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Authors: Jonathan Kissling, Olivier Bachmann, Marco R. Thali, and José Gabriel Segarra-Moragues

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**PRIMER NOTE**

**NOVEL MICROSATELLITE LOCI FOR *SEBAEA AUREA* (GENTIANACEAE) AND CROSS-AMPLIFICATION IN RELATED SPECIES**

**JONATHAN KISSLING**<sup>2,3,6</sup>, **OLIVIER BACHMANN**<sup>2</sup>, **MARCO R. THALI**<sup>4</sup>, and **JOSÉ GABRIEL SEGARRA-MORAGUES**<sup>5</sup>

<sup>2</sup>Institute of Biology, University of Neuchâtel, Rue Emile-Argand 11, 2000 Neuchâtel, Switzerland; <sup>3</sup>Department of Ecology and Evolution, University of Lausanne, 1015 Lausanne, Switzerland; <sup>4</sup>Ecogenics GmbH, Grabenstrasse 11a, 8952 Zürich-Schlieren, Switzerland; and <sup>5</sup>Centro de Investigaciones sobre Desertificación (CIDE-CSIC-UV-GV), Carretera de Moncada-Náquera, Km 4.5, E-46113 Moncada, Valencia, Spain

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*Sebaea aurea* (L. f.) Roem. & Schult. is a diploid (*2n = 28*; Kissling et al., 2008) annual herb found in the Western Cape (South Africa). *Sebaea aurea* is a diplostigmatic species, with styles showing two stigmatic areas, one each at the apex and base of the styles (see Kissling et al., 2009a). This character is suspected to provide reproductive assurance and reduce seed discounting (i.e., the formation of self-fertilized seeds from ovules that, if they had not been self-fertilized, would have been cross-fertilized) and, therefore, might provide some evolutionary advantages (Kissling et al., 2009b).

To date, no polymorphic microsatellite markers have been developed in this species or in other species for which microsatellites could be potentially transferred to *S. aurea*. Therefore, the purpose of this study was to isolate and characterize microsatellite loci in *S. aurea* to conduct reproductive biology experiments and to estimate the selfing rate in a progeny array, which will contribute to a better understanding of the functional role of diplostigamy. Furthermore, this set of microsatellite loci will contribute to the investigation of the distribution of genetic diversity across the species range.

**METHODS AND RESULTS**

Microsatellite isolation was carried out through two independent 454 pyrosequencing strategies. First, DNA was extracted from 20 individuals of *S. aurea* from the Helderberg Nature Reserve population (Appendix 1) using the DNeasy Plant Mini Kit (Qiagen, Hombrechtikon, Switzerland) following the manufacturer instructions and then pooled and concentrated. Five hundred nanograms of total DNA was used to construct 454 genomic libraries by the sequencing service from the University of Valencia (Servicio Central de Soporte a la Investigación Experimental [SCSIE], Valencia, Spain) and shotgun sequenced on a GS Junior 454 sequencer (454 Life Sciences, Roche Company, Barcelona, Spain). We obtained 120,157 reads with an average read length of 468.43 bp that were trimmed of adapter and low-quality regions and assembled into contigs using GS De Novo Assembler implemented in Newbler 2.5p1 (454 Life Sciences, a Roche Company, Madrid, Spain). Generated contigs and unique reads not assigned to contigs were subjected to BLAST analysis, and those matching organellar (chloroplast or mitochondria) sequences were discarded.

We screened all 64,422 unique reads and nonorganellar contigs with iQDD version 1.3.0.0 software (Meglécz et al., 2010). We set the script to identify all possible di-, tri-, tetra-, penta-, and hexanucleotide repeats with a minimum of five repeat units, as well as compound primers, and to directly design primers using Primer3 (Rozen and Skaletsky, 2000). After discarding reads with too short flanking sequences, primers were successfully designed for 174 reads. Of these 73, 90, and 11 corresponded to di-, tri-, and tetranucleotide repeats, respectively.

