

## **Characterization of 13 Microsatellite Markers for *Calochortus gunnisonii* (Liliaceae) from Illumina MiSeq Sequencing**

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## PRIMER NOTE

# CHARACTERIZATION OF 13 MICROSATELLITE MARKERS FOR *Calochortus gunnisonii* (LILIACEAE) FROM ILLUMINA MiSeq SEQUENCING<sup>1</sup>

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- *Premise of the study:* Microsatellite primers were designed for *Calochortus gunnisonii* (Liliaceae), a montane lily species of the central and southern Rocky Mountains, using next-generation DNA sequencing. The markers will be used to investigate population structure, genetic diversity, and demographic history.
- *Methods and Results:* Thirteen polymorphic microsatellite loci were isolated from *C. gunnisonii* using Illumina MiSeq next-generation DNA sequencing and bioinformatic screening. The mean number of alleles per locus ranged from 4.15 to 5.92 (avg. = 4.97). Observed and expected heterozygosity ranged from 0.077 to 0.871 and 0.213 to 0.782, respectively. The primers were also tested for cross-species amplification value with *C. flexuosus*, *C. nuttallii*, *C. kennedyi* var. *kennedyi*, and *C. subalpinus*.
- *Conclusions:* These primers will be useful for genetic and evolutionary studies across *C. gunnisonii*'s range within the southern and central Rocky Mountains. Furthermore, these markers have proven valuable for cross-species amplifications within *Calochortus*.

**Key words:** *Calochortus gunnisonii*; Illumina; Liliaceae; microsatellites; MiSeq.

*Calochortus* Pursh (Liliaceae) is a large genus of bulbous geophytes (ca. 70 spp.) originating in California ~7 million years ago (Patterson and Givnish, 2003). Its range includes a center of diversity in California that spreads north to British Columbia, east to the Dakotas, and south to Guatemala (Ownbey, 1940; Patterson and Givnish, 2003; Henss et al., 2013). The genus also occurs in a wide range of habitats including grasslands, deserts, vernal pools, woodland meadows, springs, montane woodlands, and forest understories, with most taxa occupying narrow geographic ranges (Ownbey, 1940; Patterson and Givnish, 2003; Fiedler and Zebell, 2012; Henss et al., 2013).

Gunnison's mariposa lily, *C. gunnisonii* S. Watson, is a North American endemic populating portions of the central and southern Rocky Mountains. *Calochortus gunnisonii* has a broad distribution encompassing northeastern Arizona, northern New Mexico, much of Colorado, eastern Utah, large portions of Wyoming, southern Montana, and western South Dakota at elevations of 1200–3300 m (Fiedler and Zebell, 2012). This species achieves some of the highest elevations for the genus in the southern portion of the Rocky Mountains. Disjunctions in the northern portions of *C. gunnisonii*'s range exist across mountain ranges of the Big Horn Mountains, Black Hills, Absaroka Range, Sierra Madre, Medicine Bow, and Laramie Range. A population genetic study of *C. gunnisonii* across multiple, disjunct populations in the central and southern Rocky Mountains is currently being conducted.

However, previous genetic studies of members within the genus are limited to amplified fragment length polymorphisms (Henss et al., 2013) and chloroplast sequence comparisons (Patterson and Givnish, 2003). Here, we report the characterization of 13 microsatellite loci that will be used to investigate the role of glacial oscillatory demographic changes in shaping genetic structure of *C. gunnisonii* across multiple montane disjunctions of the central and southern Rocky Mountains.

