MICROSATELLITE PRIMERS FOR THE GYNO DioECIOUS GRASSLAND PERENNIAL Saxifraga granulata (Saxifragaceae) 1

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• Premise of the study: Nine polymorphic and 12 monomorphic microsatellite loci (simple sequence repeats [SSRs]) were isolated and characterized for the gynodioecious grassland perennial Saxifraga granulata.

• Methods and Results: Based on genomic screening of leaf material of four individuals from four populations, a total of 21 microsatellite primer pairs were designed for S. granulata. Nine loci were polymorphic and were optimized into two PCR multiplex reactions and tested on 100 individuals from five riparian populations from central Belgium. The number of alleles of the polymorphic loci ranged from three to 18, and gametic heterozygosity ranged from 0.26 to 0.94.

• Conclusions: The markers that are presented here are the first microsatellite markers reported for S. granulata and will be used to assess how river systems shape the spatial genetic structure and diversity of riparian populations of this species.

Key words: gynodioecy; heterozygosity; polyploidy; Saxifraga granulata; Saxifragaceae; simple sequence repeat (SSR).

The genus Saxifraga L. consists of about 400 species that are mainly distributed across the arctic and northern temperate zones (Cornell, 1987). Species within this genus are morphologically very diverse and occur in a wide range of habitats, including grasslands, woodland margins, tundra vegetation, and rocky slopes. Saxifraga granulata L. is an insect-pollinated, perennial, rosette-forming herb that can reproduce sexually and clonally, by formation of small bulbils at the base of the plant, and has been described as being gynodioecious (Stevens and Richards, 1985; Stevens, 1988). Saxifraga granulata mainly occurs in mesic to dry grasslands in Western Europe and North Africa (Andersson, 1996). In Belgium, most populations can be found in riparian meadows and grasslands along river systems. In recent decades, many populations throughout Europe have become smaller and more isolated due to habitat loss and fragmentation (Walisch et al., 2012). Because of the species’ close association with riparian habitats in Belgium, rivers can be expected to be important in maintaining genetic connectivity of increasingly isolated populations. The nine polymorphic microsatellite markers presented here will be used to assess how rivers affect levels of gene flow and consequently shape the genetic diversity and structure of riparian plant populations, and to estimate whether levels of gene flow between populations are sufficient to maintain genetic diversity within populations.

METHODS AND RESULTS

Leaf samples of four individuals from four different populations were collected during the flowering season of 2012. All populations were located in central Belgium, were at least 1 km and at most 11 km apart, and contained more than 300 individuals. DNA was extracted from ~20 mg of dried plant material that was homogenized to a fine powder using a grinder (Mini Bead-Beater-16, BioSpec Products, Bartlesville, Oklahoma, USA) and 10 small ceramic beads (MagNA Lyser Green Beads, Roche, Basel, Switzerland). Genomic DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany), and DNA concentration and quality were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA). The purified genomic DNA of four individuals from different populations, was mixed in equimolar ratio and used for further analyses. The DNA was prepared for an Illumina paired-end (PE) shotgun library according to the manufacturer’s guidelines (Illumina, San Diego, California, USA) and run on a HiSeq 2000 system (Illumina). The reads were imported in PAL Finder version 0.02.04 software (Castoe et al., 2012) to extract simple sequence repeats (SSRs) and to develop primer pairs for amplification. The sequence data generated in this study has been deposited at the National Center for Biotechnology Information (NCBI) in the Sequence Read Archive (SRA) database (accession no.: SRX651652). Forty primer pairs were designed (two with tetra-, 16 with tri-, and 22 with dinucleotide repeats) and tested for amplification quality and polymorphism. A total of 21 loci, nine polymorphic and 12 monomorphic, were selected after amplification in 25 individuals from five populations (Table 1). Sampled individuals were at least 1 m apart to avoid collecting clones. The nine polymorphic primer pairs were then optimized into two PCR multiplex reactions and further tested on 100 individuals from five populations collected along the Dyle River in central Belgium (Table 2). Both PCR multiplexes were performed in a 2720 Thermal Cycler (Applied Biosystems, Carlsbad,
from 46 to 60 chromosomes, assuming a basic number of 52 chromosomes, and older data give chromosome numbers ranging from a maximum of eight different alleles. At loci Saxgra-09, Saxgra-10, Saxgra-16, Saxgra-22, Saxgra-33, and Saxgra-38, genotypes could be correctly identified. The data were manually constructed, and all data were visually checked to make sure that the genetic data were scored using GeneMapper software version 4.0 (Applied Biosystems). The raw fragments were sized on an ABI Prism and analyzed by capillary electrophoresis using the 3130-Avant Genetic Analyzer (Applied Biosystems). The raw data were analyzed using GenoDive 2.0 (Meirmans and van Tienderen, 2004). Three measures of genetic diversity (Nei, 1987) were calculated and corrected for unknown dosage of alleles: the number of alleles, the effective number of alleles, and gametic heterozygosity (Hg). Genetic diversity of the microsatellite loci studied in five populations of Saxifraga granulata is shown in Table 2. Gametic heterozygosity was high. The number of alleles varied between three and 13.6 (mean = 4.0; Table 2) and the number of effective alleles (i.e., the number of alleles in a population weighted for their frequencies) varied between 1.3 and 13.6 (mean = 4.0; Table 2). Gametic heterozygosity, which is equivalent to the expected heterozygosity in diploid species (Meirmans and Hedrick, 2011), varied between 0.26 (at locus Saxgra-16) and 0.94 (at locus Saxgra-06; Table 2). Six out of nine loci (Saxgra-06, Saxgra-10, Saxgra-16, Saxgra-22, Saxgra-23, and Saxgra-38) showed significant negative deviations from Hardy–Weinberg proportions.

