

# *Jumping through the hoops: the challenges of daffodil (Narcissus) classification*

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# **Jumping through the hoops: the challenges of daffodil classification**

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8

9 Running title: Hoop-petticoat daffodils

10

11 ABSTRACT

12

13 Hoop-petticoat daffodils are a morphologically congruent group that has two distinct lineages  
14 in the molecular phylogeny of *Narcissus*. It is possible that the morphological similarity is a  
15 product of both historic and current low-level gene flow between these lineages. For the first  
16 time we report population sampling from across the entire range of distribution covering both  
17 the Iberian Peninsula and Morocco. In total 455 samples were collected from 59 populations.  
18 Plastid DNA sequences of *matK* and *ndhF* were generated alongside 11 microsatellite loci to  
19 permit comparison between plastid and nuclear lineage history. The plastid DNA phylogeny  
20 was highly congruent with previous molecular studies and supported the recognition of these  
21 two lineages of hoop-petticoat daffodils as separate sections. Assignment of samples to  
22 sections sometimes differed between plastid DNA and (nuclear) microsatellite data. In these  
23 cases, the taxa had previously been the focus of dissent in taxonomic placement based on  
24 morphology. These discrepancies could be explained by hybridisation and introgression  
25 among the two lineages during the evolution of hoop-petticoat daffodils and shows that  
26 placement of species in sections is dependent on the source of data used. This study  
27 underlines the complex evolutionary history of *Narcissus* and highlights the discrepancies  
28 between floral morphology and phylogeny, which provides a continuing challenge for the  
29 systematics of *Narcissus*.

30

31 KEYWORDS: *Narcissus*, *matK*, microsatellites, DAPC, *N. obesus*, *N. luteolentus*

32

## 33 INTRODUCTION

34

35 Daffodils, the genus *Narcissus* L., are one of the most iconic spring flowers, yet the taxonomy of  
36 the genus, which underpins their conservation and breeding, remains in flux. The last  
37 comprehensive monographic revision of the genus, at species level, was by Baker in 1875  
38 (Burbidge & Baker, 1875), although the system of subgenera and sections proposed by Fernandes  
39 (1968, 1975) has been largely followed in subsequent publications on the genus. The genus is  
40 split into two subgenera: *Narcissus* and *Hermione* (Haw.) Spach and 10-14 sections (Figure  
41 1) (Fernandes, 1968; Aedo, 2013; Marques *et al.*, 2017). Recent changes in the delimitation  
42 of sections mean that the established approach of using morphology to allocate species to  
43 sections is no longer reliable. Some sections are best defined on DNA evidence.

44

45 *Narcissus* section *Bulbocodii* DC. has traditionally been distinguished from the rest of  
46 *Narcissus* by the large funnel-shaped corona relative to the narrow tepals, a right angled  
47 attachment of the anther to the filament, and the declinate stamens and stigma which gives  
48 rise to a zygomorphic flower (Fernandes, 1968; Blanchard, 1990). This distinctive corona  
49 shape has given rise to the English common name “hoop-petticoat daffodil”. The recognition  
50 of these daffodils dates to the late 16<sup>th</sup> and the early 17<sup>th</sup> century (David & Könyves, 2013).  
51 The integrity of this group had not been questioned by morphological studies. This distinctive  
52 group has been recognised at different taxonomic ranks, ranging from genus (*Corbularia*;  
53 Salisbury, 1812), or subgenus (*N. subgenus Corbularia* Pax, 1888), to section (*N. section*  
54 *Bulbocodii*, De Candolle, 1815) and even as a single species (Baker in Burbidge & Baker,  
55 1875). The recognition of hoop-petticoat daffodils as a distinct genus has never been  
56 accepted widely, but a number of authors have treated it at subgeneric level (Ascherson &  
57 Graebner, 1907; Maire, 1959; Mathew, 2002). However the most common treatment of hoop-

