Microsatellite Markers for Hoop-Petticoat Daffodils (Narcissus sect. Bulbocodii; Amaryllidaceae) 1

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PRIMER NOTE

MICROSATELLITE MARKERS FOR HOOP-PETTICOAT DAFFODILS (*NARCISSUS* SECT. *BULBOCODII*; AMARYLLIDACEAE)[1]

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- *Premise of the study:* Microsatellite markers were developed using hoop-petticoat daffodils (*Narcissus* sect. *Bulbocodii*; Amaryllidaceae) to aid in the taxonomic revision of the section, and to further evaluate their broad applicability for daffodil cultivar identification.
- *Methods and Results:* Three hundred fifty-one primer pairs were developed using a commercial service. Nineteen polymorphic and repeatable markers were developed by screening 67 of these primer pairs. Of these, 11 chosen markers were used to screen 317 samples; the number of alleles per locus ranged from four to 21, and the observed heterozygosity ranged from 0.101 to 0.297. There were null genotypes in some samples for six of the markers. All the microsatellites were transferable to other *Narcissus* sections.
- *Conclusions:* The results indicate that these new markers have sufficient potential variation to be used for taxonomic revision of the genus and to distinguish many commercial daffodil cultivars.

Key words: Amaryllidaceae; daffodil cultivars; horticultural taxonomy; microsatellite markers; *Narcissus* section *Bulbocodii*; polyploidy.

Narcissus L. (Amaryllidaceae) is the single most important ornamental crop for both the cut flower and the bulb trade combined. Complex breeding programs of daffodils over the past 150 yr have resulted in more than 30,000 registered cultivars (Könyves et al., 2011), but this makes the description and commercialization of new cultivars increasingly complex. Naming new cultivars requires the identification and description of discriminating features, and molecular markers, such as microsatellites, could provide fast, cheap, and easily searchable data to achieve this (Culham and Grant, 1999). To assess the use of microsatellites in *Narcissus* for cultivar identification and for taxonomic revision, we developed new microsatellite markers for *Narcissus* sect. *Bulbocodii* DC. (hoop-petticoat daffodils) as a test case. This section is an excellent study group due to its distinct floral morphology, having a large funnel-shaped corona; its limited distribution, ranging from southern Morocco to southwest France; and its long history in cultivation (David and Könyves, 2013). The section exhibits natural variation in both morphology and in chromosome number, ranging from diploid to octoploid (Fernandes, 1963), and the taxa frequently hybridize, resulting in four to 33 taxa from species down to varietal ranks, depending on taxonomic treatment. The microsatellite markers described here were developed using material from a naturally occurring population and screened using a combination of wild and cultivated plants to establish the extent of genetic variation.

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METHODS AND RESULTS

Material from the wild was collected across the natural distribution of *Narcissus* sect. *Bulbocodii* (Könyves, 2014). In total, 44 populations were sampled (Appendix 1). Total genomic DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1987). Microsatellite library development and primer design were carried out by Genoscreen (Lille, France). An equimolar DNA mix of 10 individuals of population KET (Appendix 1) was enriched with eight microsatellite probes (TG, TC, AAC, AAG, AGG, ACG, ACAT, ACTC) and sequenced according to the GS FLX protocol by Malausa et al. (2011). The resulting library consisted of 37,979 raw sequences. Of these, 5765 contained microsatellites, and primers were designed for 351 using QDD (Meglécz et al., 2010) following Malausa et al. (2011).

