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Thirteen microsatellites developed by SSR-enriched pyrosequencing for *Solanum rostratum* (Solanaceae) and related species[1]

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- *Premise of the study*: Microsatellite markers were developed using second-generation sequencing in *Solanum rostratum* as a tool to study the reproductive biology and genetic structure of this invasive species.
- *Methods and Results*: Thirteen microsatellites were successfully discovered and amplified in a single multiplexed PCR. All loci showed genetic variation in *S. rostratum*. Cross-amplification in five closely related taxa was successful for a subset of loci.
- *Conclusions*: The set of 13 microsatellite markers developed here provides a time-effective and cost-effective genetic tool to study the reproductive biology of *S. rostratum*. The demonstrated transferability of the PCR multiplex to related taxa also highlights its usefulness for evolutionary studies across *Solanum* sect. *Androceras*.

Key words: invasive species; population genetics; reproductive biology; *Solanum rostratum*; *Solanum fructu-tecto*; *Solanum heterodoxum*; *Solanum grayi* var. *grayi*; *Solanum grayi* var. *grandiflorum*; *Solanum lumholtzianum*; *Solanum* sect. *Androceras*.

Solanum rostratum Dunal (Solanaceae) is a diploid, annual, self-compatible herb with weakly zygomorphic, bee-pollinated, nectarless yellow flowers (Whalen, 1979). It forms part of a clade of 12 species of *Solanum* sect. *Androceras*, a group that has been used as a model to investigate the relationship between flower form and reproductive isolation and mating patterns (e.g., Whalen, 1979; Vallejo-Marín et al., 2009). The native range of *S. rostratum* extends from central Mexico to the United States (Whalen, 1979). However, it is now found in China, Russia, Australia, and Europe (Whalen, 1979; Lin and Tan, 2007; Vallejo-Marín, unpublished). The limited availability of genetic markers in *S. rostratum* currently thwarts studies on the reproductive biology and genetic structure of both native and invasive populations.

In this study, we describe 13 new microsatellite markers for *S. rostratum* to enable further studies on its phylogeography and reproductive biology. We used second-generation sequencing and bioinformatic tools to optimize a single microsatellite PCR multiplex (Guichoux et al., 2011) for cost-effective and time-effective amplification of these markers in *S. rostratum* and related taxa.

METHODS AND RESULTS

Seven *S. rostratum* individuals were sampled from two populations (Tehuacán, 18.480°N, 97.411°W; Mexico City, 19.313°N, 99.178°W, Mexico; Appendix 1). Genomic DNA was isolated from silica-dried leaf tissue with the DNeasy Plant Mini kit (QIAGEN, Crawley, West Sussex, UK) and sent to Genoscreen (Lille, France) for microsatellite-enriched library preparation and sequencing by 454 GS-FLX Titanium (Roche Applied Science, Indianapolis, Indiana, USA) according to Malausa et al. (2011). Briefly, the pooled sample of seven individuals was subject to genomic DNA fragmentation, ligated to standard adapters, and enriched with eight microsatellite probes (TG, TC, AAC, AAG, AGG, ACG, ACAT, ACTC). The enriched DNA was then amplified using adapter-specific primers as described in Malausa et al. (2011). The resulting library was tagged with a specific multiplex identifier (MID) tag sequence and pooled together with eight other samples in a quarter of a 454 GS-FLX Titanium run for sequencing. The resulting 33 491 reads (average length = 254 ± 107 bp; mean ± SD; Appendix S1) were analyzed with QDD version 1.3 (Meglécz et al., 2010) to design microsatellite primers using selection criteria detailed in Lepais and Bacles (2011). These criteria were chosen to optimize potential for single PCR multiplexing of the designed primers, and included limiting the length of the expected PCR product to between 90 and 400 bp, optimal primer length of 24 bp (range 21–30 bp), optimal annealing temperature of 63°C (range 60–66°C), and 50% GC content (range 40–60%). Five hundred fifty-seven microsatellites were identified, from which 355 had designed primers (Appendix S2).