58 petticoat daffodils, supported by morphological and cytological evidence, is as section  
59 *Bulbocodii* (Fernandes, 1934; Webb, 1978; Zonneveld, 2008; Aedo, 2013; Fennane, 2015).  
60  
61 Hoop-petticoat daffodils are distributed from southwest France through the Iberian Peninsula,  
62 to Morocco and northwest Algeria. Natural populations of hoop-petticoat daffodils show a  
63 great range of morphological variation (Figure 2), that has resulted in disagreement in the  
64 number of taxa and the level at which they have been recognised (Fernandes, 1963; Webb,  
65 1978; Barra & López González, 1982; Fernández Casas, 1986a, 1996; Barra Lázaro, 2002;  
66 Vázquez Pardo, 2013). In addition to the morphological diversity within the basic hoop-  
67 petticoat ground plan, along with a propensity for hybridisation between species (Blanchard,  
68 1990; Aedo, 2013), a wide range of chromosome numbers have been reported (Fernandes,  
69 1934, 1963; Zonneveld, 2008). All of these factors have led to an unstable classification  
70 indicated by the number of recognised taxa at species level or below ranging from 4-35  
71 (Könyves, 2014).  
72  
73 Recent molecular evidence has indicated that the evolutionary history and taxonomy of  
74 section *Bulbocodii* is more complex than first thought. The first molecular study of *Narcissus*  
75 (Graham & Barrett, 2004) used plastid DNA data and identified two separate clades  
76 comprising the section *Bulbocodii sensu* DC. making the section polyphyletic. Later studies  
77 with wider taxonomic sampling across *Narcissus* showed the same polyphyletic pattern in  
78 section *Bulbocodii* (Marques, 2010; Santos-Gally, Vargas, & Arroyo, 2012; Fonseca *et al.*,  
79 2016). Moreover, other sections also show polyphyly: section *Tazettae* DC. (Santos-Gally *et*  
80 *al.*, 2012), section *Jonquillae* DC. and section *Pseudonarcissi* DC. (Marques, 2010). These  
81 results suggest that extensive hybridisation and subsequent gene flow may have contributed  
82 to the complex genetic history of *Narcissus*. The most comprehensive study of the genus to

83 date, Marques *et al.* (2017), using markers from three genomes (plastid, mitochondrial, and  
84 nuclear ribosomal), found hoop-petticoat daffodils to be polyphyletic in both organellar and  
85 nuclear datasets. This led to the formal split of section *Bulbocodii* and the recognition of  
86 section *Meridionalis* I.Marques, Fuertes, Martins-Loução, Moharrek & Nieto Fel. to include  
87 some species previously in *N.* section *Bulbocodii*. These two sections are distinguished on  
88 molecular evidence and there are, as yet, no clear morphological characters to separate them.  
89  
90 *Narcissus* is most diverse in the Iberian Peninsula and Northern Africa, and current  
91 treatments of this genus in these areas are synthetic accounts. The accounts vary in their  
92 scope, breadth of new knowledge added, and the chosen breadth of species concept used to  
93 encompass morphological diversity. The most recent account of taxa occurring in the Iberian  
94 Peninsula is the treatment in *Flora iberica* (Aedo, 2013). This treatment is a detailed revision  
95 and employs broadly defined species and recognises only a few infraspecific taxa. In contrast,  
96 the treatment in *Flore Pratique du Maroc* (Fennane, 2015) is a compilation of current  
97 knowledge with a narrower species concept applied. This leads to problems in comparing  
98 different treatments of hoop-petticoat daffodils as there is only partial overlap of the taxa  
99 between the Iberian Peninsula and Morocco and the species circumscriptions, even under the  
100 same name, differ. For example, *Narcissus bulbocodium* L. is accepted in both accounts,  
101 however Aedo (2013) treats *N. bulbocodium* as a variable species including *N. obesus* Salisb.  
102 as a synonym, in contrast to the view of Webb for *Flora Europaea* (1978) who treats it as  
103 subsp. *obesus* (Salisb.) Maire, or Zonneveld (2008) who treats it as a species: *N. obesus*. A  
104 further issue is the treatment of *N. obesus* in Morocco. Maire (1959) treats it as *N.*  
105 *bulbocodium* var. *obesus* (Salisb.) Baker. Fernandes (1968) also notes its presence in  
106 Morocco, as *N. obesus*. However, Fennane (2015) treats the Moroccan *N. obesus* as a  
107 synonym of *N. tingitanus* Fern.Casas, which has now been sunk into *N. albicans* (Haw.)