Resources allowed test PCR amplification of 67 primer pairs from the 351 developed. The primers were chosen to maximize the variation in length of amplicon, motif repeat sequence, and motif length. Test amplification of primers used one sample each from populations CAT and V, and four samples from an existing living collection (accession no. SJ20597, SJ001999, BD96/198, and *Narcissus* 'Golden Bells', the most widely available cultivar in this section; Appendix 2), with the equimolar DNA mix of population KET used as a positive control. PCR reactions were performed in a 10-μL volume containing final concentrations of 1× Bioline Biomix (Bioline Reagents Ltd., London, United Kingdom), 0.1–0.2 μM of each primer, and 10 ng of DNA template. Cycling conditions were 94°C for 120 s; 40 cycles of 94°C for 45 s, 48–63°C for 30 s, 72°C for 45 s; and finally 72°C for 10 min (see Table 1). The PCR products were separated on 2% w/v agarose gels in 1× TAE buffer (pH 8.0) stained with ethidium bromide with accompanying HyperLadder 100bp (Bioline Reagents Ltd.) as a marker. Gels were photographed under ultraviolet illumination to record the presence of PCR products. Of the 67 primer pairs selected for initial trial, 39 primer pairs amplified the expected target fragments. Microsatellite variability was tested

TABLE 1. Characteristics of 19 microsatellite loci developed for *Narcissus* sect. *Bulbocodii*.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	T_a (°C)	GenBank accession no.
NSB14[a]	F: TGTGTAAGCATACTAACGTTTCG	$(ATGT)_{14}$	147–221	48	KT005774
	R: AAAAGAGCACCAAGGATGAA				
NSB52[a]	F: CAATGGTGGAGCCTCTAATAGC	$(GT)_{10}$	117–138	59	KT005775
	R: TGTCATTCTTTACTTTGTTCTCATTCA				
NSB73[a]	F: GGAGAGGAGTGAGTGGAGTGA	$(CTT)_8$	142–221	60	KT005776
	R: CAGGCTGTTCAACTATCTTGC				
NSB113[a]	F: TTGTGATAAATAAAGGTGCAACTCA	$(AGT)_6$	78–123	54	KT005777
	R: CATTGCCCGTGATAAGCTCT				
NSB122[a]	F: CAAAGTGTTTGTGAATTGCTTC	$(AC)_9$	169–198	59	KT005778
	R: GCAATGAGGAGCTTATGAATTAAC				
NSB143[a]	F: CTGTTTCTTTGTTCTGCACATT	$(GAA)_7$	244–269	59	KT005779
	R: TCCCAAAATTGCTTCTGAGC				
NSB182[a]	F: TTGTATTATACGTTGTTCTGGGGA	$(AC)_{12}$	115–121	58	KT005780
	R: GAGATGCTGACACGCAAACT				
NSB232[a]	F: CTCCACTTTGGTTGAATCCC	$(CT)_9$	110–118	63	KT005781
	R: GACTACCTCCTATTCTAAATGCCA				
NSB253[a]	F: GAGGATTACTGTAGCCAATTCCA	$(GTT)_6$	100–140	56	KT005782
	R: GGACTACAAGATGGCTTCCA				
NSB263[a]	F: CGAAGGAGGAGTCTTGGAAA	$(GAA)_6$	94–131	60	KT005783
	R: GAGCAAACTCCTGGCTGAAG				
NSB272[a]	F: GGTTCTGCCGATGGACTAAT	$(CT)_9$	122–146	60	KT005784
	R: TTATCACATCCAACGGTTTGC				
NSB23[b]	F: TTCACCCTCAACTTTTAAAACCA	$(AAC)_{11}$	146–164	51	KU300963
	R: TGCTTTTGTTACATCCATAACG				
NSB32[b]	F: GCCCCACCAAAATAGAGAAA	$(AG)_{11}$	106–121	48	KU300964
	R: TCTGGATTTTATTTCCACCCC				
NSB33[b]	F: ACCTCACTATCTCCCAAAATGC	$(CTT)_{10}$	87–93	51	KU300965
	R: CCTCTTCTTCAAAATTAGCCAAA				
NSB82[b]	F: CATCATATTCATGGATGCCAA	$(TC)_{10}$	108	54	KU300966
	R: TGACAACACAATGAGCGAGTT				
NSB152[b]	F: GTGGACAAAAGGGGTAGCTG	$(TC)_8$	250–258	60	KU300967
	R: GCAAGAGAAGCTCTTCTTTCACTT				
NSB273[b]	F: TGGAAGATGAACCCTTACCA	$(AAG)_5$	291–310	63	KU300968
	R: GGAAGTGTCATTTGACCATAACA				
NSB282[b]	F: TGTGCATTAATTCTCTAATCCCT	$(TC)_9$	104–110	51	KU300969
	R: AAATTAATGTAGCGTTTCTTCATCA				
NSB322[b]	F: ATTCTAGAAGATATGATTTGATTTGGA	$(TC)_7$	286–300	58	KU300970
	R: TTGGCCGAGCTATACAATATG				