Two screenings of 24 primer pairs were performed following the selection strategy of Lepais and Bacles (2011). In brief, microsatellite loci containing dinucleotide (AG and AC) and trinucleotide (AAC, AAG, and AGG) repeat motifs were categorized in one of six expected PCR product size classes and ranked based on the number of motif repeats. In the first screening, a selection of 24 primer pairs representing all six size classes was chosen for testing in simplex PCR format on a panel of 19 *S. rostratum* individuals. Based on the results of this first screening, a new set of 24 primer pairs was then selected to try to obtain

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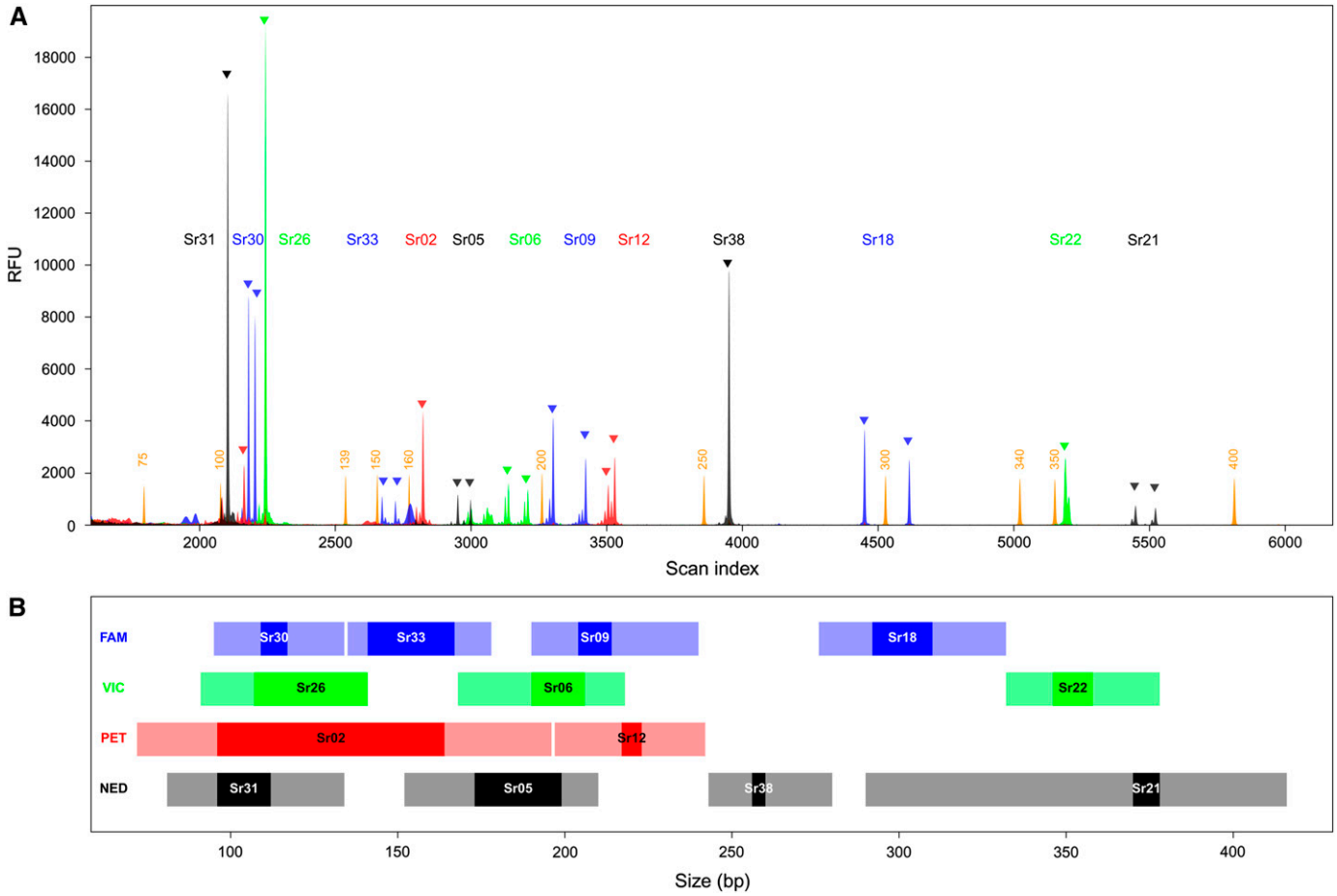

Fig. 1. Example of a typical electropherogram profile obtained for one individual with the multiplex PCR genotyping protocol presented here (A), and diagram showing the allele size range and fluorescent dyes of each of the 13 loci (B). In (A), down-turned triangles indicate alleles at each locus; fragment sizes (bp) of the 500 LIZ size standards are indicated by numbers above each corresponding peaks. In (B), dark rectangles represent the observed allele range in 34 *Solanum rostratum* individuals; light rectangles represent an arbitrary potential allele range used during the multiplex design to avoid overlap of loci with the same fluorescent dye.

