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DEVELOPMENT OF HIGHLY VARIABLE MICROSATELLITE MARKERS FOR THE TETRAPLOID *Silene stellata* (CARYOPHYLLACEAE)

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There are nearly 700 species in the genus *Silene* L. (Caryophyllaceae). *Silene* species exhibit diverse pollination specialization as manifested by their floral diversity (Kephart et al., 2006). Approximately one third of all *Silene* species in the Old and New World exhibit nocturnal pollination syndromes and usually form close interactions with noctuid moths from the genus *Hadena* (Noctuidae) (Kephart et al., 2006). *Silene stellata* (L.) W. T. Aiton is an infrequent native perennial herb that is distributed throughout the eastern part of the United States. The flowers are pollinated by *Hadena ectypa* as well as by a number of generalist nocturnal moths (Reynolds et al., 2009; Kula et al., 2013). In addition to the positive effect of pollination, oviposition by female *H. ectypa* inside the calyx results in strong negative effects through larval predation on the reproductive tissues of *S. stellata* (Kula et al., 2013). Depending on the amount of pollination service provided by the generalist moths, the net outcome of the *Silene–Hadena* interaction can range from mutualism to parasitism (Reynolds et al., 2012), making it a valuable system for understanding the evolutionary dynamics of interspecific interactions (Kephart et al., 2006; Bernasconi et al., 2009).

Ecological and evolutionary studies utilizing genetic markers of North American *Silene* species are relatively few compared with the European species (Moyle, 2006), potentially due to technical complications caused by the prevalent polyploidy: most North American *Silene* species have been shown to be polyploids, including tetra-, hexa-, and octoploids, with tetraploidy being the most common ploidy level (Popp and Oxelman, 2007). Prior to this study, we tested nine microsatellite markers developed for *S. latifolia* Poir. (Magalhaes et al., 2011) in 20 individuals of *S. stellata* and did not identify any polymorphic loci. We report the development of 18 highly variable microsatellite markers for the tetraploid *S. stellata*. These markers are being used to quantify the population genetic structure of *S. stellata*. We will also use these markers to assess individual siring abilities through paternity assignment to investigate selection on floral design through the male function of the hermaphroditic *S. stellata*. We also tested transferability of these markers to two closely related tetraploid *Silene* species: the specialist hummingbird-pollinated *S. virginica* L. and the primarily Bombus- and hawk moth–pollinated *S. caroliniana* Walter (Reynolds et al., 2009).

METHODS AND RESULTS

Genomic DNA was extracted from fresh leaf tissue of a *S. stellata* individual collected from a natural population near Mountain Lake Biological Station in Giles County, Virginia, USA (37.348296°N, 80.544301°W, elevation ca. 1100–1300 m; Appendix 1), using the DNeasy Plant Mini Kit (QIAGEN, Valencia, California, USA) following the manufacturer’s protocol. DNA libraries were prepared using the KAPA Library Preparation Kit version 2.13 (KR0453; Kapa Biosystems, Wilmington, Massachusetts, USA) following the manufacturer’s protocol. Libraries were quantified with a Qubit Fluorometric Quantitation instrument (Life Technologies, Carlsbad, California, USA) and then sequenced using an Illumina MiSeq version 3 kit (Illumina, San Diego, California, USA) to produce paired-end reads of ≤301 bases. A total of 8,557,438 reads were imported and paired by name in Geneious 7.0.6 (Biomatters, Auckland, New Zealand).