Note: T_a = annealing temperature.
[a]Marker selected; size range values based on 312–317 individuals (see Table 2).
[b]Marker not selected; size range values based on seven individuals (see Methods and Results section).

with an equimolar DNA mix of 19 samples (marked with a/b in Appendix 1 and 2) by ligating the PCR products with the M13 promoter and labeling the products with 6-FAM according to Cryer et al. (2005). Fragment analysis of amplicons was carried out by Source BioScience (Nottingham, United Kingdom). The electropherograms were analyzed using GeneMapper version 4.0 (Applied Biosystems by Life Technologies, Carlsbad, California, USA). Thirty-three of the tested primer pairs amplified multiple clean peaks. Of these, the best 24, based on the overall quality of the electropherograms, were used to genotype seven samples (two samples each of populations KET and TIG, one each of populations CAT and POR, and one of *Narcissus* 'Golden Bells'). Forward primers were labeled with fluorescent dyes 6-FAM, HEX (Sigma-Aldrich, St. Louis, Missouri, USA), NED, PET, or VIC (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Readable electropherograms were obtained for 19 primer pairs (Table 1). Of these, resources allowed the 11 best markers (most length-variable and

TABLE 2. Summary statistics of the chosen 11 microsatellites based on 312–317 hoop-petticoat daffodil samples.

Locus	No. successfully genotyped	Total no. of alleles	No. of alleles per individual	Alleles per individual (mean ± SE)	H_o	Allelic diversity	Proportion of null genotypes
NSB14	317	19	0–4	1.227 (± 0.039)	0.231	0.784	0.121
NSB52	314	11	0–3	1.404 (± 0.028)	0.243	0.799	0.002
NSB73	314	21	0–4	1.185 (± 0.025)	0.135	0.740	0.022
NSB113	317	8	1–3	1.243 (± 0.022)	0.154	0.561	0.000
NSB122	317	11	0–4	1.202 (± 0.023)	0.141	0.731	0.011
NSB143	312	8	0–3	1.125 (± 0.021)	0.111	0.718	0.027
NSB182	317	4	1–3	1.350 (± 0.025)	0.224	0.454	0.000
NSB232	317	5	1–3	1.180 (± 0.022)	0.101	0.279	0.000
NSB253	317	12	0–3	1.259 (± 0.029)	0.242	0.842	0.064
NSB263	314	11	1–3	1.268 (± 0.023)	0.173	0.665	0.000
NSB272	317	10	1–4	1.483 (± 0.029)	0.297	0.766	0.000

Note: H_o = observed heterozygosity; SE = standard error.

reproducible) to be used to genotype 317 samples of hoop-petticoat daffodils across the natural distribution range to assess the degree of polymorphism in nature (Table 2).

PCR amplifications were performed as single reactions according to the previously detailed cycling conditions. The PCR products were combined for multiplex fragment analysis. Unambiguously identifying microsatellite alleles in polyploids can be challenging, as identifying stutter peaks in samples of unknown ploidy is difficult and can lead to inclusion of noise in a data set. To avoid this, alleles were scored according to the MANUAL 8 scoring routine described by Pfeiffer et al. (2011). Moreover, as the allele dosage of polyploids is unknown, traditional population genetic techniques (e.g., deviation from Hardy–Weinberg equilibrium) cannot readily be applied. Therefore, we used a presence-absence scoring of peaks to estimate polymorphism, similar to a dominant marker (e.g., amplified fragment length polymorphism [AFLP]) data set.