successfully amplifying loci across all size classes, and screened in the same 19 individuals. Simplex PCR cycles consisted of a denaturing step of 5 min at 94°C; followed by 30 cycles of 94°C for 30 s, 58°C for 45 s, and 72°C for 45 s; eight cycles of 94°C for 30 s, 53°C for 45 s, and 72°C for 45 s; and a final elongation step of 10 min at 72°C (Lepais and Bacles, 2011). Fragment analysis was performed on an ABI 3730xl capillary sequencer (Applied Biosystems, Foster City, California, USA) at DNA Sequencing and Services (Dundee, United Kingdom) and subsequently analyzed using STRand (VGL, University of California, Davis, California, USA). Out of 48 tested primer pairs, 29 successfully amplified, and 15 were polymorphic with repeatable profiles (Appendix S2).

Thirteen loci were found to be compatible for simultaneous PCR multiplexing using Multiplex Manager (Holleley and Geerts, 2009) and were evaluated using a panel of 38 *S. rostratum* individuals from two populations (Teotihuacán, 19.683°N, 98.858°W; Plan de Fierro, 18.333°N, 97.572°W, Mexico; Appendix 1). In addition, marker transferability and multiplex applicability were tested on two individuals from each of five taxa in *Solanum* sect. *Androceras*: *S. fructu-tecto* Cav., *S. heterodoxum* Dunal, *S. grayi* Rose var. *grandiflorum* Whalen, *S. grayi* var. *grayi* Whalen, and *S. lumholtzianum* Bartlett (Appendix 1). The multiplex PCR reaction was performed using 1× QIAGEN Type-it Microsatellite PCR Kit (QIAGEN), various concentrations (Table 1) of each of the 13 fluorescent forward primers labeled with one of 6-FAM (Eurofins MWG Operon, Ebersberg, Germany), VIC, PET, or NED (Applied Biosystems) dyes and reverse primer, and approximately 5 ng of template DNA. PCR cycles consisted of a denaturing step of 5 min at 95°C, followed by 30 cycles of 95°C for 30 s, 58°C for 180 s, and 72°C for 30 s, and a final elongation step of 30 min at 60°C. Products were analyzed in an ABI 3730xl capillary sequencer (Applied Biosystems). Fluorescence profiles were analyzed using STRand and exported to MsatAllele (Alberto, 2009) in R version 2.12.0 (R Development Core Team, 2010) to determine suitable allele bin range.

All 13 loci were polymorphic in at least one population with two to 13 alleles detected (Figure 1; Table 2), and showed moderate genetic diversity with expected heterozygosity ranging from 0.00 to 0.86 (Table 2).

All loci amplified in *S. fructu-tecto*; Sr21, Sr06, and Sr02 failed to amplify in *S. heterodoxum*; Sr21 and Sr06 did not amplify in *S. grayi* var. *grayi*; Sr21, Sr06, and Sr02 did not amplify in *S. grayi* var. *grandiflorum*; and Sr21, Sr06, and Sr26 failed to amplify in *S. lumholtzianum*. It is important to note that loci that amplified in these taxa did so within the expected size range, thus demonstrating the transferability of the multiplex protocol.

CONCLUSIONS

Second-generation sequencing and novel bioinformatic approaches are very effective tools to isolate microsatellite markers in nonmodel organisms. This allows discovery of numerous microsatellites that can be combined in one or a few PCR reactions, reducing both time and cost of genotyping (Lepais and Bacles, 2011). Here we developed a set of 13 polymorphic microsatellite markers for *S. rostratum* that can be amplified in a single multiplexed PCR and demonstrated its potential use in related taxa, thus enabling future investigation of numerous ecological and evolutionary questions.

TABLE 1. Characteristics of 13 microsatellite primers developed in *Solanum rostratum* and optimized to coamplify in a single multiplex PCR.