## METHODS AND RESULTS

Next-generation sequencing was used to acquire a large quantity of genomic sequence data in search of microsatellite repeats. Genomic DNA (gDNA) was extracted from leaf tissue using a modified cetyltrimethylammonium bromide (CTAB) protocol (Friar, 2005). Two individual DNA samples collected from two separate geographic populations (Hell Canyon Road, South Dakota: 43.724753°N, 103.854112°W; La Prele Reservoir, Wyoming: 42.706796°N, 105.578531°W [Appendix 1]) were pooled and sent to the Center for Genome Research and Biocomputing at Oregon State University. A total of ~400 ng of gDNA was used for library preparation and Illumina MiSeq sequencing (Illumina, San Diego, California, USA). A single, 300-bp paired-end MiSeq sequencing run resulted in 18,332,564 reads with an average length of 301 bases. Raw sequence reads were filtered, reformatted, and trimmed using the default commands of the Trimmomatic v.0.32 program (Bolger et al., 2014). Trimmomatic yielded 12,080,556 high-quality contigs (6,040,278 forward and reverse). Contigs were de novo assembled using Trinity (v. 07-04-2014) (Grabherr et al., 2011) producing 486,538 contigs, with an average size of 516 bp (N50 = 583). MSATCOMMANDER v.1.0.8 (Faircloth, 2008) found a total of 4118 perfect microsatellite repeats from the assembled contigs: 85 hexanucleotide with at least four repeat units, 585 pentanucleotide with at least four repeat units, 481 tetranucleotide with at least five repeat units, and 2967 trinucleotide with at least five repeat units. Dinucleotide repeats were excluded to avoid stutter and subsequent scoring problems in later analyses. The inset version of Primer3 (Rozen and Skaletsky, 2000) in MSATCOMMANDER designed 1153 primer pairs using default parameters (except that GC clamp = yes and repeat motif was ≥4 for penta- and hexanucleotide repeats). The PCR product size was set to 120–400 bp. One primer of each pair was designed with a common tag at the 5' end following the procedure of Boutin-Ganache et al. (2001). Two common

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TABLE 1. Characteristics of 13 microsatellite loci designed for *Calochortus gunnisonii*.

| Locus   | Primer sequences (5'–3') <sup>a</sup>                 | 5' Tag <sup>b</sup> | Repeat motif          | Fluorescent dye | Allele size range (bp) | T <sub>a</sub> (°C) | MgCl <sub>2</sub> /MgSO <sub>4</sub> <sup>c</sup> | GenBank accession no. |
|---------|-------------------------------------------------------|---------------------|-----------------------|-----------------|------------------------|---------------------|---------------------------------------------------|-----------------------|
| CAGU_14 | F: TTGTCAGTGGGCAAGTGTC<br>*R: ATACAACGCCCGCATACC      | M13                 | (ACACC) <sub>4</sub>  | FAM             | 246–266                | 62.4                | MgCl <sub>2</sub>                                 | KR139841              |
| CAGU_15 | *F: ATCTCACTGCCCACTCAC<br>R: GTGCAGCAGATCCACATTCC     | M13                 | (AGAGG) <sub>4</sub>  | VIC             | 385–415                | 62.4                | MgCl <sub>2</sub>                                 | KR139842              |
| CAGU_22 | F: CACATGGTGTGATGCAGGG<br>*R: TTGTATCGTGCAACAGTCCC    | CAGT                | (ATCC) <sub>5</sub>   | PET             | 237–257                | 62.4                | MgCl <sub>2</sub>                                 | KR139843              |
| CAGU_31 | F: CCACCAAGAAGAGCTAAAGG<br>*R: TTCACCGTCTCCAACAC      | CAGT                | (ACATC) <sub>4</sub>  | PET             | 310–340                | 62.4                | MgCl <sub>2</sub>                                 | KR139844              |
| CAGU_35 | F: TAATACCCGTGAATCCGGC<br>*R: TGCAACCGAGTAGTGACC      | M13                 | (ATGC) <sub>5</sub>   | VIC             | 263–283                | 62.4                | MgCl <sub>2</sub>                                 | KR139845              |
| CAGU_36 | F: CCCTCGATCTCAGCGATTG<br>*R: TAAGTTAGGTACGGAGCAGGC   | M13                 | (ATCG) <sub>5</sub>   | FAM             | 378–398                | 62.4                | MgCl <sub>2</sub>                                 | KR139846              |
| CAGU_39 | F: TTACACCAAGTCCGCAG<br>R: GTCTTGCTCATGTTGCTCCC       | CAGT                | (ACTG) <sub>6</sub>   | FAM             | 335–374                | 62.4                | MgCl <sub>2</sub>                                 | KR139847              |
| CAGU_42 | *F: TCGTGTCAAGTGCTACCATCG<br>R: CTTGCTGGAATCATATCACCC | M13                 | (AAGAC) <sub>5</sub>  | PET             | 380–415                | 66.9                | MgCl <sub>2</sub>                                 | KR139848              |
| CAGU_45 | *F: TCTAGTAGAAACCAGGGTGCC<br>R: ACCCTAGCTCTTGTTCGGTG  | M13                 | (ACCG) <sub>5</sub>   | FAM             | 190–214                | 62.4                | MgCl <sub>2</sub>                                 | KR139849              |
| CAGU_46 | F: GCATCGATTTCAGAGCTGC<br>*R: TTCAAGGATGGATGGCATCG    | T7                  | (ACTAT) <sub>4</sub>  | PET             | 164–184                | 59.8                | MgSO <sub>4</sub>                                 | KR139850              |
| CAGU_47 | F: TCCCTCAGTCCCAAGTC<br>*R: GAACCTTCCTTTGTCCGCAC      | CAGT                | (AGCTCC) <sub>4</sub> | VIC             | 345–381                | 61.1                | MgCl <sub>2</sub>                                 | KR139851              |
| CAGU_48 | F: TGCACCTTAGAGACCATGG<br>*R: GTCAAAAGCATCCCGTCCTC    | CAGT                | (ACAGAT) <sub>4</sub> | FAM             | 406–448                | 61.1                | MgSO <sub>4</sub>                                 | KR139852              |
| CAGU_50 | *F: TAGGAGGGGTTCAGGAAC<br>R: TAGGTGCGGCAGGGTCTAATG    | CAGT                | (ACAGT) <sub>5</sub>  | PET             | 364–409                | 56.9                | MgCl <sub>2</sub>                                 | KR139853              |