**Key words**: Caryophyllaceae; microsatellites; plant–animal interaction; polyploidy; *Silene stellata*.
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1. Characteristics of 18 microsatellite loci developed in Silene stellata.

| Locus     | Primer sequences (5'-3') | Fluorescent dye | Repeat motif | Allele size range (bp) | T_a (°C) | GenBank accession no. |
|-----------|--------------------------|-----------------|--------------|------------------------|----------|-----------------------|
| SS1010    | F: ACACAGCGGATGTTGTCG    | HEX             | (AG)_{36}    | 174–248                | 60       | KX712873              |
|           | R: CTCTTTTCATATTCGCCC    |                 |              |                        |          |                       |
| SS62979   | F: CTTGTTGCTGCATCTCTTCG | HEX             | (AG)_{60}    | 155–239                | 55       | KX712874              |
|           | R: TGTGTTGCTGCATCTCTTCG |                 |              |                        |          |                       |
| SS63719   | F: GTACGGCTGGCTGTTTATAC | FAM             | (AT)_{16}    | 135–154                | 58       | KX712875              |
|           | R: TGATACTGCTACCGGATGC  |                 |              |                        |          |                       |
| SS64450   | F: CAATGTTGATGACGGCAAAG | FAM             | (AT)_{31}    | 220–254                | 60       | KX712876              |
|           | R: ACCGTAACCTGACTGGGTCG |                 |              |                        |          |                       |
| SS64692   | F: TGATACCTCATTAGACTGCG | HEX             | (AAAT)_{14}  | 148–265                | 60       | KX712877              |
|           | R: CCTTCTGAGCCCTGCCTTC  |                 |              |                        |          |                       |
| SS68587   | F: TCAATGCTAAGGCTGCTGGG | HEX             | (AAC)_{15}   | 191–281                | 55       | KX712878              |
|           | R: TTGGTGTTGGAATGAAATTGG |                 |              |                        |          |                       |
| SS71250   | F: TACTGAGGGTCCGGAATTGG | FAM             | (AT)_{14}    | 125–203                | 58       | KX712879              |
|           | R: TTTCCTCAACATCTCAGCCTC |                 |              |                        |          |                       |
| SS73434   | F: GAGACAGGAGAGACGGGTTG | FAM             | (AC)_{10}    | 184–210                | 60       | KX712880              |
|           | R: TGACCCAGAGACCATCTAGTGGG |             |              |                        |          |                       |
| SS78777   | F: GGTACAACTAGCATGGG    | HEX             | (AC)_{16}    | 156–248                | 60       | KX712881              |
|           | R: TCGAGGCTATAGTAGGGCCAC |                |              |                        |          |                       |
| SS85007   | F: TACACCTCCACATCAAGGGC | FAM             | (AAAT)_{14}  | 149–356                | 55       | KX712882              |
|           | R: AGATTGTTCTCTGGCTCCC  |                 |              |                        |          |                       |
| SS89178   | F: TCACTACCCCACCTTCTAGC | FAM             | (AG)_{8}     | 254–374                | 55       | KX712883              |
|           | R: ATCCCCATAGCTGCCGACG |                 |              |                        |          |                       |
| SS100056  | F: GCCCTTGCTGCTGCCAGC   | FAM             | (AAG)_{37}   | 91–205                 | 60       | KX712884              |
|           | R: GGCTATCTCTGACCTCCACTC |                |              |                        |          |                       |
| SS115569  | F: CCCAGCTACTCCCAAGCAATC | FAM             | (AAC)_{11}   | 377–399                | 55       | KX712885              |
|           | R: ACCCAACCAAGAAATCAACAC |                |              |                        |          |                       |
| SS122722  | F: ACAATGTTGTTCTGAGTGCG | HEX             | (AAAT)_{15}  | 176–287                | 60       | KX712886              |
|           | R: ATTTATCTCTGCGCTCCAC  |                 |              |                        |          |                       |
| SS129528  | F: AGTGACCGGATCATCAGGAG | HEX             | (AAAT)_{13}  | 96–226                 | 60       | KX712887              |
|           | R: CATCATACTGCTCTCTTCTC |                 |              |                        |          |                       |
| SS131968  | F: ACCCAACATAGCATCACTCTC | FAM             | (AAAT)_{16}  | 154–205                | 55       | KX712888              |
|           | R: GAGCGGTTGGAATGATGTTG |                 |              |                        |          |                       |
| SS131990  | F: TCAGCTACCCCACTTCTGTCG | HEX             | (AT)_{19}    | 186–282                | 55       | KX712889              |
|           | R: CCCAACCACTTAACTGACACC |                |              |                        |          |                       |
| SS141347  | F: GGAGAGAAATGGAGGCTGTC | HEX             | (ACC)_{14}   | 132–192                |          | KX712890              |
|           | R: GGAGACATCGACGGACGCGT |                 |              |                        |          |                       |

