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Characterization of 42 polymorphic microsatellite loci in *Mimulus ringens* (Phrymaceae) using Illumina sequencing[1]

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- *Premise of the study:* Microsatellite markers were isolated and characterized in *Mimulus ringens* (Phrymaceae), a herbaceous wetland perennial, to facilitate studies of mating patterns and population genetic structure.
- *Methods and Results:* A total of 42 polymorphic loci were identified from a sample of 24 individuals from a single population in Ohio, USA. The number of alleles per locus ranged from two to nine, and median observed heterozygosity was 0.435.
- *Conclusions:* This large number of polymorphic loci will enable researchers to quantify male fitness, patterns of multiple paternity, selfing, and biparental inbreeding in large natural populations of this species. These markers will also permit detailed study of fine-scale patterns of genetic structure.

Key words: Illumina paired-end; microsatellite; *Mimulus ringens*; PAL-finder; PCR primers; simple sequence repeat markers.

Mimulus ringens L. (Phrymaceae) is a herbaceous perennial native to wetlands of central and eastern North America. This species and several congeners have become model systems for field and laboratory studies of mating system evolution (Dudash et al., 2005; Wu et al., 2008; Karron et al., 2009; Karron and Mitchell, 2012). *Mimulus ringens* plants produce showy displays of one to 15 large (2 cm), hermaphroditic flowers that are primarily pollinated by bumblebees (*Bombus*; Mitchell et al., 2004). The self-compatible flowers have a mixed mating system, with considerable variation in selfing rate (0–100%; Karron et al., 2009) and number of pollen donors/fruit (typically 2–8; Mitchell et al., 2005). Siring success and functional gender vary markedly as a function of increasing floral display size (Karron and Mitchell, 2012). Here we describe 42 polymorphic microsatellite markers for a single population of *M. ringens*, and discuss how these highly polymorphic markers will enhance studies of mating patterns in this species.

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METHODS AND RESULTS

Twenty-five individuals were sampled for genetic analyses in a single large population of *M. ringens* located in Panzner Wetland Wildlife Preserve (41.06796N, 81.60928W) near Akron, Ohio, USA. A voucher specimen was deposited at the University of Georgia (GA; *Mimulus ringens* L., *D. W. Trapnell 381*, Panzner Wetland Wildlife Preserve, Summit County, Ohio [41.06796N, 81.60928W]). Total DNA was extracted from leaf tissue of one *M. ringens* plant following the protocol of Varadarajan and Prakash (1991) for isolation of microsatellite loci. An Illumina paired-end shotgun library was then prepared by shearing 1 μg of DNA using a Covaris S220 (Covaris, Woburn, Massachusetts, USA) and following the standard protocol of the Illumina TruSeq DNA Library Kit (Illumina, San Diego, California, USA), using a multiplex identifier adapter index. This library was pooled with those of other species and Illumina sequencing was conducted on the HiSeq with 100-bp paired-end reads. Five million of the resulting reads were analyzed with the program PAL_FINDER_v0.02.03 (Castoe et al., 2012) to extract those reads that contained di-, tri-, tetra-, penta-, and hexanucleotide microsatellites. Once positive reads were identified in PAL_FINDER_v0.02.03, they were batched to a local installation of the program Primer3 version 2.0.0 (Rozen and Skaletsky, 2000; http://frodo.wi.mit.edu/) for primer design. To avoid issues with copy number of the primer sequence in the genome, loci for which the primer sequences only occurred one or two times in the 5 million reads were selected. Ninety-six loci of the 2610 that met this criterion were chosen. For each primer pair, a universal CAG tag was added to the 5′ end of one primer and the sequence GTTT was added to the 5′ end of the other primer.

DNA was extracted from leaf tissue of each of the 24 remaining individuals using the QIAGEN Plant Mini Kit (QIAGEN, Valencia, California, USA).