Note: T<sub>a</sub> = PCR annealing temperature.  
<sup>a</sup>The primer tagged with either M13R, CAGT, or T7 is indicated by an asterisk.  
<sup>b</sup>The 5' tag used for incorporation of the fluorescent tag: M13R (AGGAACAGCTATGACCAT), T7 (GCTAGTTATTGCTCAGCGG), or CAGT (ACAGTCGGGCGTCATCA).  
<sup>c</sup>MgCl<sub>2</sub> or MgSO<sub>4</sub> used in PCR reactions.

TABLE 2. Allelic diversity at 13 variable microsatellite loci for three populations of *Calochortus gunnisonii*.

| Locus   | Dixon |      |                |                             | Sand Lake |      |                |                             | Shavano Camp |      |                |                             |
|---------|-------|------|----------------|-----------------------------|-----------|------|----------------|-----------------------------|--------------|------|----------------|-----------------------------|
|         | N     | A    | H <sub>o</sub> | H <sub>e</sub> <sup>a</sup> | N         | A    | H <sub>o</sub> | H <sub>e</sub> <sup>a</sup> | N            | A    | H <sub>o</sub> | H <sub>e</sub> <sup>a</sup> |
| CAGU_14 | 31    | 4    | 0.469          | 0.469                       | 31        | 3    | 0.233          | 0.376*                      | 33           | 4    | 0.559          | 0.493                       |
| CAGU_15 | 31    | 8    | 0.563          | 0.607                       | 32        | 4    | 0.419          | 0.348                       | 34           | 3    | 0.571          | 0.519                       |
| CAGU_22 | 31    | 5    | 0.125          | 0.252*                      | 32        | 5    | 0.387          | 0.356                       | 33           | 5    | 0.441          | 0.440                       |
| CAGU_31 | 31    | 6    | 0.531          | 0.582*                      | 32        | 5    | 0.645          | 0.674                       | 34           | 5    | 0.457          | 0.602                       |
| CAGU_35 | 30    | 6    | 0.419          | 0.601                       | 29        | 5    | 0.241          | 0.405*                      | 28           | 6    | 0.393          | 0.695*                      |
| CAGU_36 | 30    | 5    | 0.387          | 0.455*                      | 18        | 3    | 0.118          | 0.213                       | 34           | 4    | 0.294          | 0.407*                      |
| CAGU_39 | 30    | 6    | 0.452          | 0.655                       | 32        | 5    | 0.806          | 0.691                       | 34           | 6    | 0.600          | 0.666*                      |
| CAGU_42 | 31    | 7    | 0.250          | 0.750*                      | 30        | 4    | 0.448          | 0.542                       | 25           | 5    | 0.077          | 0.643*                      |
| CAGU_45 | 31    | 7    | 0.344          | 0.616*                      | 32        | 4    | 0.548          | 0.575                       | 33           | 5    | 0.500          | 0.599*                      |
| CAGU_46 | 28    | 4    | 0.517          | 0.633                       | 32        | 3    | 0.516          | 0.533                       | 33           | 4    | 0.618          | 0.478                       |
| CAGU_47 | 30    | 5    | 0.387          | 0.363                       | 31        | 4    | 0.400          | 0.575                       | 33           | 3    | 0.118          | 0.452*                      |
| CAGU_48 | 30    | 8    | 0.871          | 0.763                       | 28        | 5    | 0.692          | 0.648                       | 34           | 6    | 0.686          | 0.782                       |
| CAGU_50 | 29    | 6    | 0.633          | 0.554                       | 30        | 4    | 0.586          | 0.691                       | 34           | 7    | 0.714          | 0.738                       |
| Mean    | 30.23 | 5.92 | 0.458          | 0.562                       | 29.92     | 4.15 | 0.465          | 0.510                       | 32.46        | 4.85 | 0.464          | 0.578                       |