Table 1. Characterization of 21 microsatellite loci developed for Saxifraga granulata.

| Locus   | Primer sequences (5′-3′) | Repeat motif | Allele size range (bp) | A Tc (°C) | Multiplex Fluorescent label | GenBank accession no. |
|---------|-------------------------|--------------|------------------------|----------|------------------------------|-----------------------|
| Saxgra-01 | F: CGCTTAATCTTGTGCAATCTCGG | (TTC)24 | 140 – 154 | 1 54 | (TTC)24 | KJ187803 |
|         | R: TTTGAAAGCTGAGCAAACGCG |             |           | 1 54 | TTTGAAAGCTGAGCAAACGCG | KJ187804 |
| Saxgra-02 | F: CAATACATATATAATAGAAGTACGACCC | (TTC)24 | 100 – 154 | 1 54 | (TTC)24 | KJ187805 |
|         | R: AAGTATGACCTCAACGATTCTGGG |             |           | 1 54 | AAGTATGACCTCAACGATTCTGGG | KJ187806 |
| Saxgra-03 | F: GCACACCTTAAAGCTCGGACG | (TC)24(TC)12 | 145 – 154 | 1 54 | (TC)24(TC)12 | KJ187807 |
|         | R: ACCCAACACCTTCTGCCCC |             |           | 1 54 | ACCCAACACCTTCTGCCCC | KJ187808 |
| Saxgra-04 | F: AAAATGCTAAGCTAAAAGACTGTC | (GA)14 | 245 – 154 | 1 54 | (GA)14 | KJ187809 |
|         | R: ATGATGTAAGCTAAGTGGG |             |           | 1 54 | ATGATGTAAGCTAAGTGGG | KJ187810 |
| Saxgra-06 | F: TTTCACTCTTGGAATGTAGTTAATGC | (GT)36 | 235 – 287 | 18 54 | (GT)36 | VIC | KF680947 |
|         | R: CACTGTGATATCTGGCTAAGAACC |             |           | 1 54 | CACTGTGATATCTGGCTAAGAACC | VIC | KF680948 |
| Saxgra-09 | F: AGGTAGATCTTTGGAAGCGT | (AG)26 | 284 – 314 | 8 54 | (AG)26 | NED | KF680949 |
|         | R: CTCACACCGTTGATGAGG |             |           | 1 54 | CTCACACCGTTGATGAGG | KJ187807 |
| Saxgra-10 | F: AGGTAGAAGCCCTGCAATTCGG | (ACT)31 | 158 – 191 | 4 58 | (ACT)31 | VIC | KF680950 |
|         | R: TGTATCTCCAGTGATTGCCACG |             |           | 1 54 | TGTATCTCCAGTGATTGCCACG | KJ187808 |
| Saxgra-11 | F: CTAATCAATTTCAATCTATACC | (GTG)31 | 320 – 154 | 1 54 | (GTG)31 | PET | KF680951 |
|         | R: CTTATTAAAGTTGACGACCC |             |           | 1 54 | CTTATTAAAGTTGACGACCC | KJ187809 |
| Saxgra-16 | F: CCGTGGAGTCTAAACCTATCG | (ATC)34 | 190 – 215 | 4 58 | (ATC)34 | VIC | KF680952 |
|         | R: TCTACTGCACTGTCCTCGGG |             |           | 1 54 | TCTACTGCACTGTCCTCGGG | KF680953 |
| Saxgra-17 | F: CTCGCAAATCAGATATTCTACG | (ATG)34 | 200 – 154 | 1 54 | (ATG)34 | VIC | KF680954 |
