

**NEW MICROSATELLITE LOCI FOR *NARCISSUS PAPYRACEUS*  
(AMARILLYDACEAE) AND CROSS-AMPLIFICATION IN OTHER  
CONGENERIC SPECIES<sup>1</sup>**

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- *Premise of the study:* Microsatellite loci from a genomic library of the species *Narcissus papyraceus* were optimized and characterized for studies of population genetics.
- *Methods and Results:* Eleven markers that were successfully amplified showed polymorphism when tested on 50 individuals from two populations in southern Spain and northern Morocco. Overall, the number of alleles per locus ranged between 4 and 15. Between 8 and 11 loci successfully amplified in other eight *Narcissus* species.
- *Conclusions:* These markers will enable genetic diversity studies of *N. papyraceus* across its distribution range and conduct paternity analyses among individuals differing in flower morphology.

**Key words:** *Narcissus papyraceus*; Amarillydaceae; microsatellite; cross-amplification.

The genus *Narcissus* L. (Amarillydaceae) is well known for its morphological diversity due to the vast amount of within- and among-species floral variation affecting both perianth and sex organs. Both polymorphisms make the genus a good model system to study floral evolution and style polymorphism (Barrett et al., 1996; Pérez-Barrales et al., 2006). The genus is circummediterranean, and the highest diversity is found in the Iberian Peninsula and Northwestern Africa. One of the most common species occurring in the core of the center of diversity of the genus (southern Spain and northern Morocco) is *Narcissus papyraceus* Ker-Gawler. The species is self-incompatible and exhibits style dimorphism and a differential geographic distribution of style morph types (Arroyo et al., 2002) and perianth traits (Pérez-Barrales et al., 2007, 2009). Here we characterize 11 new polymorphic microsatellite loci for *N. papyraceus* and their cross-amplification in eight other *Narcissus* species collected in the field. These markers will be used to assess the genetic differentiation and structure across the species' distribution range, and conduct paternity analyses to investigate the ecological and genetic mechanisms underlying variation in style polymorphism.

**METHODS AND RESULTS**

Microsatellite libraries were developed following Jones et al. (2002) by Genetic Identification Services (www.genetic-id-services.com). Extracted DNA (approximately 100 µg) was digested with different standard restriction enzymes. Fragments were ligated and cloned into an *E. coli* strain. After incubation, a total of 100 randomly chosen recombinant clones were selected, purified, and sequenced. Finally, primer pairs were designed for all 100 clones. After visual inspection of the sequences, we selected a total of 29 primer pairs. Overall 50 individuals from two populations were employed to test the amplification and polymorphism of primer pairs. Populations were located in southern Spain (Cádiz; 36°8'N, 5°35'W; N = 35) and northern Morocco (Ouezzane; 34°49'N, 5°32'W; N = 15).

For primer testing, DNA was isolated from silica-dried leaf samples using a previously described protocol (Bernartzky and Tanksley, 1986) without mercaptoethanol. Polymerase chain reactions were performed in 25 µl of reaction mixture containing 50 ng/µl of template genomic DNA, 1× PCR buffer, 1.5 mM MgCl<sub>2</sub>, 1 mg/ml BSA, 0.034 µM forward primer, 0.25 µM reverse primer, 0.4 µM forward dye-labeled M13 primer, 0.05 mM each dNTP and 1.25 U Taq polymerase. Samples were incubated in a Touch-Down PCR in a Biometra TGradient Thermal Cycler, with an initial 5 min of denaturation at 94°C, 27 cycles at 94°C for 30 s, annealing at 67–43°C for 30 s (1°C decrease in each cycle), and extension at 72°C for 30 s, 17 cycles at 94°C for 30 s, 53°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 10 min. Polymerase chain reaction products were analyzed on an ABI 3130 × 1 Genetic Analyzer and sized using GeneMapper 4.0 (Applied Biosystems, Foster City, CA) and LIZ 500 size standard. Cross-amplification was conducted on two field-collected samples of eight *Narcissus* species (Fig. 1) following the same protocol described above.

After excluding those that did not amplify or were monomorphic, we selected 11 primer pairs that showed polymorphism (Table 1). The number of observed alleles per locus (A), observed heterozygosity ( $H_o$ ), gene diversity ( $H_e$ ) and tests for Hardy-Weinberg equilibrium (HWE) were calculated using GenAlEx v.6.3 software (Peakall and Smouse, 2006). Tests for linkage disequilibrium were performed using FSTAT v.2.9.3 software (Goudet, 1995).