Additionally, size-selected fragments from genomic DNA were enriched for microsatellites by Ecogenics GmbH (Zürich-Schlieren, Switzerland) using streptavidin-coated magnetic beads and biotin-labeled (CT)13 and (GT)13, repeat

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6 Author for correspondence: jonathan.kissling@unine.ch

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probes. The simple sequence repeat (SSR)–enriched library was sequenced on a Roche 454 platform using the GS FLX Titanium reagents at Ecogenics GmbH. The 34,992 reads sequenced had an average length of 427 bp. Of these, 996 contained a microsatellite repeat with a tetra- or a trinucleotide motif of at least six repeat units, or a dinucleotide motif of at least 10 repeat units. Suitable primer design using Primer3 (Rozen and Skaletsky, 2000) was possible in 180 reads. Thirty-nine and 36 loci from the first and second sequencing strategies, respectively, were tested for functionality and polymorphism using at least seven individuals.

Amplifications were carried out in 10-μL reactions containing 1–3 ng of template DNA, 1x GoTaq Flexi Buffer (Promega Corporation, Neuchâtel, Switzerland), 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.04 μM forward primer with a 5′ M13 tail, 0.16 μM F6-labeled M13 primer, 0.16 μM reverse primer, and 0.5 U HotStarTaq DNA Polymerase (QIAGEN). PCRs were performed in a Dyad Cycler (Bio-Rad GmbH, Cressier, Neuchâtel, Switzerland), and the PCR cycling conditions consisted of an initial activation step of 5 min at 95°C; followed by 30 cycles each of 45 s at 95°C, 60 s at 55°C, and 60 s at 72°C; eight cycles each of 45 s at 95°C, 60 s at 53°C, and 60 s at 72°C; and a final extension step of 30 min at 72°C. PCR products were run and sized as in the aforementioned pilot study.

We detected a total of 164 different SSR alleles for the 12 polymorphic microsatellite loci in the three analyzed populations of *S. aurea*. The number of alleles ranged from a minimum of three alleles for locus Seba19849 to a maximum of 29 alleles for locus Seba13, and the mean number of alleles per locus was 13.67 ± 7.13. One locus (Seba19849) was monomorphic in the Somerset West population (Table 2). H₀ ranged from 0.0 (locus Seba28206) to 0.945 (locus Seba28206) (Table 2). Almost half of the loci in each population showed significant deviation from Hardy–Weinberg equilibrium toward heterozygote deficiency, whereas those remaining in each population showed either nonsignificant heterozygote deficiency or excess. Global population inbreeding estimates gave significant heterozygote deficiency in all three populations. A likely explanation for this result may be the presence of null alleles. In fact, 17 out of the 36 loci per population comparison gave estimated frequencies of null alleles higher than 0.05. Nonetheless, local population substructure or high selling rates within populations could also result in high inbreeding coefficient (Fᵢ)'