The number of alleles per locus ranged from four to 21, the observed heterozygosity (H_o) ranged from 0.101 to 0.297, and allelic diversity (calculated as: $1-\sum\{p_i(p_i-1)/N(N-1)\}$, where p_i is the frequency with which the ith allele was detected) ranged from 0.279 to 0.842. In addition to the allelic variation, there were null genotypes for six of the markers, with frequency ranging from 0.002 to 0.121, confirmed by repeating PCR amplifications. The presence of null genotypes was expected due to incomplete transferability of these markers in section *Bulbocodii*. However, in a presence-absence data set these are valuable characters that allow samples with a null-allele data set for some individual markers to be included.

Two populations each of the most widely sampled species (*N. bulbocodium* L. [CAT, ALD], *N. cantabricus* DC. [SDF, HOR], and *N. romieuxii* Braun-Blanq. & Maire [KET, OUL]) were used to calculate genotypic diversity estimators (Table 3). The total number of different alleles per population across all loci (A) ranged from 26 to 42, the number of private alleles per population across all loci (A_p) was between zero and three, proportion of observed heterozygotes averaged per locus (H_o) was from 0.19 to 0.35, proportion of null genotypes carried by each individual averaged across all loci (F_{g0}) ranged from 0 to 0.21, and the genotypic richness was 0.94 or 1 (calculated as: R = G – 1/N – 1; where G is the number of multilocus genotypes and N is the number of genotyped samples; Dorken and Eckert, 2001).

Broader transferability of these markers was tested using 18 species belonging to seven of the nine (Blanchard, 1990) other *Narcissus* sections. The success of the transfer was assessed using fragment analysis. The 11 markers were all transferable to other *Narcissus* sections to some degree, ranging from 39% to 100% (Table 4).

CONCLUSIONS

The microsatellite markers developed in this study are sufficiently variable to allow species-level and population-level variation of hoop-petticoat daffodils to be investigated. The markers show potential to be used to develop molecular identification tools for daffodil cultivars, and to contribute toward the

TABLE 3. Results of initial genotypic variability screening among populations of *Narcissus* sect. *Bulbocodii*.

Population	N	A	A_p	H_o	F_{g0}	G	R
N. bulbocodium							
CAT	20	42	2	0.22	0.064	20	1
ALD	18	39	3	0.32	0.21	18	1
N. cantabricus							
SDF	18	28	0	0.23	0	17	0.94
HOR	17	26	0	0.19	0	16	0.94
N. romieuxii							
KET	16	40	1	0.35	0	16	1
OUL	12	40	0	0.31	0	12	1

Note: A = total number of different alleles across all loci; A_p = number of private alleles across all loci; F_{g0} = proportion of null genotypes carried by each individual averaged across all loci; G = number of multilocus genotypes; H_o = proportion of observed heterozygotes per loci; N = number of individuals; R = genotypic richness.

TABLE 4. Transferability of the chosen 11 microsatellite loci in 18 *Narcissus* species.