The mean number of alleles per locus was 9.55 (range: 4–15) and 8.27 (range: 5–14) for the Spanish and Moroccan populations, respectively (Table 2). On average, observed heterozygosity was 0.47 (range: 0.16–0.91) and 0.41 (range: 0.07–0.93) for the Spanish and Moroccan populations, respectively (Table 2). Mean gene diversity was 0.710 (range: 0.436–0.885) and 0.734

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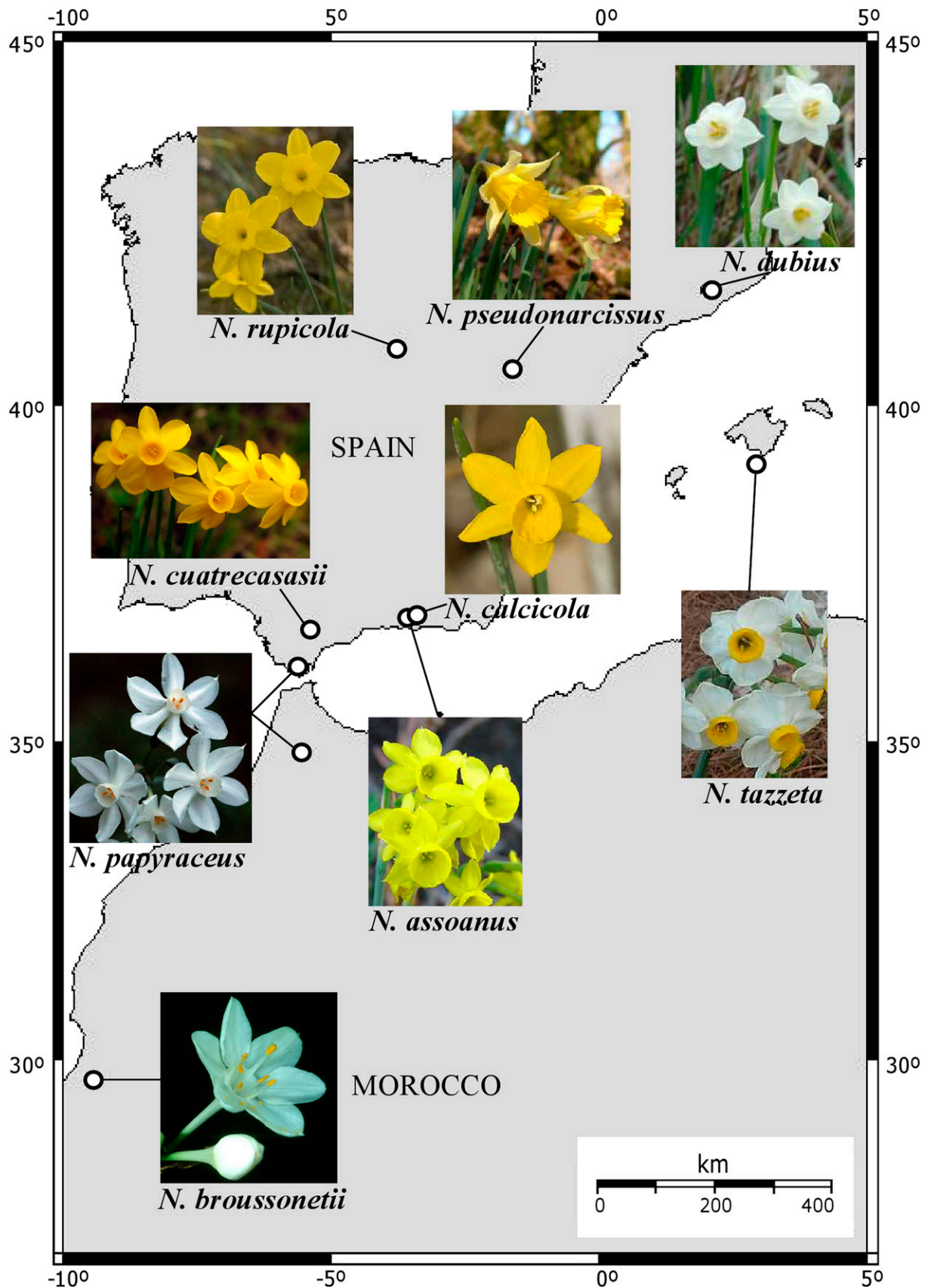


Fig. 1. Geographic location of sampling populations for *Narcissus papyraceus* and the other congeneric species of study in Spain and Morocco.

TABLE 1. Characteristics of 11 microsatellite loci of *Narcissus papyraceus*. GenBank accession numbers (below loci names), repeat motifs, forward (F) and reverse (R) primer sequences, allele size ranges and optimal annealing temperatures ( $T_a$ ) are given.