|         | R: TTGTAATATCTGCCTCTGACC |             |           | 1 54 | TTGTAATATCTGCCTCTGACC | KJ187810 |
| Saxgra-18 | F: CCCATTGACCTTCTGCAACC | (TGG)32 | 130 – 154 | 1 54 | (TGG)32 | PET | KJ187811 |
|         | R: ACTGAGCACTGAAACCGAGG |             |           | 1 54 | ACTGAGCACTGAAACCGAGG | KJ187812 |
| Saxgra-22 | F: CACTGAAATCTGAAACCC | (ATC)34 | 190 – 215 | 4 58 | (ATC)34 | VIC | KF680955 |
|         | R: CAGAGAATCTTAAATAGCCTTAGG |             |           | 1 54 | CAGAGAATCTTAAATAGCCTTAGG | KF680956 |
| Saxgra-23 | F: GCATATCATCGATGTGATTTGG | (TA)34 | 114 – 146 | 7 58 | (TA)34 | VIC | KF680957 |
|         | R: GCTGTAGGCTATTTGGG |             |           | 1 54 | GCTGTAGGCTATTTGGG | VIC | KF680958 |
| Saxgra-26 | F: AGTAACTTCCACAGCTAGTACC | (AGT)31 | 180 – 154 | 1 54 | (AGT)31 | VIC | KF680959 |
|         | R: TCACCTCTTTCTTACATCGGACC | (GCTT)31 | 130 – 154 | 1 54 | TCACCTCTTTCTTACATCGGACC | VIC | KF680960 |
| Saxgra-27 | F: CATCTGTGTTAGATGCC | (GTT)34 | 155 – 207 | 4 58 | (GTT)34 | VIC | KF680961 |
|         | R: CACGTAAGGTGGATGATACTC | (GTT)34 | 155 – 207 | 4 58 | (GTT)34 | VIC | KF680962 |
| Saxgra-33 | F: TCCGGTAACTTCTACCTGTTATACAGG | (GAT)34 | 160 – 190 | 5 58 | (GAT)34 | VIC | KF680963 |
|         | R: TCTCTCTTTCTCCGAGGG |             |           | 1 54 | TCTCTCTTTCTCCGAGGG | VIC | KF680964 |
| Saxgra-34 | F: TGGTTGACATTGTTGATGTTGCTC | (AAC)32 | 120 – 154 | 1 54 | (AAC)32 | VIC | KF680965 |
|         | R: TGCTCAATCTATGATCCGGG |             |           | 1 54 | TGCTCAATCTATGATCCGGG | VIC | KF680966 |
| Saxgra-35 | F: TCAACTCTCTTTATATAGCCTTCCC | (TA)14(GA)12 | 150 – 154 | 1 54 | (TA)14(GA)12 | VIC | KF680967 |
|         | R: GATATACTCAAGTATGTTTATAAGCAGGG |             |           | 1 54 | GATATACTCAAGTATGTTTATAAGCAGGG | VIC | KF680968 |
| Saxgra-36 | F: TCTTCTGTGTTATAGTTAAGTGGG | (TC)14 | 220 – 154 | 1 54 | (TC)14 | VIC | KF680969 |
|         | R: TGCTGTTGGTATAGTTAAGTGGG | (TC)14 | 220 – 154 | 1 54 | (TC)14 | VIC | KF680970 |
| Saxgra-38 | F: GGTCTGATGGAGCTCGG | (GTT)34 | 243 – 271 | 7 58 | (GTT)34 | PET | KF680971 |
|         | R: CAGGAACTCTTCGTCAAGAGG | (GTT)34 | 243 – 271 | 7 58 | (GTT)34 | PET | KF680972 |

Note: A = number of alleles; Tc = annealing temperature.