Section	Species	NSB14	NSB52	NSB73	NSB113	NSB122	NSB143	NSB182	NSB232	NSB253	NSB263	NSB272
Apodanthi A. Fern.	*N. cuatrecasasii* Fern. Casas, M. Laínz & Ruíz Rejón	+	+	+	+	+	+	+	+	+	+	+
	N. rupicola Dufour	+	+	+	+	+	+	—	+	+	+	+
	N. scaberulus Henriq.	+	+	—	+	+	+	—	+	—	+	+
Aurelia (Gay) Baker	*N. broussonetii* Lag.	+	—	—	+	—	—	+	+	—	+	+
Braxireon (Raf.) Valdés	*N. cavanillesii* (Cav.) Barra & G. López	+	+	—	+	—	+	+	+	+	+	+
Ganymedes (Haw.) Shult. f.	*N. triandrus* L.	+	+	+	+	+	+	+	+	+	+	+
Jonquillae DC.	*N. assoanus* Dufour	+	+	+	+	+	+	+	+	—	+	+
	N. jonquilla L.	+	+	—	+	+	+	+	+	+	+	+
	N. viridiflorus Schousb.	+	+	+	+	+	—	+	+	+	+	+
Pseudonarcissi DC.	*N. asturiensis* Pugsley	+	—	—	+	+	+	+	+	—	+	+
	N. hispanicus Gouan	+	—	—	+	+	+	+	+	+	+	+
	N. lobularis Schult. f.	+	+	—	+	+	+	+	+	+	+	+
	N. perez-chiscanoi Fern. Casas	+	—	—	+	+	+	+	+	+	+	+
	N. segurensis S. Ríos, D. Rivera, Alcaraz & Obón	+	—	+	+	+	+	—	+	+	+	+
	N. yepesii S. Ríos, D. Rivera, Alcaraz & Obón	+	+	—	+	+	+	+	+	+	+	+
Tazettae DC.	*N. dubius* Gouan	+	—	+	+	+	—	+	+	+	—	+
	N. elegans (Haw.) Spach	—	—	—	+	—	—	+	+	—	+	+
	N. papyraceus Ker Gawl.	—	—	—	—	—	—	+	+	—	+	+
Percentage transferability (%)		89	61	39	94	83	78	83	100	72	94	100

Note: + = successful fragment analysis; — = unsuccessful fragment analysis.

taxonomic revision of section *Bulbocodii*. The high degree of transferability suggests that these markers have the potential to distinguish many *Narcissus* cultivars in most sections of the genus.

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Appendix 1. Voucher information and geographic location of *Narcissus* sect. *Bulbocodii* samples. All voucher specimens are deposited at the University of Reading Herbarium (RNG), Reading, United Kingdom.