TABLE 1. Characteristics of 42 polymorphic microsatellite loci primers developed in *Mimulus ringens*.[a]

Locus	Primer sequences (5′–3′)	Repeat motif	Size range (bp)	T_a (°C)[b]	T_m (°C)[c]
Miri3	F: *AACTGGCAATCTTTCGCTCC	TTC	139–145	TD65	68
	R: CAATTCCTTCATCTCCAACTCC				56
Miri6	F: *TTGTTGCCCATCAGAAATGG	AAAG	169–181	TD65	67
	R: TTCTAGAAGGAAAGTTGGAGCG				56
Miri7	F: *TCTATAATAACGAGAAGCCTGTTGC	TTC	181–217	TD65	66
	R: TCAGAATTAGTGATGCGCCC				57
Miri8	F: *AACTGGCAATCTTTCGCTCC	TTC	222–228	TD65	68
	R: GCAGGTAATGGCTTTCTGTGG				59
Miri9	F: *TGTCGATCCTTCTTTGTAAATACACC	AAAG	207–219	An65	66
	R: AGGGCCTCGTCCTGATGG				60
Miri10	F: *TGTTCCGAGGTCAGTGAATAGC	AAAG	222–230	TD65	68
	R: CCCATGGTTACTGGTCTGGG				60
Miri11	F: *CGATATAATTTGATTTCCAGTCCG	TCG	198–219	An65	65
	R: TAAACGGCTCGGGATAGAGG				57
Miri12	F: *CATCAAGGCTAACAAACAACATCC	AAAT	174–178	TD65	66
	R: TACCCATTTGGTTTGCAGGC				58
Miri13	F: *AATTTGCGAACTCCTTCCTCC	TC	171–193	TD65	68
	R: AAATGTGCATTTGTTGTAGAGGG				55
Miri15	F: *CAAAGAAGGATGCATTAGATCAGG	ATAC	130–142	TD65	66
	R: CGAGGATCCTAAGAAGATTTAGATGC				57
Miri16	F: *CGTTCGCAGTTTACTATCATTGC	AAAT	204–212	TD65	67
	R: CCGGACCATTAACACAAATCG				57
Miri22	F: *TGCCATATGGTCCTCTAGTTTGC	ATAC	184–196	TD65	68
	R: TTATCACGAGCGTAACCATGC				56
Miri23	F: *TGAGTGATTCATGCATTCCG	TTC	160–178	TD65	66
	R: ATATTTGATGTCCCTACTTTATAATAGCC				54
Miri24	F: *GAACGTGCTCCTGTGAATGC	TTC	240–267	TD65	69
	R: TTGGATCTTATTGCAAACAATGG				54
Miri26	F: *CCTCTGAACACCATTTCCTCC	ATC	184–208	TD65	68
	R: GTTACCCTCGAGGTGTTGGC				60
Miri27	F: *GCACTGGTTTAAAGATGAATGTCC	TTC	146–176	TD65	67
	R: CGTGAAGTGCACGTGATATGC				59
Miri28	F: *AAAGATTGGAAGATACGAGCCC	ATC	132–156	TD65	67
	R: TGGTGCATAAACATCACAGGG				57
Miri38	F: *CCCTGAAAGATGCAATACTCTATCC	AAAG	227–235	TD65	67
	R: GACTCAACAAGTAACTCATGAATAAATGC				57
Miri40	F: *GATAGTTTGCTGTGAGAAGAATGC	TTC	138–174	TD65	66
	R: TTTCACCCAGAGACTGCG				56
Miri45	F: *AGGATTTCCAGGTTGTGAATTAGG	AAAG	193–210	An65	67
	R: GGATTCTTCCGCAGATGACG				58
Miri46	F: *TTGATGATTAGGCGAGTCCG	TTC	230–273	TD65	67
	R: TAAACGCATCCATCATTGGG				55
Miri47	F: *AAAGGCTCAACACTTGTCCC	AAAG	318–322	TD65	68
	R: TCATGTAGGAATTGTTATTTAAACCC				53
Miri50	F: *TCCAGTAGCTATTCGGTGATGG	ATC	465–477	TD65	67
	R: AAATTTGCACACCAGTAGACGC				57
Miri51	F: *TTAGAATAATCTAGGTTGAATGACCG	ATT	147–171	TD65	64
	R: AATTTCAACACGTTAACATTGTCC				54
Miri52	F: *TTGCCAATATGCTTTCACGC	TTC	232–238	TD65	67
	R: ACATGGGAATCACGACAAGC				56
Miri58	F: *CATATATGTGGCTCCTTGACCC	ATC	138–168	TD65	67
	R: CATGATATGCTGCAAGATGTGG				57
Miri61	F: *TAGCAAATCGTGGTTCGTGC	ATT	133–163	TD65	68
	R: CCGGAATGAATTTGAGTTTGG				55
Miri64	F: *CAAGGCTTCTCCCAAGACC	AAC	235–241	TD65	68
	R: CGTCAGTGCACGAGATATGC				58
Miri65	F: *AAACTAACGCTTACGATACTACTGTGG	TTC	168–180	TD65	66
	R: CTAGCCTCCACCACTCTGCC				61
Miri66	F: *ACAAAGAGTGTATTTATCTCTTCAGATTCC	TTC	198–225	TD65	65
	R: CTGGCACTCAATCTATTCATCTAACC				57
Miri67	F: *CTTGATCCAACCATCCTGGG	ATT	214–241	TD65	69
	R: GAAGACCATCCATGTGAAATGC				57
Miri69	F: *TTCGTGCTTGAACATGATTGC	ATC	297–312	TD65	67
	R: GCAAGCATAGTTGGAATTGGC				58
Miri70	F: *GCATTCGATTGTTCATTAAGCC	TTC	306–322	TD65	66
	R: GCAGATTTACTATCTACCTGCGAACC				59
Miri78	F: *TTGAGTACCGCTTTCTGGGC	TTC	118–128	TD65	69
	R: GTACACTGCGCTTCGACTGC				61
Miri82	F: *GCCACTAGCAGCAATACAACC	ATT	157–169	TD65	68
	R: TCCTACACAGAGGCTCGGG				59

