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PRIMER NOTE

The development of eight microsatellite loci in the wild daffodil *Narcissus triandrus* (Amaryllidaceae)

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Abstract

We report microsatellite primer pairs for the wild tristylous daffodil, *Narcissus triandrus* (Amaryllidaceae). From enriched libraries, we identified 58 unique microsatellite loci. We designed primer pairs for 27 of these loci and screened genomic DNA from 38 to 40 adults from a single population. For eight polymorphic loci, the number of alleles per locus ranged from five to 17. As six primers also amplified loci in three other *Narcissus* species, including two horticultural varieties, we expect that some of these markers will be transferable to other *Narcissus* species.

Keywords: daffodils, mating asymmetries, microsatellites, *Narcissus triandrus*, SSR, tristyly

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Narcissus represents one of the most economically important ornamentals with more than 20 000 registered names resulting from over a century of breeding effort (Blanchard 1990). In addition, this genus has been of interest to evolutionary biologists because it possesses remarkable diversity in floral design, pollination biology and sexual systems (Barrett & Hodgins 2006). *Narcissus triandrus* is a bee-pollinated, diminutive perennial, common in the Iberian Peninsula. The species is the only member of the genus that is tristylous where populations are composed of three floral morphs. Our interest in *N. triandrus* stems from the occurrence of consistently biased morph ratios in populations. Recent theoretical work has demonstrated that asymmetrical mating patterns among the morphs could explain these unusual morph ratios (Barrett & Hodgins 2006). To test these theoretical predictions, we developed microsatellite markers to enable us to study mating patterns among the morphs.

We extracted genomic DNA using the Puregene DNA isolation kit (Gentra Systems) from leaf material from a single individual (population 270, Lamosa, Portugal). Three libraries were generated and enriched for $(GA)_n$ and $(GT)_n$, $(CAA)_n$ and $(AGG)_n$, as well as $(GCC)_n$ and $(GATA)_n$ repeats following a protocol modified from Hamilton *et al.* (1999). For each library, we first digested 4 μg of genomic DNA with *Rsa*I and ligated SuperSNX linkers to facilitate cloning. Libraries were then enriched by hybridization to biotinylated microsatellite oligonucleotides which were captured on Dynabeads (Dynal Biotech Inc.). We amplified the enriched libraries by polymerase chain reaction (PCR) using primers homologous to the SuperSNX linker sequences (Glenn & Schable 2005). We then cloned the PCR product using TOPO TA Cloning system (Invitrogen). The resulting ligation mixtures were transformed into chemically competent *Escherichia coli* DH5α cells and plated onto LB agar plates supplemented with 100 μL/mL ampicillin, 50 μg/mL kanamycin and X-gal for blue/white selection. We picked and dipped individual white colonies into a standard 25 μL PCR mixture containing 0.1 μM M13F and M13R primers to amplify the inserts. The cycling conditions were 1 cycle of 94 °C for 4 min; 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2.5 min; and 1 cycle of 72 °C for 5 min. We selected PCR products of 500–1000 bp for sequencing. We performed sequencing reactions with the Genome Laboratory DTCS QSK (Beckman Coulter) run on the Beckman CEO 8000 Genetic Analysis System. The sequencing reactions contained 2.5 μL of enzymatically cleaned PCR product, 2.5 μL of Beckman dye terminator cycle sequencing mix, 1.5 μL of 1× PCR buffer with 1.5 mM $MgCl_2$, 3 μL of water, and either 0.5 μL (0.5 μM) of modified T3 primer (5′-GCCAAGCTCAGAATTAACCCTCACTAAAGG) or modified T7 primer (5′-CGACGGCCAGTGAATTGTAATACGACTC). The cycling conditions were 96 °C for 20 s, 55 °C for 20 s, and 60 °C for 4 min, repeated 50 times. We cleaned the products by ethanol precipitation

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Table 1 Variable microsatellite loci in *Narcissus triandrus*. Locus designations are given along with GenBank Accession numbers, repeat motifs (based on the original clone sequences used for primer design), forward (F) and reverse (R) primer sequence (5′–3′ direction) as well as the allele size range. Optimal annealing temperatures (T_a) for PCR are provided with estimates of allele number (A), observed (H_O) and expected (H_E) heterozygosity derived from PCR analysis of population 270 from Lamosa, Portugal (40.87607°N and 7.61841°W). N is the total number of individuals for each sample