Population	Species	*N*	Country	GPS coordinates	Voucher no.
AGE	*N. romieuxii* Braun-Blanq. & Maire	4	Morocco	32°55′N, 5°32′W	KK#016
AINL	*N. romieuxii*	5	Morocco	33°23′N, 5°15′W	KK#015
AKE	*N. bulbocodium* L.	2	Spain	42°43′N, 8°42′W	BD#1001
ALD	*N. bulbocodium*[a]	19	Spain	39°17′N, 6°18′W	KK#023
ALM	*N. cantabricus* DC.[a]	17	Spain	36°52′N, 4°32′W	KK#021
AMA	*N. romieuxii*	3	Morocco	31°13′N, 8°01′W	KK#001
BD13-7	*N. bulbocodium*	1	Spain	40°37′N, 4°02′W	BD#1102
BD13-8	*N. bulbocodium*	1	Spain	40°44′N, 4°01′W	BD#1101
CAT	*N. bulbocodium*[b]	20	Spain	42°40′N, 8°43′W	KK#027
HOR	*N. cantabricus*	17	Spain	38°34′N, 6°05′W	KK#022
HUE	*N. hedraeanthus* Colmeiro subsp. *luteolentus* (Barra & G. López) Aedo[a]	13	Spain	38°28′N, 3°46′W	KK#019
IDA	*N. cantabricus*	10	Morocco	30°44′N, 9°20′W	KK#003
JD11-1	*N. bulbocodium*[a]	1	Spain	39°02′N, 4°32′W	JDES#1101
JD11-10	*N. cantabricus*	1	Spain	38°28′N, 4°04′W	JDES#1110
	N. ×*litigiosus* Amo	1	Spain	38°28′N, 4°04′W	JDES#1111
JD11-14	*N. hedraeanthus* subsp. *luteolentus*	1	Spain	38°23′N, 3°27′W	JDES#1114
	N. ×*cazorlanus* Fern. Casas	1	Spain	38°23′N, 3°27′W	JDES#1115
JD11-16	*N.* ×*cazorlanus*	1	Spain	38°27′N, 3°19′W	JDES#1116
JD11-17	*N. hedraeanthus* subsp. *luteolentus*	2	Spain	38°31′N, 2°46′W	JDES#1117
JD11-19	*N. bulbocodium*	1	Spain	40°24′N, 1°26′W	JDES#1119
JD11-20	*N. bulbocodium*[a]	1	Portugal	40°16′N, 8°02′W	JDES#1120
JD11-3	*N. bulbocodium*[a]	1	Spain	39°01′N, 4°33′W	JDES#1103
	N. ×*fosteri* Lynch	2	Spain	39°01′N, 4°33′W	JDES#1104
JD11-7	*N.* ×*fosteri*[a]	1	Spain	39°07′N, 4°33′W	JDES#1107
JD11-8	*N. bulbocodium*	1	Spain	38°38′N, 4°05′W	JDES#1108
	N. cantabricus	1	Spain	38°38′N, 4°05′W	JDES#1109
JD12-8	*N. hedraeanthus* subsp. *hedraeanthus*	1	Spain	37°50′N, 3°03′W	JDES#1208
JOA	*N. bulbocodium*	10	Portugal	39°29′N, 8°50′W	JA#01
JTAZ	*N. romieuxii*	10	Morocco	34°03′N, 4°08′W	KK#012
JTIZ	*N. romieuxii*	7	Morocco	35°00′N, 4°53′W	KK#007
KET	*N. romieuxii*[b]	16	Morocco	34°57′N, 4°40′W	KK#008
LEUH	*N. romieuxii*	5	Morocco	33°25′N, 5°12′W	KK#014
MOUSS	*N. romieuxii*[b]	8	Morocco	33°07′N, 5°47′W	KK#017
NOR	*N. bulbocodium*[a]	11	Portugal	40°37′N, 8°10′W	KK#026
OUL	*N. romieuxii*	12	Morocco	33°22′N, 6°00′W	KK#018
OUR	*N. bulbocodium*	10	Morocco	31°20′N, 7°45′W	KK#002
POR	*N. obesus* Salisb.[a]	14	Spain	37°12′N, 7°04′W	KK#024
RIF	*N. cantabricus*	7	Morocco	35°01′N, 4°09′W	KK#010
SDA	*N. obesus*	9	Portugal	38°27′N, 9°01′W	KK#025
SDF	*N. cantabricus*	19	Spain	37°14′N, 2°16′W	KK#020
TAFR	*N. bulbocodium*	4	Morocco	29°43′N, 8°50′W	KK#006
TAN	*N. cantabricus*	13	Morocco	30°44′N, 9°21′W	KK#004
TARQ	*N. cantabricus*	8	Morocco	34°58′N, 4°23′W	KK#009
THAR	*N. cantabricus*	4	Morocco	34°40′N, 4°12′W	KK#011
THEO	*N. bulbocodium*	1	Spain	37°10' N; 7°43' W	TS#01
TIG	*N. cantabricus*[b]	13	Morocco	29°32′N, 9°21′W	KK#005
V	*N. bulbocodium*[a]	2	Spain	42°40′N, 7°15′W	KK#028
ZEKA	*N. romieuxii*	5	Morocco	34°03′N, 4°09′W	KK#013

Note: *N* = number of individuals.
[a] Population used in initial variability screening.
[b] Two individuals used from the same population in initial variability screening.

Appendix 2. Voucher information of samples from the living hoop-petticoat daffodil (*Narcissus* sect. *Bulbocodii*) collection at University of Reading (RNG), Reading, United Kingdom.

Species	Accession no.	*N*
N. bulbocodium	SJ001999[a]	1
N. cantabricus	SJ20597	1
N. romieuxii	BD96/198	1
N. 'Golden Bells'	GB_W[b]	1

Note: *N* = number of individuals.
[a] Sample used in initial variability screening.
[b] Sourced from Walkers Bulbs, Spalding, United Kingdom.