Locus (GenBank accession)	Repeat motif	Primer sequence (5' to 3')	Product size (bp)	$T_a$ <sup>a</sup>
A5 (GU271106)	AC <sub>23</sub>	F-CCACGATTCCAATATGAATTTG R-TATGCACACCTGGTATGTCAAG	238-298	58
A109 (GU271107)	TA <sub>10</sub> CA <sub>14</sub>	F-GATTGTCAACAAGCATGATATG R-ATGTCGAGTGGATATGGTTATG	100-132	57
A116 (GU271108)	CA <sub>26</sub>	F-GCCATGTTTTATGCCTGA R-ATCCTCACCGGAATCAAC	262-316	58
A121 (GU271109)	GT <sub>27</sub>	F-GGGAGGACCCATAATCAAGTA R-GCCTAATAAAGCTGCTATCCC	156-202	58
A131 (GU271110)	GT <sub>11</sub>	F-AGCTCTCTGTGTGTGTTTAC R-GGTGACCGTGTCAATTACAC	119-129	58
A134 (GU271111)	GT <sub>22</sub>	F-ACCTCGCTTATGGGTGAG R-ATTTGATACTCGTGGATGGATA	276-306	58
B7 (GU271112)	GA <sub>15</sub>	F-AACCGTTGTCTCCTCTATG R-TTCTCCCTCTCTTTCATTTT	136-184	57
B104 (GU271113)	GA <sub>16</sub>	F-CTGCTACACCATAGAGACACC R-ACATCCACTGGTAACAATCTG	156-176	59
B109 (GU271114)	TC <sub>10</sub>	F-TTCCAACAAGATAACGAACCT R-AAACCGAACCTACACTAAGAGG	179-191	58
B112 (GU271115)	TC <sub>18</sub>	F-CCATTCGACCACACCTACCT R-CCAAGCTCAAATCTTCGTC	286-332	59
B131 (GU271116)	GA <sub>24</sub>	F-AAACCCACCTTCAAACGA R-TGGAACTTGTGCCCATAC	162-186	59

<sup>a</sup> Temperature of annealing ( $T_a$ ) is given for nonlabeled primers.

(range: 0.570–0.883) for the Spanish and Moroccan populations, respectively (Table 2). A total of eight and nine microsatellite loci significantly departed from Hardy-Weinberg equilibrium for the Spanish and Moroccan populations, respectively (Table 2). The species' self-incompatible breeding system accounts for this result. None of the 11 microsatellite loci exhibited significant linkage disequilibrium ( $P > 0.002$  in all cases; nominal level = 0.0009). Between 8 and 11 microsatellite loci also amplified in the other *Narcissus* species (Table 3).

## CONCLUSIONS

These microsatellites will be adequate for genetic diversity studies across the species' distribution range in the Iberian

Peninsula and northwestern Africa, separated by the Strait of Gibraltar. This region has proved to harbor high biodiversity and biogeographical significance due to its complex history (Rodríguez-Sánchez et al., 2008). This will provide insights into the geographic structure of genetic diversity that reflects the evolutionary history of the species and the paleogeographical setting of the region. Furthermore, the characteristics of these markers make them suitable to conduct paternity analysis among *N. papyraceus* individuals, which will permit the understanding of the dynamics of flower morphology evolution. Finally, the successful cross-amplification of these markers allows the study of similar questions on other *Narcissus* species.

TABLE 2. Results of initial primer screening in two populations of *Narcissus papyraceus*. Sample size (N), number of alleles (A), observed heterozygosity ( $H_o$ ), gene diversity ( $H_e$ ) and  $P$ -values for the Hardy-Weinberg equilibrium (HWE) test are given for each marker and population.

Locus	Cádiz (Spain)					Ouezzane (Morocco)				
	N	A	$H_o$	$H_e$	HWE	N	A	$H_o$	$H_e$	HWE
A5	30	15	0.400	0.878	0.000*	15	14	0.933	0.882	0.378
A109	10	4	0.200	0.575	0.034*	14	12	0.214	0.883	0.000*
A116	26	15	0.385	0.885	0.000*	11	7	0.091	0.806	0.000*
A121	30	10	0.833	0.839	0.752	15	9	0.667	0.716	0.016*
A131	31	4	0.161	0.506	0.003*	15	5	0.067	0.598	0.000*
A134	34	13	0.912	0.731	0.466	15	7	0.733	0.591	0.031*
B7	21	12	0.190	0.885	0.000*	11	7	0.273	0.570	0.002*
B104	27	10	0.667	0.869	0.226	15	8	0.333	0.824	0.002*
B109	15	4	0.400	0.436	0.013*	12	5	0.333	0.646	0.004*
B112	26	10	0.308	0.482	0.000*	12	9	0.250	0.854	0.002*
B131	31	8	0.710	0.724	0.000*	15	8	0.600	0.704	0.182

\* Significant departure from HWE ( $P < 0.05$ ).