### Table 1. Characteristics of 16 microsatellite loci developed in *Sebaea aurea*.

| Locus | Primer sequences (5′–3′)* | Repeat motif | Allele size (bp) | Tᵢ (°C) | Multiplex group | GenBank accession no. |
|-------|--------------------------|--------------|-----------------|----------|----------------|----------------------|
| Seba04 | F: VIC-ATCTCATCGTTACAGGCC | (AG)₁₀ | 130 | 56 | I | KF128835 |
| R: AATTTAGAAGGGTGCCG | | | | | | |
| Seba10 | F: PET-CTTTATTGGAACGGGGAAG | (CT)₄ | 135 | 56 | I | KF128836 |
| R: AATGATCCAATCATTGCC | | | | | | |
| Seba11 | F: 6FM-TGCCGTTGCTACGTTGAA | (CT)₄ | 136 | 56 | I | KF128837 |
| R: GCCATGATTTCCTACGCG | | | | | | |
| Seba13 | F: VIC-CGAAAGTACTGGTACATGAG | (GA)₈ | 317 | 56 | I | KF128838 |
| R: GGAAAGGGTGTTTTTATT | | | | | | |
| Seba17 | F: 6FM-AGTTGATACCGGACACATC | (AC)₇ | 256 | 56 | I | KF128839 |
| R: TCGTGAATGTTAGCTGGG | | | | | | |
| Seba21 | F: VIC-CTCTTGAAGGGCGCCCAG | (TG)₇ | 259 | 56 | I | KF128840 |
| R: GCAAACAGGTAGTGACAG | | | | | | |
| Seba05119 | F: NED-GCAGAATTTTACGCACAC | (GC)₇ | 227 | 56 | I | KF128845 |
| R: GAGACGGAGTACGAGAC | | | | | | |
| Seba11349 | F: VIC-ATGGGAGCAGGGTTTACTG | (TGC)₈ | 231 | 56 | I | KF128843 |
| R: TGACAGTGGCCCTATCATC | | | | | | |
| Seba12491 | F: ATGGTTTCAAGGAGCTGTTGG | (TTG)₉ | 248 | 56 | I | KF128847 |
| R: AGCTTTGAAAGGGATGCG | | | | | | |
| Seba19849 | F: 6FM-GTGCGGAAAAGATCTACGTTAG | (ATA)₁₂ | 174 | 56 | II | KF128841 |
| R: CCGGAAATGCTAGGACGAG | | | | | | |
| Seba28414 | F: NED-AGCTTTCGAGCGAGGATGAG | (TTG)₉ | 136 | 56 | II | KF128844 |
| R: ACTCTCTCTCAGCAAAAAAC | | | | | | |
| Seba09440 | F: CTTTACCTGTGCTCCTCCC | (GT)₁₂ | 232 | 56 | I | KF128848 |
| R: AGACGACTAATATACGCTGTCG | | | | | | |
| Seba11773 | F: GAAAGTGGCAGCGTCG | (GTG)₁₂ | 247 | 56 | II | KF128846 |
| R: CTTCAAGTCCGTGTTAGG | | | | | | |
| Seba28206 | F: PET-ACAACTGCAGTGGACTAC | (CT)₁₂ | 176 | 56 | II | KF128846 |
| R: CTGTGTTCTGGTATGAGG | | | | | | |
| Seba31211 | F: 6FM-CCATCTACCGGTAGCG | (CA)₁₅ | 250 | 56 | II | KF128842 |
| R: CTGTTGTTTACGTTGAGG | | | | | | |
| Seba31409 | F: GCAGAGTTAGGCGCATGAG | (CA)₁₅ | 241 | 56 | I | KF128850 |
| R: TCGCGATTAGCTGCGAG | | | | | | |

*Note:* NI = not included in multiplex groups; Tᵢ = annealing temperature.

*For each forward primer, the fluorescent label is indicated at the 5′ end.

Genotypic data were obtained from three populations of *S. aurea* (Helderberg Nature Reserve, N = 38; Somerset West, N = 25; Paradyskloof, N = 30; Table 2). Appendix 1) for the 12 microsatellite loci (Tables 1 and 2). Number of alleles (A), observed heterozygosity (Hₒ), and unbiased expected heterozygosity (Hₑ) (Nei, 1978) were calculated with GENETIX version 4.05 (Belkhir et al., 2004). Linkage disequilibrium between pairs of microsatellite loci and between pairs of microsatellite loci and populations, using 10,000 permutations, were calculated with GENEPOP version 4.1.4 software (Rousset, 2008). This same software was used to estimate the frequency and 95% confidence intervals of estimated null allele frequencies of each locus in each population (Dempster et al., 1977). Of the 186 available pairwise comparisons between loci and populations, only seven showed significant linkage disequilibrium (P < 0.05), whereas of the 66 available pairwise comparisons between loci only two showed significant linkage disequilibrium (P < 0.05). Of these, only the pair Seba1-Seba13 was consistent between the Helderberg and Paradyskloof populations and the global estimate of linkage disequilibrium; however, none of the pairwise comparisons were significant after Bonferroni correction.

