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Source: Applications in Plant Sciences, 2(9)

Published By: Botanical Society of America

URL: https://doi.org/10.3732/apps.1400040

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Applications in Plant Sciences 2014 2(9): 1400040

PRIMER NOTE

MICROSATELLITE PRIMERS FOR THE GYNODIOECIOUS 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 (Gornall, 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 (IPE) 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.: SRX665162). 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 Dijle River in central Belgium (Table 2). Both PCR multiplexes were performed in a 2720 Thermal Cycler (Applied Biosystems, Carlsbad,

[1]Manuscript received 14 May 2014; revision accepted 23 June 2014.

The authors would like to thank J. S. van Zweden and K. Van Acker for assistance in the field and T. Reijnders for assistance in the laboratory. This work was supported by the Flanders Research Foundation (FWO, project 11G1715N, S.M.) and the European Research Council (ERC starting grant 260601, MYCASOR, H.J.).

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doi:10.3732/apps.1400040

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TABLE 1. Characterization of 21 microsatellite loci developed for *Saxifraga granulata*.[a]

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	*A*	T_a (°C)	Multiplex	Fluorescent label	GenBank accession no.
Saxgra-01	F: CGCCTAATCTTCGTCATCTTCG	$(TTC)_{24}$	140	1	54			KJ817803
	R: TTTGAAAATGATGCAAACCGC							
Saxgra-02	F: CAATACATAATAAATAGAACTAGCGCC	$(TTC)_{24}$	100	1	54			KJ817804
	R: AAAAGTACACTCAAAGATTCATGGG							
Saxgra-03	F: GCAACACCTAAGCTCGAACG	$(TC)_{24}(TC)_{12}$	145	1	54			KJ817805
	R: ACCAAACCACACTTCGTCCC							
Saxgra-04	F: AAAATGCATGACCTAAAACTAGTGC	$(GA)_{14}$	245	1	54			KJ817806
	R: ATGATGGTAACGATGGTGGC							
Saxgra-06	F: TTCAACTTTGTGAATGTGAGTAATGC	$(GT)_{26}$	235–287	18	58	1	VIC	KF680947
	R: CACTTGTTATATCCGTGAACTAGAAACC							
Saxgra-09	F: AGGATGAATCTTGGGCATGG	$(AG)_{20}$	284–314	8	58	1	NED	KF680948
	R: CTCTACACCGCTGGTGATGC							
Saxgra-10	F: AGGTGAAGGCTTGAACTTTGG	$(ACT)_{21}$	158–191	4	58	2	NED	KF680949
	R: TGTATTTCCACTGAGTGCCAGC							
Saxgra-11	F: CGTATCTATTTCAACTATATCACCCC	$(GGT)_{33}$	320	1	54			KJ817807
	R: TTTAATAAAAGTTAGGCCAATCCC							
Saxgra-16	F: CCTGGGGATCTAAAACTCATCG	$(GAT)_{21}$	262–275	3	58	2	PET	KF680950
	R: TCATCTCAGTCTTCCCGTCG							
Saxgra-17	F: CGTCGAATACAGAATTTCATCGC	$(AAT)_{36}$	200	1	54			KJ817808
	R: TTGCTAATAGCCTTCTAGCGACC							
Saxgra-18	F: CCCTTTGAATCTTTTCAGCACC	$(TG)_{22}$	130	1	54			KJ817809
	R: ACTGAGCGATGAAACCCAGG							
Saxgra-22	F: CACTCAATTTCAAACCAACCCC	$(ATC)_{24}$	190–215	4	58	1	6-FAM	KF680951
	R: CAGGAAGATCCATTAAATGTCTGG							
Saxgra-23	F: GGCAATTAAAGCAAGGTAATGGG	$(TA)_{18}$	114–146	7	58	2	6-FAM	KF680952
	R: GCATTTGGATCATTGTTTGTGG							
Saxgra-26	F: AAGTAATCCACACGTAGTTCACG	$(AGT)_{21}$	180	1	54			KJ817810
	R: TCAATTCTTTTCATTCCAAGTCC							
Saxgra-27	F: CATCCTGTTAAGGATCGCC	$(GCTT)_{24}$	340	1	54			KJ817811
	R: CAACGGTAAGGCTATAAATCCC							
Saxgra-29	F: CGTCACCGATCACACTAAAGTCC	$(GAT)_{24}$	155–207	4	58	2	VIC	KF680953
	R: TCAATTTCAACTTTCCCCTCG							
Saxgra-33	F: TCCGTTAACTTACAACTGTTATACAAGG	$(GAT)_{24}$	160–190	5	58	1	VIC	KF680954
	R: TCTTCCTTTTCTCGACGGG							
Saxgra-34	F: TGGTTTAGCATTGGTGATGTCC	$(AAT)_{72}$	120	1	54			KJ817812
	R: TGGTCTAACTTGTTTATTGACAGGG							
Saxgra-35	F: TCAACTCTCTTTTATAGCAGCTCCC	$(TA)_{44}(GA)_{12}$	150	1	54			KJ817813
	R: GATATGCAAATAGGTTATAAGCAGCG							
Saxgra-36	F: TTCTTTGGTTATTAAGCTAATGCG	$(TC)_{18}$	220	1	54			KJ817814
	R: TGGCTGTTTTAGCTGTGTGC							
Saxgra-38	F: GTTTGGATTGGGACCGGG	$(GGT)_{24}$	243–271	7	58	1	PET	KF680955
	R: CACGAATCGTCTCAGAAGCG							