108 Spreng. (Fernández Casas, 2016). There is strong molecular evidence for the acceptance of  
109 *obesus* as a distinct taxon (Fonseca *et al.*, 2016; Marques *et al.*, 2017) at species or  
110 subspecies level, based on predominantly Iberian material. *Narcissus cantabricus* DC. is also  
111 treated differently in the two accounts: Fennane (2015) recognises two subspecies in his  
112 account of Moroccan *Narcissus*, while Aedo (2013) does not for his account of species of the  
113 Iberian Peninsula, even though one of the subspecies in Morocco has also been reported from  
114 SE Spain (Fernandes, 1968).

115

116 A particularly controversial taxon is the entity originally described as *N. cantabricus* subsp.  
117 *luteolentus* Barra & G.López (Barra & López González, 1982), that has been treated as a  
118 species, *N. blancoi* Barra & G.López (Barra Lázaro & López González, 1992) or included in  
119 *N. albicans* (Fernández Casas, 2016) or as a possible synonym of *N. hedraeanthus* (Webb &  
120 Heldr.) Colmeiro (Fernández Casas, 1984). The taxon was transferred to *N. hedraeanthus*  
121 subsp. *luteolentus* (Barra & G.López) Aedo by Aedo (2013) based on morphology. However,  
122 Fonseca *et al.* (2016) supported its assignment to *N. cantabricus* based on plastid DNA  
123 evidence.

124

125 For the remainder of the section Fennane (2015) accepts three species, all endemic to  
126 Morocco. *Narcissus romieuxii* Braun-Blanq. & Maire is treated as an ancient allopolyploid  
127 hybrid of *N. bulbocodium* and *N. cantabricus* (Fernandes, 1959). The other two species, *N.*  
128 *peroccidentalis* Fern.Casas and *N. tingitanus* were described by Fernández Casas (1996),  
129 however he has since reduced these to synonymy with *N. albicans*. This is a confused name  
130 applied to two different entities, a hoop-petticoat daffodil and a trumpet daffodil (Kington,  
131 2008). Fernández Casas (2016) typified the name *N. albicans* on the hoop-petticoat daffodil.  
132 Furthermore, two species from southern Morocco, *N. jacquemoudii* Fern.Casas and *N.*



133 *jeanmonodii* Fern.Casas, have been accepted by some (Mathew, 2002; Rankou *et al.*, 2015;  
134 Marques *et al.*, 2017), but are treated as synonyms of *N. romieuxii* by Fennane (2015).

135

136 To confound matters further, there are natural hybrids with other sections (Kington, 2008;  
137 Aedo, 2013). Our study includes hybrids between a number of hoop-petticoat daffodils and  
138 *N. triandrus* L. (sect. *Ganymedes* (Haw.) Schult.f.). Furthermore, there are crosses with other  
139 sections with limited geographic range mostly occurring in the Iberian Peninsula (Fernández  
140 Casas, 1986b, 1993).

141

142 The understanding of daffodil dispersal and the establishment of hybrid populations is based  
143 on a very small number of studies. In a study of *N. longispathus* (subsection *Pseudonarcissi*)  
144 Barrett, Cole, & Herrera (2004) reported limited pollen flow and seed dispersal. However, in  
145 a study of *N. cavanillesii* hybrids (section *Braxireon*) F1 progeny were found to have a  
146 fitness advantage in establishment and later vegetative propagation (Marques *et al.*, 2011),  
147 that allows the long-term perennation of these populations in the wild. No such studies are  
148 yet published for *N.* section *Bulbocodii sensu* Marques and *N.* section *Meridionalis*.

149

150 The various treatments of the hoop-petticoat daffodils both within the Iberian Peninsula, and  
151 for Iberia and North Africa together, illustrate clearly the challenges to finding a consistent  
152 taxonomy of the group. Interpretation of findings of previous molecular studies have been  
153 constrained by limited geographic and within-species sampling. There is a need for  
154 population level sampling across the entire range of these species. Here we use novel  
155 microsatellite and plastid DNA data to examine populations of sections *Bulbocodii* and  
156 *Meridionalis* throughout most of their distribution at a level of detail not previously  
157 attempted.