http://www.bioone.org/loi/apps
Table 2. Results of initial primer screening of 12 polymorphic microsatellite loci in three populations of Sebaea aurea.\(^{ab}\)

| Locus   | Helderberg Nature Reserve (N = 38) | Somerset West (N = 25) | Paradykloof (N = 30) |
|---------|-----------------------------------|------------------------|----------------------|
|         | A                   | H\(_o\) | H\(_e\) | F\(_IS\) | A | H\(_o\) | H\(_e\) | F\(_IS\) | A | H\(_o\) | H\(_e\) | F\(_IS\) |
| Seba04  | 10 | 0.444 | 0.874 | 0.496\(^{***}\) | 7 | 0.200 | 0.858 | 0.776\(^{***}\) | 13 | 0.360 | 0.917 | 0.612\(^{***}\) |
| Seba10  | 5  | 0.658 | 0.693 | 0.052\(^{**}\)  | 5 | 0.720 | 0.764 | 0.059\(^{**}\)  | 6  | 0.633 | 0.688 | 0.080\(^{**}\)  |
| Seba11  | 11 | 0.528 | 0.847 | 0.380\(^{***}\) | 11 | 0.571 | 0.893 | 0.366\(^{**}\)  | 9  | 0.448 | 0.844 | 0.473\(^{***}\) |
| Seba13  | 16 | 0.868 | 0.775 | −0.122\(^{**}\) | 9  | 0.480 | 0.642 | 0.256\(^{**}\)  | 18 | 0.833 | 0.829 | −0.005\(^{**}\) |
| Seba17  | 6  | 0.067 | 0.756 | 0.915\(^{***}\) | 7  | 0.182 | 0.806 | 0.778\(^{***}\) | 10 | 0.296 | 0.855 | 0.638\(^{***}\) |
| Seba21  | 9  | 0.290 | 0.822 | 0.650\(^{***}\) | 8  | 0.160 | 0.777 | 0.797\(^{***}\) | 10 | 0.621 | 0.859 | 0.281\(^{**}\)  |
| Seba05119 | 20 | 0.278 | 0.824 | 0.669\(^{***}\) | 8  | 0.286 | 0.849 | 0.669\(^{***}\) | 8  | 0.280 | 0.813 | 0.660\(^{***}\) |
| Seba11349 | 6  | 0.800 | 0.736 | −0.090\(^{**}\) | 4  | 0.583 | 0.664 | 0.124\(^{**}\)  | 5  | 0.633 | 0.691 | 0.085\(^{**}\)  |
| Seba19849 | 2  | 0.050 | 0.050 | 0.000  | 1  | 0.000 | 0.000 | 0.000  | —  | 0.069 | 0.068 | −0.009\(^{**}\) |
| Seba28414 | 5  | 0.850 | 0.739 | −0.156\(^{**}\) | 8  | 0.792 | 0.823 | 0.039\(^{**}\)  | 6  | 0.500 | 0.774 | 0.358\(^{**}\)  |
| Seba28206 | 4  | 0.000 | 0.686 | 1.000\(^{***}\) | 6  | 0.227 | 0.610 | 0.635\(^{***}\) | 19 | 0.571 | 0.945 | 0.401\(^{***}\) |
| Seba3121 | 5  | 0.750 | 0.745 | −0.007\(^{**}\) | 5  | 0.522 | 0.693 | 0.251\(^{**}\)  | 7  | 0.536 | 0.684 | 0.220\(^{**}\)  |
| Average  | 7.17 | 0.465 | 0.712 | 0.353\(^{***}\) | 6.58 | 0.394 | 0.698 | 0.443\(^{***}\) | 9.50 | 0.482 | 0.747 | 0.360\(^{***}\) |

Note: A = number of alleles per locus; F\(_IS\) = inbreeding coefficient; H\(_e\) = unbiased expected heterozygosity; H\(_o\) = observed heterozygosity; N = number of individuals.

a See Appendix 1 for population locality information.

b Deviations from HWE were statistically significant at *P < 0.05, **P < 0.01, ***P < 0.001; ns = not significant.

values. The analysis of progeny arrays would be required to definitely rule out or confirm the presence of null alleles.