Note: *A* = number of alleles; T_a = annealing temperature.

[a] Values are based on 25 samples representing five European populations located in central Belgium (*N* = 5 for each).

California, USA) with Saxgra-06, Saxgra-09, Saxgra-22, Saxgra-33, and Saxgra-38 in the first multiplex and Saxgra-10, Saxgra-16, Saxgra-23, and Saxgra-29 in the second multiplex. The total PCR multiplex reaction volume contained 5 µL of QIAGEN Multiplex PCR Master Mix (QIAGEN), 3 µL of RNase-free water, 1 µL of one of the two multiplexed primer combinations, and 1 µL of template DNA. Both PCR multiplexes followed the same thermocycler program with initial denaturation of 15 min at 95°C; 27 cycles of 30 s at 95°C, 1.5 min at 58°C, and 1 min at 72°C; and a final elongation of 30 min at 60°C. Then, 1 µL of the PCR reaction was added to a solution of 8.8 µL formamide and 0.2 µL of GeneScan 500 LIZ Size Standard (Applied Biosystems). Fragments were sized on an ABI Prism and analyzed by capillary electrophoresis using the 3130-Avant Genetic Analyzer (Applied Biosystems). The raw genetic data were scored using GeneMapper software version 4.0 (Applied Biosystems) using the default settings for microsatellites. Panels and bins were manually constructed, and all data were visually checked to make sure that the loci were identified correctly.

Genotypes at locus Saxgra-06 consisted of a maximum of eight different alleles, whereas genotypes at loci Saxgra-22, Saxgra-33, and Saxgra-38 consisted of no more than six alleles. At loci Saxgra-09, Saxgra-10, Saxgra-16, Saxgra-23, and Saxgra-29, we found genotypes that consisted of three to five alleles. It was known that *S. granulata* is polyploid; Redondo et al. (1996) mention 52 chromosomes, and older data give chromosome numbers ranging from 46 to 60 chromosomes, assuming a basic number of $x = 8$ (Philp, 1934; Darlington and Wylie, 1955). In many polyploid systems, diploid and polyploid cytotypes differ in geographical distribution (Lewis, 1980). Based on the maximum number of alleles per locus per genotype, we assumed that *S. granulata* is octoploid in the study area for further analyses. Furthermore, as allele combinations were completely random, *S. granulata* is most likely an autopolyploid rather than an allopolyploid. Allopolyploids usually display disomic inheritance due to pairing of homologue chromosomes (i.e., fixed heterozygosity), while autopolyploids show random chromosome pairing with one of its seven homologues in octoploid species (Trapnell et al., 2011).