158

## 159 MATERIALS AND METHODS

160

### 161 PLANT MATERIALS

162

163 Material was collected in the form of either silica dried leaf material or as living plants across  
164 the natural distribution of *Narcissus* sections *Bulbocodii sensu* Marques and *Meridionalis*.  
165 Herbarium vouchers were collected for each sampled population. Collecting permits were  
166 obtained from the local authorities (Universities in Morocco; National Parks and Regional  
167 Governments in Spain and Portugal). In total 455 samples were collected from 59  
168 populations (Table S1). Populations were sampled according to accessibility or by haphazard  
169 sampling (Lowe, Harris, & Ashton, 2004) ensuring at least 5 metres between samples to limit  
170 sampling of ramets. To maximise genetic variation, a minimum of 10 individuals were  
171 collected per population where possible. The sampling strategy was designed to sample  
172 genetic variation without endangering small populations. A combination of descriptions from  
173 Blanchard (1990) and Aedo (2013) was used for identification of samples.

174

175 To help elucidate the relationship of hoop-petticoat daffodils, 78 additional samples  
176 representing 24 taxa from other sections were also collected following the same procedures  
177 or purchased (suppliers listed in Table S2). Sequences for one additional daffodil, *N. tazetta*  
178 L., and three *Sternbergia* Waldst. & Kit. species for outgroups, were downloaded from  
179 GenBank (*N. tazetta*: HM011047 & HM011012; *S. greuteriana* Kamari & R.Artelari:  
180 HM011031 & HM010997; *S. lutea* (L.) Ker Gawl. ex Spreng.: HM011025 & HM010992; *S.*  
181 *sicula* Tineo ex Guss.: HM011014 & HM010984, *matK* and *ndhF* respectively), voucher  
182 specimens given in Gage *et al.* (2011).

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## MICROSATELLITE METHODS

Total genomic DNA was extracted using a modified CTAB protocol (Doyle & Doyle, 1987). Eleven of the 15 hoop-petticoat daffodil microsatellite markers reported in Könyves *et al.* (2016) were amplified following their protocol. The allele dosage of polyploids cannot readily be established, therefore traditional population genetic techniques which rely on correct allele frequencies (e.g. STRUCTURE, Pritchard *et al.*, 2000) cannot be applied. 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 and used discriminant analysis of principal components to identify clusters within the microsatellite dataset (DAPC, Jombart *et al.*, 2010). To characterise the variability of the selected markers the total number of alleles per marker, the number of alleles per individual, the mean number of alleles per individual and the observed heterozygosity ( $H_o$ ) were calculated. To assess the variation between markers and within samples, and therefore the preliminary identification power of the markers, an allelic diversity was calculated as the complement of Simpson's diversity ( $D=1 - \sum\{p_i(p_i - 1)/N(N - 1)\}$ ), where  $p_i$  is the frequency with which the  $i$ th allele was detected). As some of the markers exhibited no alleles in some samples (null genotypes) the proportion of null genotypes was also calculated. The presence of null genotypes was confirmed by repeated PCR amplifications using both a labelled and unlabelled forward primer, to rule out user error or possible adverse effect of the dye labelling. The presence of null genotypes was expected due to incomplete transferability of these markers in hoop-petticoat daffodils. Five samples (two samples from population *Hue*, one sample each from populations *Ald*, *JTiz*, and *Sdf*; Table S1), that had failed fragment analysis after successful PCR of one or more loci, were removed. In total 450 hoop-petticoat

208 samples were included in the analysis. DAPC was conducted using the package *adegenet* ver.  
209 1.3–9.2 (Jombart & Ahmed, 2011) in the statistical program *R* ver. 3.0.2 (R Development  
210 Core Team, 2013).

211

212 The optimal number of clusters in the dataset was determined using the guidelines of Jombart  
213 (2013). The model was run for  $10^6$  iterations to search for convergence, retaining principal  
214 components that explained 95% of the variance. All eigenvalues were retained, as the number  
215 of the clusters was small. The clustering analysis identified four clusters. The two most  
216 populous ones, Clusters 1 and 3, were further analysed using DAPC to elucidate any  
217 additional genetic structure.