Table 1. Continued.

Locus	Primer sequences (5′–3′)	Repeat motif	Size range (bp)	T_a (°C)[b]	T_m (°C)[c]
Miri84	F: *CACAATCCTGATTATTATGAGTCGC	AGT	139–145	TD65	65
	R: TCTTGTCCTAAATATGGCAAGTGG				56
Miri85	F: *ACTGATGACGATCGCTGTGC	ATC	135–142	TD65	69
	R: TGATACTTTGCTCCATCTTTCCC				56
Miri86	F: *GAAGCGAGAACGGACTAGGG	ATC	473–503	TD65	69
	R: TGCTGGAATGAAGTTGCTGG				57
Miri90	F: *TGAGTATAGCCAAACAAACATAGCC	ATT	187–202	TD65	66
	R: TGTTATTACTTCTGTTGAATAGTTGAGGG				56
Miri92	F: *AAGGTTAGGAAACTGGAAGAGTGG	ATC	306–321	TD65	67
	R: TTATAGCTCCACATATTTGAGACATGG				56
Miri94	F: *TGGTGAACATCTTCCATTCTTCC	ATT	210–223	TD65	67
	R: TTCCTCAAAGCCACAAACCC				57
Miri95	F: *GGAGGGAGAACAAGAAGTAATGC	CGG	235–250	TD65	67
	R: TTGTGTTAACAAGCTAAGAAACCC				55

Note: T_a = annealing temperature; T_m = melting temperature.

* Indicates CAG tag (5′-CAGTCGGGCGTCATCA-3′) label.

[a] Data are deposited in the Dryad Repository: http://dx.doi.org/10.5061/dryad.cr3g5v4f .

[b] TD65 refers to the touchdown 65 protocol and An65 to a standard protocol (see text).

[c] T_m of the forward (top) and reverse (bottom) primers.