Note: A = number of alleles; H<sub>e</sub> = expected heterozygosity; H<sub>o</sub> = observed heterozygosity; N = sample size.  
<sup>a</sup> Statistical significance associated with departure from Hardy–Weinberg equilibrium (HWE) is indicated with an asterisk (\*P ≤ 0.05).

tags, M13R (GGAAACAGCTATGACCAT) and CAGT (CAGTCGGGCGT-CATCA), and one custom tag, T7 (GCTAGTTATTGCTCAGCGG), were used. Fifty loci with optimum primer conditions were screened for amplification success and within-population variability. Microsatellite loci were amplified in 12-μL reaction volumes that included 2.5 mM of tagged primer, 25 mM of nontagged primer, 25 mM of fluorescently labeled tag (6-FAM, PET, or VIC; Applied Biosystems, Wilmington, Delaware, USA), 5× Colorless GoTaq Flexi Buffer (Promega Corporation, Madison, Wisconsin, USA), 2.5 mM each dNTP, 0.02 units GoTaq Flexi DNA Polymerase (Promega Corporation), 0.1 μL 100× bovine serum albumin, 25 mM MgCl<sub>2</sub> or 100 mM MgSO<sub>4</sub> (Table 1), ~20–50 ng of gDNA, and brought to volume with purified water. Thermal cycling conditions were 94°C for 3 min; 35 cycles of 94°C for 30 s, 56–63°C for 30 s, and 72°C for 30 s; followed by an extension for 10 min at 72°C; and a final 30-min extension at 72°C. PCR products were mixed with 1× SYBR Green (LONZA, Rockland, Maine) and visualized on a 1% agarose gel. Successful PCR products were diluted with water and mixed with Hi-Di formamide and GeneScan 500 LIZ Size Standard (Applied Biosystems) before electrophoresis on an Applied Biosystems 3730 Genetic Analyzer at Arizona State University. Fragments were sized using the microsatellite plug-in of Geneious v.8.0.4 (created by Biomatters, <http://www.geneious.com/>).

Of the 50 initial screenings, 22 failed to yield reliable PCR product via gel electrophoresis. Twenty-eight loci yielded consistent products and were amplified. Preliminary sizing of the products revealed 13 primer pairs that consistently yielded amplicons within the target size range (120–400 bp) (Table 1). Three sample populations of *C. gunnisonii* were selected to evaluate variability in the isolated loci (Table 2). One population represents central Colorado (Shavano Campground; n = 34), the second represents a low-elevation population in northern Colorado (Dixon Reservoir; n = 31), and the third is a population from south-central Wyoming near the northern edge of the southern Rockies (Sand Lake; n = 32).

All 13 microsatellite loci were variable and polymorphic among sampled populations. Population statistics and deviations from Hardy–Weinberg equilibrium (HWE) were calculated using GenAlEx v.6.5 (Peakall and Smouse, 2012). The mean number of alleles per locus ranged from 4.15 to 5.92, with an average of 4.97 (Table 2). The observed and expected heterozygosity ranged from 0.077 to 0.871 and 0.213 to 0.782, respectively. Significant deviations from HWE were observed for individual loci, but no consistent pattern across multiple populations was observed, suggesting the deviations are due to population processes (Table 2). Observed and expected heterozygosities are high when examined across all populations, indicating the value of these markers for future studies.

All 13 loci were screened for amplification success in four additional *Calochortus* species spread throughout the *Calochortus* phylogeny (Patterson and Givnish, 2003). Cross-amplification success was high in the closely related *C. nuttallii* Torr. & A. Gray (11), moderate in *C. kennedyi* Porter var. *kennedyi* (8), and low in *C. flexuosus* S. Watson (7) and *C. subalpinus* Piper (4) (Appendix 2).

CONCLUSIONS

We identified 13 *C. gunnisonii* microsatellite loci that are variable and informative. These markers will be used to

investigate the population genetic structure and levels of genetic variability of *C. gunnisonii* in the central and southern Rocky Mountains. Intra- and intermontane patterns of gene flow and divergence will be inferred within *C. gunnisonii*. Cross-species amplification was high in a closely related taxon, *C. nuttallii*, and decreased in more divergent sampled taxa.