Note: T_a = annealing temperature.

*All values are based on 95 samples representing three populations in Virginia.

New Zealand). A de novo assembly was performed on the first 1,000,000 sequences for which both reads of any pair were ≥200 bases.

A total of 99,506 consensus sequences between 200 and 400 bp were extracted and screened for potential microsatellite loci using MSATCOMMANDER 1.0.8 beta (Faircloth, 2008) with default settings. Primers were designed for sequences with perfect di-, tri-, and tetranucleotide repeats in Primer3 software (Rozen and Skaletsky, 1999). We designed primers for 153 out of a total of 946 loci identified as containing microsatellites. One hundred fourteen primer pairs were first tested with seven randomly selected S. stellata samples for amplification success and polymorphism. For each primer pair, we modified the forward primer by attaching a CAG tag (5’-CAGTCGGGCGTCATCA-3’) preceding the 5’ end to enable the cost-efficient fluorescent labeling system of PCR products described by Boutin-Ganache et al. (2001) and Glenn (2001). Ten microtiter PCR reactions were performed using the QIAGEN Type-it Microsatellite PCR Kit. Each reaction contained the following components: -10 ng of genomic DNA, 5 µL of the 2x Multiplex PCR Master Mix, 0.02 µM of the modified forward primer, 0.2 µM of reverse primer, and 0.2 µM of the fluorescently labeled CATG primer (5’-6FAM, 5’-HEX). A touchdown PCR protocol was used to test all primer pairs: 5 min of denaturing at 95°C; five cycles of 95°C for 30 s, 60°C for 1.5 min, and 72°C for 30 s; followed by 28 cycles of 95°C for 30 s, 55°C for 1.5 min, and 72°C for 30 s; and a final extension at 60°C for 30 min. Of the 114 primer pairs tested, 50 produced bands consistently on an agarose gel. Amplicons of these primers were analyzed using an ABI Prism 3730 Genetic Analyzer (Applied Biosystems, Foster City, California, USA), then visualized and scored in Geneious 7.0.6 (Biomatters). Eighteen loci showed clear peak patterns and were polymorphic (Table 1). These 18 polymorphic loci were further characterized using a total of 95 S. stellata individuals collected from three local populations near Mountain Lake Biological Station (Meadow, Woodland, and Windrock) within 8 km of one another. To investigate marker transferability, these 18 loci were also tested on six individuals from one population each of S. virginica from Newport, Virginia, and S. caroliniana from Potomac, Maryland (Appendix 1).

We report the following parameters for the three populations of S. stellata: sample size, number of alleles, number of private alleles, observed heterozygosity, and expected heterozygosity. The parameters were estimated using GenoDive version 2.0b27 (Meirmans and Van Tienderen, 2004) while correcting for unknown dosage of alleles for partial heterozygotes. Across the three populations, the number of alleles ranged from six to 45, and expected heterozygosity ranged from 0.511 to 0.951 (Table 2).

Of the 18 loci tested in S. virginica and S. caroliniana, 10 loci were successfully amplified in both species. Genotyping results showed two loci were monomorphic in both species, one locus was polymorphic in S. virginica but monomorphic in S. caroliniana, five loci were polymorphic in both species, and two loci showed multiple bands (Table 3). Vouchers for the Silene species were deposited at the Norton-Brown Herbarium (University of Maryland, College Park, Maryland, USA; Appendix 1).