Ninety-six primer pairs were tested for amplification and polymorphism using DNA obtained from four individuals. To avoid running out of samples, the set of four individuals used for initial screening was rotated, but all came from the same set of 24 individuals. All PCR amplifications were performed in a 12.5 μL volume (10 mM Tris [pH 8.4], 50 mM KCl, 25.0 μg/mL BSA, 0.4 μM unlabeled primer, 0.04 μM tag-labeled primer, 0.36 μM universal FAM dye-labeled primer, 3.0 mM $MgCl_2$, 0.8 mM dNTPs, 0.5 units AmpliTaq Gold Polymerase [Applied Biosystems, Foster City, California], and 20 ng DNA template using a GeneAmp 9700 PCR machine [Applied Biosystems]). A touchdown thermal cycling program (Don et al., 1991) encompassing a 10°C span of annealing temperatures ranging between 65–55°C (TD65) was used for nearly all loci. Touchdown cycling parameters consisted of an initial denaturation step of 5 min at 95°C followed by 20 cycles of 95°C for 30 s, highest annealing temperature (decreased 0.5°C per cycle) for 30 s, and 72°C for 30 s; and 20 cycles of 95°C for 30 s, lowest annealing temperature for 30 s, and 72°C for 30 s. For other loci, a standard thermal cycling program was used with an initial denaturation step of 5 min at 95°C followed by 40 cycles of 95°C for 30 s, 65°C for 30 s, and 72°C for 30 s. PCR products were subjected to fragment analysis using an ABI 3130xl sequencer (Applied Biosystems) and sized with Naurox size standard prepared as described in DeWoody et al. (2004), except that unlabeled primers started with GTTT. Results were analyzed using GeneMapper version 3.7 (Applied Biosystems).

Forty-two of the 96 primer pairs tested amplified high-quality PCR products that exhibited polymorphism (Table 1). Variability of the 42 polymorphic loci was assessed in all 24 individuals. Characteristics of each locus are summarized in Table 1, and patterns of genetic polymorphism are summarized in Table 2. The number of alleles ranged from two to nine, median observed heterozygosity was 0.435, and fragment size ranged from 118 to 503 base pairs. The number of alleles per locus and observed heterozygosity were estimated using GenAlEx version 6.4 (Peakall and Smouse, 2006).

CONCLUSIONS

Identification of 42 polymorphic loci in a single population of *M. ringens* provides important new opportunities for linking pollinator movements with resulting patterns of paternity. The total probability of paternal exclusion across the 12 most polymorphic loci is 0.999 (Peakall and Smouse, 2006), facilitating detailed studies of the effects of floral traits on siring success. These markers will also greatly enhance studies of biparental inbreeding, a parameter that has been very difficult to measure accurately in natural populations (Karron et al., 2012). Finally, these markers will be useful for studies of mate diversity within fruits, permitting detailed studies of the ecological and evolutionary factors influencing variation in mate diversity within and among individuals.

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TABLE 2. Results of initial polymorphic primer screening in 24 *Mimulus ringens* individuals from a single population.

Locus	*N*	*A*	H_o
Miri3	24	2	0.500
Miri6	24	3	0.500
Miri7	24	6	0.667
Miri8	23	2	0.522
Miri9	18	3	0.167
Miri10	24	2	0.333
Miri11	15	3	0.400
Miri12	21	2	0.429
Miri13	18	7	0.556
Miri15	24	3	0.250
Miri16	23	2	0.261
Miri22	22	2	0.045
Miri23	14	4	0.571
Miri24	19	7	0.632
Miri26	23	5	0.522
Miri27	22	5	0.636
Miri28	23	7	0.435
Miri38	21	2	0.048
Miri40	21	5	0.571
Miri45	22	3	0.273
Miri46	21	7	0.524
Miri47	22	2	0.091
Miri50	18	3	0.556
Miri51	24	4	0.333
Miri52	21	3	0.429
Miri58	23	9	0.696
Miri61	23	5	0.478
Miri64	23	2	0.435
Miri65	24	5	0.167
Miri66	23	3	0.391
Miri67	22	4	0.318
Miri69	23	3	0.435
Miri70	22	4	0.545
Miri78	10	3	0.600
Miri82	20	5	0.700
Miri84	23	3	0.348
Miri85	21	5	0.524
Miri86	21	4	0.333
Miri90	23	4	0.522
Miri92	22	5	0.364
Miri94	23	5	0.348
Miri95	21	6	0.571

Note: *A* = number of alleles; H_o = observed heterozygosity; *N* = number of individuals screened.