0.933 | 0.897 | 10 | 1.000 | 0.867 | 5 | 1.000 | 0.692 | 8 | 1.000 | 0.806 |
| Mean  | 7.95 | 0.969 | 0.798 | 8 | 0.977 | 0.816 | 6.05 | 0.946 | 0.736 | 8.45 | 0.919 | 0.804 |

Note: A = total number of alleles per locus; H_o = expected heterozygosity; H_e = observed heterozygosity; N = sample size for each population.

\textsuperscript{a}Location and voucher information are provided in Appendix 1.

\textsuperscript{b}Significant departure from Hardy–Weinberg equilibrium (P < 0.01).

\textsuperscript{c}Null allele present.

In the four populations, observed heterozygosity values ranged from 0.222 to 1.000, whereas expected heterozygosity values varied between 0.211 and 0.952 (Table 2). Of the 20 loci, 11 showed significant deviation from HWE in one or more populations, and only one locus (Law25) showed significant deviation from HWE in all four populations (Table 2). Fourteen locus pairs showed significant LD (P < 0.01) across the four populations (Appendix 2). After removing the loci Law12 and Law19, only three locus pairs showed significant LD (P < 0.01).

CONCLUSIONS

The 20 validated microsatellite primer pairs in this study will be useful for further studies on population genetics, phylogenetics, and evolution of the large genus Gentiana, especially G. lawrencei var. farreri and G. veitchiorum. The 6381 microsatellites obtained in this study offer a foundation for further research on this large genus, such as marker-assisted breeding and functional characterization of genes related to trait formation.

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APPENDIX 1. Sample information of four Gentiana populations used in this study.*

| Taxon                  | Population code | Voucher no. | N  | Location                        | Geographic coordinates | Altitude (m) |
|------------------------|-----------------|-------------|----|---------------------------------|------------------------|--------------|
| Gentiana lawrencei var. farreri (Balf. f.) T. N. Ho | HY              | Fu2016022   | 18 | Ganzi, Sichuan Province, China  | 32°14′58″N, 102°29′21″E | 3597         |
|                        | GZ              | Fu2016051   | 18 | Ganzi, Sichuan Province, China  | 31°39′44″N, 99°44′02″E | 3495         |
|                        | XGLL            | Fu2016146   | 18 | Xianggelila, Yunnan Province, China | 28°18′59″N, 99°45′09″E | 3881         |
| Gentiana veitchiorum Hemsl. | SP              | Fu2016029   | 18 | Songpan, Sichuan Province, China | 32°59′36″N, 103°41′35″E | 3386         |

Note: N = sample size for each population.

*Vouchers are stored in the Herbarium of Luoyang Normal University, Henan, China.
### APPENDIX 2. Results of linkage disequilibrium between all primer pairs in four *Gentiana* populations.

| Locus | Law4 | Law5 | Law12 | Law19 | Law24 | Law25 | Law32 | Law34 | Law37 | Law41 | Law43 | Law45 | Law54 | Law57 | Law70 | Law71 | Law77 | Law87 | Law88 | Law95 |
|-------|------|------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Law4  | +    |      | *     |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| Law5  |      |      |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| Law12 |      |      | *     |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| Law19 |      |      | +     |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| Law24 |      |      |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| Law25 |      |      |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| Law32 |      |      |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| Law34 |      |      |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| Law37 |      |      |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| Law41 |      |      |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| Law43 |      |      |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| Law45 |      |      |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| Law54 |      |      |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| Law57 |      |      |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| Law70 |      |      |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| Law71 |      |      |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| Law77 |      |      |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| Law87 |      |      |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| Law88 |      |      |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| Law95 |      |      |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |

Note: + = significant linkage disequilibrium (P < 0.01); — = nonsignificant linkage disequilibrium; * = no data available.