CONCLUSIONS

We developed 18 novel microsatellite loci for S. stellata. These loci showed high variability in S. stellata and therefore are suitable for future paternity analysis. Five of these markers are polymorphic in the related S. virginica and S. caroliniana. These microsatellites will also be useful for studying the
### Table 2. Genetic diversity of the 18 polymorphic microsatellites of Silene stellata.4

| Locus         | Total (n = 95) | Meadow (n = 55) | Windrock (n = 20) | Woodland (n = 20) |
|---------------|---------------|----------------|-------------------|-------------------|
|               | A | H0 | Hp | A | A0 | H0 | Hp | A | A0 | H0 | Hp |
| SS1010        | 31 | 0.847 | 0.894 | 28 | 10 | 0.824 | 0.903 | 17 | 0 | 0.840 | 0.912 | 19 | 2 | 0.886 | 0.876 |
| SS62979       | 36 | 0.828 | 0.913 | 28 | 12 | 0.664 | 0.925 | 17 | 4 | 0.823 | 0.918 | 15 | 4 | 0.996 | 0.894 |
| SS63719       | 18 | 0.468 | 0.877 | 14 | 2 | 0.527 | 0.858 | 12 | 2 | 0.364 | 0.891 | 12 | 2 | 0.512 | 0.885 |
| SS64450       | 15 | 0.486 | 0.712 | 10 | 4 | 0.393 | 0.626 | 7 | 2 | 0.474 | 0.724 | 9 | 3 | 0.591 | 0.782 |
| SS64692       | 38 | 0.707 | 0.906 | 29 | 11 | 0.658 | 0.909 | 17 | 4 | 0.712 | 0.879 | 19 | 4 | 0.737 | 0.915 |
| SS68587       | 27 | 0.866 | 0.932 | 25 | 5 | 0.883 | 0.931 | 17 | 1 | 0.835 | 0.932 | 16 | 1 | 0.879 | 0.933 |
| SS71250       | 26 | 0.999 | 0.918 | 24 | 4 | 0.999 | 0.921 | 18 | 1 | 0.989 | 0.912 | 18 | 1 | 1.010 | 0.922 |
| SS73434       | 13 | 0.210 | 0.875 | 10 | 2 | 0.305 | 0.890 | 7 | 2 | 0.154 | 0.842 | 9 | 0 | 0.172 | 0.889 |
| SS78777       | 34 | 0.892 | 0.919 | 33 | 12 | 0.975 | 0.931 | 17 | 0 | 0.770 | 0.911 | 17 | 1 | 0.933 | 0.915 |
| SS80007       | 45 | 0.621 | 0.921 | 38 | 17 | 0.716 | 0.922 | 18 | 3 | 0.585 | 0.926 | 20 | 3 | 0.555 | 0.902 |
| SS90178       | 37 | 0.826 | 0.887 | 26 | 9 | 0.903 | 0.866 | 19 | 5 | 0.677 | 0.866 | 18 | 3 | 0.931 | 0.951 |
| SS100056      | 35 | 0.821 | 0.951 | 33 | 8 | 0.799 | 0.961 | 22 | 1 | 0.883 | 0.943 | 21 | 1 | 0.781 | 0.948 |
| SS115569      | 6 | 0.394 | 0.511 | 6 | 4 | 0.458 | 0.551 | 2 | 0 | 0.397 | 0.538 | 2 | 0 | 0.328 | 0.442 |
| SS122722      | 38 | 0.733 | 0.944 | 31 | 9 | 0.807 | 0.957 | 18 | 4 | 0.547 | 0.916 | 19 | 2 | 0.856 | 0.959 |
| SS129528      | 35 | 0.896 | 0.937 | 29 | 7 | 0.825 | 0.924 | 20 | 3 | 0.982 | 0.933 | 23 | 2 | 0.883 | 0.954 |
| SS131968      | 18 | 0.779 | 0.761 | 17 | 3 | 0.699 | 0.730 | 14 | 1 | 0.760 | 0.704 | 11 | 0 | 0.869 | 0.844 |
| SS131990      | 30 | 0.440 | 0.939 | 27 | 8 | 0.458 | 0.937 | 15 | 0 | 0.407 | 0.943 | 15 | 3 | 0.455 | 0.937 |
| SS141347      | 17 | 0.993 | 0.891 | 16 | 2 | 0.998 | 0.884 | 15 | 0 | 0.989 | 0.895 | 14 | 1 | 0.990 | 0.891 |