Locus	Repeat type	EMBL Accession No.	Primer sequences (5′–3′)	Fluorescent dye	[Primer] (nM)	Size range (bp)
Sr09	$(AC)_8$	FR846150	F: TCACTTTGAGACCCCTAACACCTC R: TAAGAGGAACAGGAAGAAGAGGGC	FAM	170	204–214
Sr18	$(CA)_6$	FR846159	F: AATCACCCACCTACTGTGACGTTT R: ATCCAGTGCTTGTGTTGATAGGCT	FAM	170	292–310
Sr30	$(TC)_8$	FR846171	F: ATGCTCCCCATTTTCCATTTTC R: ATCTGCTGAGAAGTTGAATTTCCG	FAM	120	109–117
Sr33	$(GT)_6$	FR846174	F: ATACTTCATTTGTTGCAGGAGCTG R: CAAAAGCTAAAACCCAAGACAGGA	FAM	340	141–167
Sr06	$(AG)_8$	FR846147	F: ATGAGGACCCAGTTGAGTTTCTTG R: CTTTAAATTCCTCCCATCCAGCTC	VIC	340	190–206
Sr22	$(AAC)_6$	FR846163	F: CTAACAATTTCTCCAACAACCTTGG R: CCAAAACTTTCACCAGAAAACTCAC	VIC	170	346–358
Sr26	$(CT)_9$	FR846167	F: GCTATTTCCCCTACTCCGGTTCTT R: GTAGGTGCCCAAATATTGATCCAG	VIC	120	107–141
Sr05	$(TC)_9$	FR846146	F: CTGAATGTTGTAATTGGGTGTCCA R: ACAAGAACCGAAAACGAAGAACAG	NED	340	173–199
Sr21	$(AAC)_8$	FR846162	F: GGTCGATTGCCTCTATCTACTGTTG R: TGGTAGTGGTAAGGTCTGCGTACA	NED	200	370–378
Sr31	$(TC)_7$	FR846172	F: AACTCAGCCATAGTTCCAGACACC R: AGAGGTGCTGGAGTTGAGAAAAGA	NED	170	96–112
Sr38	$(GAA)_6$	FR846179	F: GATCTCAAAGAAGGGTCTCCCCTA R: AGTGCAGAAAATGAAGTGCTCTGG	NED	170	256–260
Sr02	$(CT)_{13}$	FR846143	F: GGAATAGAGGGAGTTATACAGAATACACGA R: GGCGAGACCAGTTCTTGTCATATT	PET	200	96–164
Sr12	$(TC)_7$	FR846153	F: GGTTAGGCCCAAACGTTGAAATAA R: ACCAGAGATGGATCAAACTTCAGC	PET	170	217–223

Notes: Shown for each primer pair are the repeat motif type, the accession number at the European Molecular Biology Laboratory—Nucleotide Sequence Database (EMBL; http://www.ebi.ac.uk/embl/), the forward and reverse primer sequences, the fluorescent dye added to the 5′ end of the forward primer, the final primer concentration ([Primer]) in the PCR mixture (nM), and the allele size range (bp).

TABLE 2. Results of initial loci screening in two populations of *Solanum rostratum*.

	Population 1[a] (N = 15)		Population 2[b] (N = 23)		Total
Loci	N_a	H_e	N_a	H_e	N_a
Sr09	2	0.238	3	0.343	4
Sr18	2	0.186	6	0.783	6
Sr30	3	0.476	3	0.573	5
Sr33	4	0.612	4	0.489	5
Sr06	4	0.667	5	0.612	6
Sr22	4	0.352	3	0.606	4
Sr26	4	0.531	5	0.501	6
Sr05	8	0.852	6	0.754	12
Sr21	2	0.457	3	0.625	3
Sr31	3	0.440	6	0.792	8
Sr38	1	0.00	2	0.417	2
Sr02	7	0.660	9	0.862	13
Sr12	3	0.676	5	0.543	5

Notes: N = number of genotyped individuals; N_a = number of alleles; H_e = expected heterozygosity.

[a] Population 1 = Teotihuacán, Estado de México.

[b] Population 2 = Plan de Fierro, Puebla.

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APPENDIX 1. Voucher information for taxa used in this study. All vouchers are deposited at the University of Stirling (STIU).

Taxon	Locality	Accession No.
Solanum rostratum Dunal	Mexico, Tehuacán, Puebla	08s104
Solanum rostratum Dunal	Mexico, Mexico City, Distrito Federal	10s110
Solanum rostratum Dunal	Mexico, Plan de Fierro, Puebla	TP-8
Solanum rostratum Dunal	Mexico, Teotihuacán, Estado de México	TEM-19
Solanum fructu-tecto Cav.	Mexico, Atitalaquia, Hidalgo	AH-9
Solanum heterodoxum Dunal	Mexico, Fresnillo, Zacatecas	FZ-24
Solanum grayi Rose var. *grandiflorum* Whalen	Mexico, Los Zapotes, Sinaloa	07s197
Solanum grayi Rose var. *grayi* Whalen	Mexico, Los Álamos, Sonora	07s189
Solanum lumholtzianum Bartlett	Mexico, El Progreso, Sinaloa	07s41