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APPENDIX 1. Voucher information for *Calochortus* species used in this study.

| Species                                              | Collector information <sup>a</sup> | Voucher specimen accession no. <sup>b</sup> | Collection locality <sup>c</sup>         | Geographic coordinates       | N  |
|------------------------------------------------------|------------------------------------|---------------------------------------------|------------------------------------------|------------------------------|----|
| <i>C. gunnisonii</i> <sup>d</sup>                    | MEM 608                            | GREE 23640                                  | Dixon Reservoir; Larimer County, CO      | 40.54135329°N, 105.1343894°W | 31 |
| <i>C. gunnisonii</i> <sup>d</sup>                    | RSF 47                             | GREE 23643                                  | Sand Lake; Carbon County, WY             | 41.33943317°N, 106.1658852°W | 32 |
| <i>C. gunnisonii</i> <sup>d</sup>                    | MEM 669                            | GREE 23639                                  | Shavano Campground; Chaffee County, CO   | 38.586457°N, 106.215402°W    | 34 |
| <i>C. gunnisonii</i> <sup>d</sup>                    | RSF 36                             | GREE 23642                                  | Hell Canyon Road; Custer County, SD      | 43.7246205°N, 103.854076°W   | 1  |
| <i>C. gunnisonii</i> <sup>d</sup>                    | MEM 621                            | GREE 23644                                  | La Prele Reservoir; Converse County, WY  | 42.70679583°N, 105.5785312°W | 1  |
| <i>C. nuttallii</i> <sup>d</sup>                     | MEM 620                            | GREE 23646                                  | Hell Canyon Road; Custer County, SD      | 43.727663°N, 103.847306°W    | 20 |
| <i>C. nuttallii</i> <sup>d</sup>                     | MEM 631                            | GREE 23647                                  | Shirley Basin; Carbon County, WY         | 42.341401°N, 106.411052°W    | 15 |
| <i>C. flexuosus</i> <sup>d</sup>                     | MEM 644                            | GREE 23645                                  | Big Gypsum Valley; San Miguel County, CO | 38.068645°N, 108.791213°W    | 10 |
| <i>C. kennedyi</i> var. <i>kennedyi</i> <sup>e</sup> | Lehto, Hensel & Pinkava 11039      | GREE 20646                                  | Oracle; Pima County, AZ                  | NA                           | 1  |
| <i>C. subalpinus</i> <sup>e</sup>                    | L. E. Detling 3581                 | GREE 20658                                  | Gold Hill; Lane County, OR               | 44.1687015°N, 122.4452742°W  | 1  |

Note: N = number of individuals; NA = not available.  
<sup>a</sup>MEM = Mitchell E. McGlaughlin; RSF = Ryan S. Fuller.  
<sup>b</sup>Vouchers deposited at the University of Northern Colorado Herbarium (GREE), Greeley, Colorado, USA.  
<sup>c</sup>Locality, county, and state in USA (AZ = Arizona; CO = Colorado; SD = South Dakota; WY = Wyoming).  
<sup>d</sup>Fresh tissue used in DNA extractions, microsatellite population statistics, and cross-species amplifications.  
<sup>e</sup>Herbarium specimen tissue used in cross-species amplifications.

APPENDIX 2. Cross-species amplification information for 13 microsatellite loci developed for *Calochortus gunnisonii* with four related *Calochortus* species.

| Locus   | <i>C. flexuosus</i> | <i>C. nuttallii</i> | <i>C. kennedyi</i> var. <i>kennedyi</i> | <i>C. subalpinus</i> |
|---------|---------------------|---------------------|-----------------------------------------|----------------------|
| CAGU_14 | —                   | —                   | —                                       | —                    |
| CAGU_15 | —                   | +                   | +                                       | +                    |
| CAGU_22 | +                   | +                   | +                                       | —                    |
| CAGU_31 | —                   | +                   | +                                       | —                    |
| CAGU_35 | +                   | +                   | +                                       | +                    |
| CAGU_36 | +                   | +                   | +                                       | +                    |
| CAGU_39 | +                   | +                   | +                                       | —                    |
| CAGU_42 | +                   | +                   | +                                       | +                    |
| CAGU_45 | —                   | +                   | —                                       | —                    |
| CAGU_46 | —                   | +                   | —                                       | —                    |
| CAGU_47 | —                   | —                   | +                                       | —                    |
| CAGU_48 | +                   | +                   | —                                       | —                    |
| CAGU_50 | +                   | +                   | —                                       | —                    |

Note: + = amplification product fell within the product size range for the locus; — = amplification product was absent or did not fall within the product size range expected for the locus.