Note: A = number of alleles; A0 = number of private alleles; Hp = expected heterozygosity; H0 = observed heterozygosity; n = number of individuals sampled.

*Locality and voucher information are provided in Appendix 1.

### Table 3. Genetic diversity of six microsatellite loci developed in Silene stellata in two related native Silene species.4

| Locus         | S. virginia (n = 6) | S. caroliniana (n = 6) |
|---------------|---------------------|------------------------|
|               | Allele size range (bp) | A | H0 | Hp | Allele size range (bp) | A | H0 | Hp |
| SS6492        | 154–196             | 7 | 1 | 0.94 | 151–214 | 8 | 1 | 0.861 |
| SS68587       | 188–191             | 2 | 0.5 | 0.369 | 161–191 | 5 | 0.667 | 0.614 |
| SS12150       | 113–129             | 5 | 0.833 | 0.754 | 117–165 | 6 | 1 | 0.662 |
| SS78777       | 198–254             | 11 | 0.833 | 0.969 | 184–244 | 12 | 1 | 0.939 |
| SS131968a     | —                   | — | — | — | 157–169 | 3 | 0.333 | 0.489 |
| SS141347      | 144–180             | 9 | 0.5 | 0.867 | 147–168 | 7 | 1 | 0.826 |

Note: A = number of alleles; Hp = expected heterozygosity; H0 = observed heterozygosity; n = number of individuals sampled.

*Locality and voucher information are provided in Appendix 1.

bLocus SS131968 was monomorphic in S. virginica.
**APPENDIX 1. Geographic location and voucher information of *Silene* populations used in this study.**

| Species         | Location         | Population | Geographic coordinates | Voucher no.  |
|-----------------|------------------|------------|------------------------|--------------|
| *S. stellata* (L.) W. T. Aiton | Newport, VA, USA | Meadow     | 37.348296°N, 80.544301°W | MARY1012978  |
| *S. stellata*   | Newport, VA, USA | Meadow     | 37.348296°N, 80.544301°W | MARY1012979  |
| *S. stellata*   | Newport, VA, USA | Windrock   | 37.413889°N, 80.519444°W | MARY1012974  |
| *S. stellata*   | Newport, VA, USA | Windrock   | 37.413889°N, 80.519444°W | MARY1012975  |
| *S. stellata*   | Newport, VA, USA | Woodland   | 37.355415°N, 80.553469°W | MARY1012977  |
| *S. stellata*   | Newport, VA, USA | Woodland   | 37.355415°N, 80.553469°W | MARY1012978  |
| *S. caroliniana* Walter | Potomac, MD, USA | Great Falls | 38.989511°N, 77.246713°W | MARY1012980  |
| *S. caroliniana* | Potomac, MD, USA | Great Falls | 38.989511°N, 77.246713°W | MARY1012981  |
| *S. virginica* L. | Newport, VA, USA | Windrock   | 37.413889°N, 80.519444°W | MARY1012982  |
| *S. virginica*  | Newport, VA, USA | Windrock   | 37.413889°N, 80.519444°W | MARY1012983  |

*Herbarium vouchers were deposited at the University of Maryland Herbarium (MARY).*  
*Individual used for DNA extraction.*