* Values are based on 25 samples representing five European populations located in central Belgium (N = 5 for each).
equilibrium (HWE) based on calculations of inbreeding coefficient $G_{st}$, performed with 9999 permutations. Negative $G_{st}$ values indicate an excess of heterozygous genotypes. Loci Saxgra-09, Saxgra-29, and Saxgra-33 showed no significant deviation from HWE.

**CONCLUSIONS**

The nine newly developed microsatellite markers are the first reported for *S. granulata* and are especially suitable for population genetic studies due to the highly polymorphic character of the loci. The markers will be used for studying genetic diversity and spatial genetic structure of populations along river systems and to assess levels of gene flow between populations. We expect that these microsatellite markers will provide critical insights into the processes affecting genetic diversity and therefore will contribute to the conservation of this declining species in Europe.

**LITERATURE CITED**

ANDERSSON, S. 1996. Floral variation in *Saxifraga granulata*: Phenotypic selection, quantitative genetics and predicted response to selection. *Heredity* 77: 217–223.

CASTOE, T. A., A. W. POOLE, A. P. J. DE KONING, K. L. JONES, D. F. TOMBASK, S. J. OYLER-MCCANCE, J. A. FIKE, ET AL. 2012. Rapid Microsatellite Identification from Illumina paired-end genomic sequencing in two birds and a snake. *PLoS ONE* 7: e30953.

DARLINGTON, C. D., AND A. P. WYLLIE. 1955. Chromosome atlas of flowering plants. Allen & Unwin, London, United Kingdom.

GORNALL, R. J. 1987. An outline of a revised classification of *Saxifraga*. *Botanical Journal of the Linnean Society* 95: 273–292.

LEWIS, W. H. 1980. Polyploidy in species populations. In W. H. Lewis [ed.], Polyploidy: Biological relevance, 104–143. Plenum Press, New York, New York, USA.

MERRMANS, P. G., AND P. W. HEEDRICK. 2011. Assessing population structure: $F_{ST}$ and related measures. *Molecular Ecology Resources* 11: 5–18.

MERRMANS, P. G., AND P. H. VAN TIENDEREN. 2004. GENOTYPE and GENODIVE: Two programs for the analysis of genetic diversity of asexual organisms. *Molecular Ecology Notes* 4: 792–794.

MOODY, M. E., L. D. MUELLER, AND D. E. SOULTS. 1993. Genetic variation and random drift in autotetraploid populations. *Genetics* 134: 649–657.

NEIL, M. 1987. Molecular evolutionary genetics. Columbia University Press, New York, New York, USA.

PHILP, J. 1934. Note on the cytology of *Saxifraga granulata* L., *S. rosacea* Moench, and their hybrids. *Journal of Genetics* 29: 197–201.

REDONDO, N., M. HORIZALES, S. BROWN, AND C. VILLAVICENCIO. 1996. Biometric and cytometric study of nuclear DNA within *Saxifraga granulata* L. *Boletim da Sociedade Broteriana* 67: 287–301.

STEVEN, D. P. 1988. On the gynodioecious polymorphism in *Saxifraga granulata* L. (*Saxifragaceae*). *Biological Journal of the Linnean Society* 35: 15–28.

STEVEN, D. P., AND A. J. RICHARDS. 1985. Gynodioecy in *Saxifraga granulata* L. (*Saxifragaceae*). *Plant Systematics and Evolution* 151: 43–54.

TRAPNELL, D. W., J. L. HAMRICK, C. K. PARKER, K. W. BRAUNGART, AND T. C. GLENN. 2011. Evaluating the utility of microsatellites for investigations of autoploid taxa. *Journal of Heredity* 102: 473–478.

WADE, T. J., G. COLLING, M. PONCELET, AND D. MATTHEWS. 2012. Effects of inbreeding and interpopulation crosses on performance and plasticity of two generations of offspring of a declining grassland plant. *American Journal of Botany* 99: 1300–1313.