TABLE 3. Amplification of 12 microsatellite loci in eight *Narcissus* species. Plus and minus signs mean successful and unsuccessful amplifications, respectively.

Locus	Species <sup>a</sup>							
	<i>N. pseudonarcissus</i> subsp. <i>eugeniae</i>	<i>N. tazetta</i>	<i>N. assoanus</i>	<i>N. rupicola</i>	<i>N. cuatrecasasii</i>	<i>N. dubius</i>	<i>N. broussonetii</i>	<i>N. calcicola</i> <sup>c</sup>
A5	+	+	+	+	+	+	+	+
A109	+	—	—	—	—	+	+	—
A116	—	+	—	—	—	+	+	—
A121	+	+	+	+	+	+	+	+
A131	+	+	+	+	+	+	+	+
A134	+	+	+	+	+	+	+	+
A136 <sup>b</sup>	+	—	+	+	+	+	+	+
B7	+	+	+	+	+	+	+	+
B104	+	+	+	+	+	+	+	+
B109	+	—	—	—	+	—	—	+
B112	+	+	+	—	+	+	+	+
B131	—	+	—	+	+	+	+	—

<sup>a</sup> Geographical coordinates of populations: *N. pseudonarcissus* subsp. *eugeniae*: 40°29'N, 1°35'W; *N. tazetta*: 39°8'N, 2°56'E; *N. assoanus*: 36°51'N, 3°29'W; *N. rupicola*: 40°48'N, 3°46'W; *N. cuatrecasasii*: 36°41'N, 5°22' W; *N. dubius*: 41°37'N, 2°1'E; *N. broussonetii*: 29°39'N, 9°26'W; *N. calcicola*: 36°53'N, 3°24'W.

<sup>b</sup> This marker was monomorphic in *N. papyraceus*. Primer pairs: F-ACTTTGAGTCCGCTTCAG and R-ACACCCTTTATGTTGAGTGC; motif: (CA<sub>13</sub>) GATATA (CA<sub>9</sub>); product size: 182 bp; temperature of annealing: 58°C.

<sup>c</sup> The population sampled of *N. calcicola* is highly isolated from the core range of the species in central Portugal.

LITERATURE CITED

ARROYO, J., S. C. H. BARRETT, R. HIDALGO, AND W. COLE. 2002. Evolutionary maintenance of stigma-height dimorphism in *Narcissus papyraceus* (Amarillydaceae). *American Journal of Botany* 89: 1242–1249.

BARRETT, S. C. H., D. G. LLOYD, AND J. ARROYO. 1996. Styler polymorphisms and the evolution of heterostyly in *Narcissus* (Amarillydaceae). In: D. G. Lloyd and S. C. H. Barrett [eds.], *Floral biology: Studies on floral evolution in animal-pollinated plants*, 339–376. Chapman & Hall, New York, USA.

BERNARTZKY, R., AND S. TANKSLEY. 1986. Genetics of acting-related sequences in tomato. *Theoretical and Applied Genetics* 72: 314–324.

GOUDET, J. 1995. FSTAT (Version 1.2): a computer program to calculate F-statistics. *The Journal of Heredity* 86: 485–486.

JONES, K. C., K. F. LEVINE, AND J. D. BANKS. 2002. Characterization of 11 polymorphic tetranucleotide microsatellites for forensic applications in California elk (*Cervus elaphus canadensis*). *Molecular Ecology Notes* 2: 425–427.

PEAKALL, R., AND P. E. SMOUSE. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* 6: 288–295.

PÉREZ-BARRALES, R., J. ARROYO, AND W. S. ARMBRUSTER. 2007. Differences in pollinator faunas may generate geographic differences in floral morphology and integration in *Narcissus papyraceus* (Amarillydaceae). *Oikos* 116: 1904–1918.

PÉREZ-BARRALES, R., R. PINO, R. G. ALBALADEJO, AND J. ARROYO. 2009. Geographic variation of flower traits in *Narcissus papyraceus* (Amarillydaceae): Do pollinators matter? *Journal of Biogeography* 36: 1411–1422.

PÉREZ-BARRALES, R., P. VARGAS, AND J. ARROYO. 2006. New evidence for the Darwinian hypothesis of heterostyly: Breeding systems and pollinators in *Narcissus* sect. Apodanthi. *The New Phytologist* 171: 553–567.

RODRÍGUEZ-SÁNCHEZ, F. J., R. PÉREZ-BARRALES, F. OJEDA, P. VARGAS, AND J. ARROYO. 2008. The strait of Gibraltar as a melting pot for plant biodiversity. *Quaternary Science Reviews* 27: 2100–2117.