Locus (GenBank)	Repeat array	Primer sequences (5′–3′)	Allele size range (bp)	T_a (°C)	A	H_E	H_O	N
NT26	$(GA)_4CA(GA)_{10}$	F:CATGCAAGATTGCTCTTATTCAACG	465–487	59	12	0.87	0.80	39
DQ925475		R:CGCTTGGTTTGATACGACTTAGGAC						
NT55	$(GA)_9$	F:TTGAAGCATCTATGTACCACGTGCACC	374–388	62	6	0.52	0.15*	40
DQ925476		R:CTGCCGTTAACTTTTTCCAAGAACC						
NT63	$(CT)_{12}$	F:CTTGGGTTAGAGCCTTGAATGGATTATTCC	244–262	59	10	0.78	0.65	40
DQ925477		R:AGGTGGAGGCTGACATTGGCATG						
NT113	$(GT)_{11}$	F:CCCCTGAAATCCCAATCTGTGC	268–306	59	17	0.90	0.74*	39
DQ925478		R:TCGTGAATGTTCGGTCTCAATTTGC						
NT154	$(CT)_9$	F:GTGATAACGCCGATAACAAGTTTGG	277–285	59	5	0.59	0.68	38
DQ925479		R:ATTACTGTAGTGCGTCAGAATAACATGTG						
NT155	$(GTT)_7$	F:TTAGTGGTGTCTGTCGCCATGCAAATG	100–124	59	5	0.48	0.46	40
DQ925480		R:CATGGACACATGACATGATATAAATATAGTGACAC						
NT183	$(AC)_{11}$	F:CATGTTACAAGGAGATCACGAGCCT	106–130	63	6	0.65	0.28*	39
DQ925481		R:AGTACTGAGCTGGGCATCTCAATTG						
NT230	$(GA)_{14}$	F:GGGTTTTGATAAGGTTATATACATAGTTGG	162–234	50	11	0.87	0.55*	40
DQ925482		R:ACACCAGATTTGGCTTAATCGTAGAC						

*Significant deviation from Hardy–Weinberg equilibrium ($P < 0.05$).

according to the manufacturer's instructions. In total, we sequenced 446 colonies in one direction and then sequenced colonies with microsatellites in the opposite direction.

Overall, we were able to design primers for 27 microsatellite loci and optimize 15 of these primers using eight other *N. triandrus* DNA samples. We performed DNA amplification using the following conditions: 50 ng of genomic DNA in a 25 μL PCR volume, along with 0.1 μM of each primer (Beckmen WellRED dyes, Invitrogen; primer concentration was doubled for primer NT230), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1.25 U *Taq* Polymerase (Fermentas) and 1 × PCR buffer with $(NH_4)_2SO_4$. Betaine (1 M final concentration) was added to reactions amplifying locus NT230 and DMSO (5% final concentration) was added to reactions amplifying NT26. Cycling conditions were: 1 cycle of 94 °C for 5 min; 40–50 cycles of 94 °C for 30 s, 50 °C to 63 °C for 30 s (see Table 1 for annealing temperatures), and 72 °C for 30 s; and 1 cycle of 72 °C for 5 min. We sized PCR products for all loci on the CEQ 8000 genetic analysis system (Beckman) using a 400-bp size standard according to the manufacturer's instruction. We estimated fragment sizes using the BECKMAN COULTER 8000 fragment analysis software. Eight of the 15 loci were polymorphic and produced clear peaks (Table 1).

For the eight polymorphic loci, the number of alleles ranged from five to 17 (mean = 9.0). Observed heterozygosity ranged from 0.15 to 0.80. Expected heterozygosity ranged from 0.48 to 0.91 (mean = 0.71). We tested for deviations from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) between all pairs of polymorphic loci with FSTAT (Goudet 2001), and implemented Bonferroni procedures to correct for multiple tests. Four loci (NT55, NT183, NT113 and NT230) showed significant deviations from Hardy–Weinberg expectations. These deviations were due to a deficit of heterozygotes suggesting the presence of null alleles or population structure. None of the 28 pairwise comparisons among loci exhibited significant linkage disequilibrium. We used the program CERVUS (Marshall *et al*. 1998) and determined that these loci have a combined exclusion probability of 0.981 when neither parent is known and 0.998 when the genotype of one parent is known.

To examine the utility of microsatellite primers developed for *N. triandrus* in other *Narcissus* species, we tested genomic DNA from eight individuals from a single population of *N. assoanus* (population 3, Montpellier, France), and four individuals from each of two horticultural cultivars of *N. pseudonarcissus* (Trumpet Daffodil) and *N. papyraceus* (paperwhite *Narcissus*) purchased in Toronto. Six of the eight polymorphic loci that amplified in *N. triandrus* also amplified in these other *Narcissus* species (Table 2). Therefore, we expect that these loci could facilitate evolutionary and population genetic studies as well as breeding and cultivar development in other *Narcissus* species.

Table 2 Amplification of eight microsatellite primers developed for *Narcissus triandrus* in *N. assoanus* and two horticultural cultivars — *N. papyraceus* and *N. pseudonarcissus*. '+' denotes amplification of a band in the same size range as *N. triandrus* and '–'denotes no amplification

Locus	*N. assoanus*	*N. papyraceus*	*N. pseudonarcissus*
NT26	+	+	+
NT55	–	–	–
NT63	+	+	+
NT113	+	+	+
NT154	+	+	+
NT155	+	+	+
NT183	+	+	+
NT230	–	–	–

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