The two multiplex reactions were also tested on three to six individuals of seven different species of Sebaea (Appendix 1) to assess for potential cross-amplification of SSR loci. Cross-amplification was only successful in S. ambigua Cham. and in S. minutiflora Schinz. All 12 microsatellite loci amplified in these two species and were polymorphic. Both of these species belong to the S. aurea clade, which exclusively contains the tetramerous Sebaea (Kissing et al., unpublished data). No successful amplification was obtained in any of the five remaining species for the 12 assayed loci.

CONCLUSIONS

Twelve microsatellite loci were characterized to investigate the reproductive biology and population structure in S. aurea. These markers will serve to estimate outcrossing rates in progeny arrays and therefore help to understand the reproductive function of secondary stigmas in S. aurea. Cross-species transferability experiments to S. ambigua and S. minutiflora expand the usefulness of this set of SSR loci to other species of Sebaea.

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### APPENDIX 1

Species, collection locality, geographical coordinates, and herbarium voucher of species of *Sebaea* analyzed in this study. All specimens are deposited at the Université de Neuchâtel herbarium (NEU), Neuchâtel, Switzerland.

| Species                  | Location                                      | Geographical coordinates | Altitude (m) | Voucher no.     | N  |
|--------------------------|-----------------------------------------------|--------------------------|--------------|----------------|----|
| *S. ambigua* Cham.       | South Africa: Western Cape, Pringle Bay       | 34°22.627′S, 18°49.814′E  | 40           | Kissling and   | 4  |
| *S. aurea* (L. f.) Roem. & Schult. | South Africa: Western Cape, Helderberg Nature Reserve | 34°03′43″S, 18°52′24″E  | 139          | Zeltner 45/2005 |    |
|                          | South Africa: Western Cape, Somerset West     | 34°02′54″S, 18°50′00″E    | 212          | Kissling 33/2010 | 25 |
|                          | South Africa: Western Cape, Paradyskloof      | 33°58′06″S, 018°52′43″E   | 233          | Kissling 22/2010 | 30 |
| *S. exacoides* (L.) Schinz | South Africa: Western Cape, Franschoek Pass along R45 | 33°55.041′S, 19°09.518′E | 665          | Kissling and   | 4  |
|                          | South Africa: Eastern Cape, Naude’s Nek       | 30°43.125′S, 28°08.394′E  | 2513         | Wuerfel 6/2008 |    |
| *S. marlothii* Gilg      | South Africa: Western Cape, Helderberg Nature Reserve | 34°03.647′S, 18°52.258′E | 156          | Kissling 15/2010 | 4  |
| *S. micrantha* Schinz    | South Africa: Western Cape, Gordon’s Bay, unconstructed plot in front of BP station, along R44 | 34°08.513′S, 018°51.112′E | 3            | Kissling 14/2010 | 6  |
| *S. minutiflora* Schinz  | South Africa: Western Cape, Pringle Bay       | 34°22.627′S, 018°49.814′E | 4            | Kissling and   | 4  |
| *S. spathulata* Steud.   | Lesotho: road to Sehlabathebe lodge            | 29°52.692′S, 029°05.342′E | 2599         | Zeltner 46/2005 | 3  |
| *S. thomasii* Schinz     | Lesotho: ca. 1 km from Schelabathebe lodge     | 29°52.058′S, 029°06.966′E | 2461         | Kissling and   | 3  |
|                          |                                               |                          |              | Zeltner 29/2005 |    |

*Note:* *N* = number of samples analyzed.