218

## 219 PLASTID DNA METHODS

220

221 A section of the *matK* region was amplified with primers *matK* 2.1 and *matK* 5 or, in the case  
222 of weak amplification, with primers *matK* X and *matK* 5 (Ford *et al.*, 2009). PCR reactions  
223 were performed in 30 $\mu$ l volumes containing final concentrations of 1 $\times$  Bioline Biomix  
224 (Bioline Reagents Ltd., London, UK), 0.35 $\mu$ M of each primer, 0.13mg/ml BSA (bovine  
225 serum albumin), 2.67% v/v DMSO (dimethyl sulfoxide) and 15ng DNA template. A few  
226 samples failed to amplify with any of the *matK* primer combinations. For these *trnK* was  
227 amplified, using primers *trnK* 570F and *trnK* 1710R (Samuel *et al.*, 2005), as these flank the  
228 entire *matK* region. The PCR protocol for *trnK* was 1 $\times$  Bioline Biomix, 0.35 $\mu$ M of each  
229 primer, 0.2 g/ml BSA, 4% v/v DMSO and 15ng DNA template in 50 $\mu$ l total volume. The 3'  
230 end of *ndhF* was amplified with primers *ndhF* 745F and *ndhF* 2110R (Terry, Brown, &  
231 Olmstead, 1997) for 45 samples representing all recovered *matK* haplotypes. PCR reactions  
232 were each performed in 50 $\mu$ l volumes containing final concentrations of 1 $\times$  Bioline Biomix,

233 0.35 $\mu$ M of each primer, 0.2 mg/ml BSA (bovine serum albumin), and 15ng of DNA template.  
234 PCR cycling conditions for all amplified regions are listed in Table 1.

235

236 The PCR products were separated on 1% agarose gels in 1 $\times$  TAE buffer stained with 0.3  
237  $\mu$ gml<sup>-1</sup> ethidium bromide. Gels were illuminated with UV light and photographs were taken  
238 to record the presence of PCR amplicons. Approximate size and concentration of the PCR  
239 amplicons was determined using HyperLadder™ 1kb (Bioline Reagents Ltd, London, UK).  
240 Direct sequencing of PCR products was carried out by Beckman Coulter (UK) Ltd, High  
241 Wycombe, UK; Source BioScience, Nottingham, UK; and Macrogen Europe, Amsterdam,  
242 Netherlands in both forward and reverse direction. Sequence trace files were assembled and  
243 edited using Seqman II (DNASTar, Inc., Madison, WI, USA). Sequences were aligned with  
244 the MUSCLE algorithm using the default parameters (Edgar, 2004) implemented in ebioX  
245 1.6 (Martínez Barrio *et al.*, 2009). The ends of the alignments were trimmed to the point  
246 where all sequences were present and base calls were unambiguous.

247

248 To explore the relationships between the sampled populations and other sampled *Narcissus* a  
249 statistical parsimony network was constructed from the *matK* dataset using TCS ver. 1.21  
250 (Clement, Posada, & Crandall, 2000) under the 95% statistical parsimony criterion.

251 Phylogenetic trees were constructed through Bayesian inference (BI) in MrBayes ver. 3.2  
252 (Ronquist *et al.*, 2012) according to the best-fit model of evolution identified by MrModeltest  
253 ver. 2.3 (Nylander, 2004). To avoid overfitting of the model, BI analysis of the *matK* dataset  
254 was performed using only the haplotypes identified by TCS. BI analysis of the combined  
255 dataset including the *matK* haplotypes and the corresponding *ndhF* sequences was used to  
256 improve the resolution of the *matK* phylogenetic tree. The incongruence of the *matK* and  
257 *ndhF* datasets was assessed with the incongruence length difference (ILD) test in PAUP\*

258 4.0b 10 (Swofford, 2003). All BI analyses were conducted with two separate runs, each of  
259 four chains. The analyses for the *matK* and the combined datasets were run for 2,500,000 and  
260 5,000,000 generations respectively, sampling every 1000 generations. Autocorrelation of the  
261 sampled generations was tested in Microsoft Excel 2011 by checking the correlation between  
262 subsequent generations. Burn-in was identified by assessing convergence with Tracer ver. 1.5  
263 (Rambaut & Drummond, 2009). Trees from the first 25% of the sampled generations were  
264 discarded.

265

## 266 GENETIC DIVERSITY ESTIMATORS

267

268 For each taxon, the number of haplotypes, the total number of different alleles across all loci  
269 ( $A$ ), the number of private alleles across all loci ( $A_p$ ) and the genotypic richness ( $R=G-1/N-1$ ;  
270 where  $G$  is the number of multilocus genotypes and  $N$  is the number of genotyped samples,  
271 Dorken & Eckert 2011) were calculated. Multilocus genotypes for each sample were  
272 identified using the *R*-library *polysat* ver. 1.3-2 (Clark & Jasieniuk, 2011). Samples with zero  
273 distance were considered to belong to the same multilocus genotype.