As a result of the polyploid nature of *S. granulata* in the study area, population genetic data were analyzed using the program 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 (H_S; Moody et al., 1993). Genetic diversity of the microsatellite loci studied in five populations of *S. granulata* was high. The number of alleles varied between three and 18 (mean = 6.7; 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

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TABLE 2. Characterization of nine polymorphic loci tested in five *Saxifraga granulata* populations.[a,b]

	Arenberg				Doode Bemde				Korbeek-Dijle 1				Korbeek-Dijle 2				Sint-Joris-Weert			
Locus	*N*	*A*	A_e	H_S	*N*	*A*	A_e	H_S	*N*	*A*	A_e	H_S	*N*	*A*	A_e	H_S	*N*	*A*	A_e	H_S
Saxgra-06	20	17	12.0	0.93	20	14	10.8	0.92	20	17	11.8	0.93	20	18	13.6	0.94	20	15	11.6	0.92
Saxgra-09	20	6	5.3	0.84	20	10	4.5	0.80	20	9	3.9	0.77	20	8	2.5	0.62	20	7	4.2	0.79
Saxgra-10	20	5	2.8	0.67	20	3	1.5	0.33	20	6	2.4	0.60	20	3	1.5	0.34	20	5	1.7	0.43
Saxgra-16	20	5	1.6	0.37	20	3	1.4	0.30	20	5	1.9	0.49	20	3	1.6	0.39	20	4	1.3	0.26
Saxgra-22	20	5	3.5	0.72	20	4	3.8	0.75	20	4	3.6	0.73	20	4	3.8	0.74	20	4	3.9	0.76
Saxgra-23	20	9	3.4	0.73	20	7	2.1	0.53	20	8	3.6	0.74	20	6	3.0	0.68	20	6	2.5	0.62
Saxgra-29	20	5	2.7	0.65	20	5	2.7	0.64	20	4	3.4	0.73	20	6	2.7	0.65	20	4	3.3	0.72
Saxgra-33	20	4	1.4	0.30	20	5	1.8	0.45	20	5	3.1	0.70	20	5	2.1	0.53	20	6	3.3	0.71
Saxgra-38	20	6	3.8	0.75	20	7	3.6	0.74	20	8	5.4	0.83	20	6	4.3	0.78	20	7	3.7	0.74
Mean	20	6.9	4.1	0.66	20	6.4	3.6	0.61	20	7.3	4.4	0.72	20	6.6	3.9	0.63	20	6.4	3.9	0.66

Note: *A* = number of alleles; A_e = effective number of alleles; H_S = gametic heterozygosity; *N* = number of samples.

[a] Geographic coordinates for the populations: Arenberg = 50.8633111°N, 4.6843361°E; Doode Bemde = 50.8173833°N, 4.6464944°E; Korbeek-Dijle 1 = 50.8396111°N, 4.6495750°E; Korbeek-Dijle 2 = 50.8493083°N, 4.6578889°E; Sint-Joris-Weert = 50.7957667°N, 4.6351028°E. All five populations are located in Belgium.

[b] Herbarium vouchers (and herbarium of deposit) of *Saxifraga granulata* specimens from each studied population: Arenberg = *van der Meer 1* (BR); Doode Bemde = *van der Meer 2* (BR); Korbeek-Dijle 1 = *van der Meer 3* (BR); Korbeek-Dijle 2 = *van der Meer 4* (BR); Sint-Joris-Weert = *van der Meer 5* (BR).

equilibrium (HWE) based on calculations of inbreeding coefficient G_{IS}, performed with 9999 permutations. Negative G_{IS} 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.

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