274

## 275 RESULTS

276

### 277 MICROSATELLITE VARIATION

278

279 A summary of the variability within microsatellite markers is given in Table 2. The number  
280 of alleles per locus ranged from five to 25, while the observed heterozygosity ( $H_o$ ) was  
281 between 0.138 and 0.424. Most of the samples appeared homozygous with the mean number  
282 of alleles per individual ranging from 1.099 to 1.532. The allelic diversity of the amplified

283 markers was between 0.353 and 0.832, while the frequency of null genotypes ranged from  
284 0.4% to 30%.

285

286 The DAPC of 450 individuals revealed a separation of the microsatellite dataset into four  
287 main clusters (Figure 3 A). *Narcissus bulbocodium* populations were assigned to clusters 1, 2  
288 and 3. *Narcissus cantabricus* and *N. romieuxii* were in Cluster 3. The two subspecies of *N.*  
289 *hedraeanthus* formed Cluster 4. *Narcissus obesus* was indistinguishable from *N.*  
290 *bulbocodium* samples included in Cluster 1. The hybrid individuals were assigned to clusters  
291 including the hoop-petticoat daffodil parent (apart from one individual of *N. × fosteri* Lynch,  
292 a hybrid of *N. bulbocodium* and *N. triandrus*, and one individual of *N. × cazorlanus*  
293 Fern.Casas, hybrid of *N. hedraeanthus* and *N. triandrus*, which were assigned to Cluster 3  
294 with *N. cantabricus* and *N. romieuxii*). A further DAPC conducted on Cluster 1 detected two  
295 sub-clusters (Figure 3 B). These represented the *N. bulbocodium* *N. × fosteri* (Sub-cluster 1.1)  
296 and *N. obesus* populations (Sub-cluster 1.2). The further DAPC conducted on Cluster 3  
297 detected three sub-clusters (Figure 3 C). The three sub-clusters do not appear to correlate  
298 with established taxonomic groups.

299

### 300 PLASTID DNA VARIATION

301

302 The total aligned and analysed length of the *matK* dataset was 836bp including a 6bp  
303 insertion. TCS identified 46 different haplotypes (GenBank accession numbers: XXXXXX -  
304 XXXXXX) and created two unconnected networks, corresponding to subgenus *Hermione*  
305 (h43-h46) and subgenus *Narcissus* (Figure 4 A).

306

307 The hoop-petticoat daffodil sequences were grouped into 22 haplotypes, the remaining 20  
308 haplotypes belonged to other daffodil samples in subgenus *Narcissus*. *Narcissus* section  
309 *Bulbocodii sensu* Marques and section *Meridionalis* appeared as two distantly related groups,  
310 separated by a minimum of 22bp differences. The *matK* (Figure S1), and the combined *matK*  
311 and *ndhF* BI analyses (Figure 5) recovered a topology congruent with Marques *et al.* (2017).  
312 The correspondence of the DAPC and plastid DNA results is shown in Figure 5. The  
313 correspondence of floral morphology and sectional classification is shown in Figure 4.  
314  
315 The DAPC results (Figure 3 A, B) showed *N. obesus* to be grouped with *N. bulbocodium* in  
316 section *Bulbocodii sensu* Marques, however, the plastid DNA analyses placed it in section  
317 *Meridionalis*. *Narcissus hedraeanthus* subsp. *luteolentus* was placed in the same cluster as *N.*  
318 *hedraeanthus* subsp. *hedraeanthus* by DAPC, but it was indistinguishable from *N.*  
319 *cantabricus* and *N. romieuxii* in the plastid DNA analysis. The plastid DNA analyses and  
320 DAPC differ in the placement of some samples carrying haplotypes H29, H32 and H34: the  
321 former placing them in section *Bulbocodii*, the latter in section *Meridionalis* (Figure 4). Three  
322 of these populations occur in southern Morocco and two in central Spain. The geographic  
323 distribution of the plastid DNA haplotypes and the DAPC clusters is shown in Figure 6.

324

## 325 DIVERSITY ESTIMATORS

326

327 *Narcissus* section *Bulbocodii sensu* Marques, comprising purely *N. bulbocodium* samples in  
328 our analysis, contained 13 different plastid DNA haplotypes, and 34 private microsatellite  
329 alleles. In comparison section *Meridionalis*, comprising five taxa, also had 13 haplotypes, but  
330 only 15 private alleles. Genotypic richness (*R*) was 1 in all cases except *N. cantabricus*,  
331 which was 0.95 (Table 3).



332

## 333 DISCUSSION

334

335 The DAPC and plastid DNA analyses, presented here, each recovered two separate hoop-  
336 petticoat daffodil groups. These two groups correspond with the two hoop-petticoat daffodil  
337 sections recognised by Marques *et al.* (2017). Our results support the recognition of these  
338 sections by recovering the divergent groups through analysis of microsatellite markers, which  
339 have broader coverage of the nuclear genome than ITS used by Marques *et al.* (2017). The  
340 recognition of the two sections is strongly supported by molecular evidence, but the putative  
341 morphological markers for these sections are not definitive. The two distinct lineages (Figure  
342 5) have an apparently identical range of floral morphology (Figure 2 A-H section  
343 *Meridionalis*, Figure 2 I-L as section *Bulbocodii sensu* Marques). This emphasis on floral  
344 morphology to distinguish taxa has led to other characters being overlooked that may  
345 distinguish these sections. A parallel can be drawn with the relationships within *Scilla* L. *s.l.*  
346 where taxonomy based on floral characters is inconsistent with the taxa recognised using  
347 non-floral characters (Speta 1998a; Speta 1998b), the latter appearing to be supported by  
348 molecular data (Ali *et al.*, 2012).

349

350 The sampling employed by Fonseca *et al.* (2016) and Marques *et al.* (2017) focused mostly  
351 on Iberian hoop-petticoat daffodils. Our study includes much wider sampling in Morocco and  
352 in section *Meridionalis* than these earlier studies, which gives a more comprehensive picture  
353 of the species relationships among hoop-petticoat daffodils. As the two widely sampled  
354 species, *N. cantabricus* and *N. romieuxii*, could not be separated with plastid DNA analyses,  
355 there were no grounds, based on these data, to evaluate their infraspecific taxa. *Narcissus*  
356 *romieuxii* is endemic to Morocco and is an ancient allotetraploid hybrid of *N. bulbocodium*  
357 and *N. cantabricus* (Fernandes, 1959). Its flower colour appears intermediate, ranging from

358 white to whitish-yellow (Fernandes, 1959). The results of the microsatellite analyses show  
359 the same pattern, a cluster including *N. cantabricus* and *N. romieuxii* (Cluster 3), without  
360 clear separation between them. The plastid DNA results indicate that *N. cantabricus* was the  
361 seed parent of *N. romieuxii*, while the microsatellite results indicate introgression of *N.*  
362 *romieuxii* and *N. cantabricus*. Due to the limited information provided in the original  
363 descriptions of *N. peroccidentalis* and *N. tingitanus*, it is uncertain whether samples  
364 attributable to these species were collected and analysed for this study. From their brief  
365 descriptions, and the more recent synonymy with *N. albicans*, as circumscribed by Fernández  
366 Casas (2016), they would likely be part of section *Meridionalis*.

367

368 Thirteen taxa have been described below species level in *N. bulbocodium*, more than in any  
369 other species of hoop-petticoat daffodil and this variation seems to be reflected in the genetic  
370 diversity measured by plastid DNA. This species had the highest haplotype diversity,  
371 carrying 13 different haplotypes, but the DAPC showed more uniform genetic structure, with  
372 most samples assigned to sub-cluster 1.1. However, two *N. bulbocodium* populations (*Ald,*  
373 *Edc*) in central Spain were assigned to Cluster 2. These plants also carried *matK* haplotypes  
374 exclusive to them, but they appeared morphologically similar to the rest of the *N.*  
375 *bulbocodium* samples. So far, no corresponding morphological discontinuity has been  
376 identified to explain this variation. Apart from the treatment of *N. obesus* (discussed later), *N.*  
377 *bulbocodium* is widely treated as a single variable species (Webb, 1978; Aedo, 2013;  
378 Fennane, 2015; Fonseca *et al.*, 2016; Marques *et al.*, 2017). However, recently Fernández  
379 Casas (2017a,b) delineated two species that broadly belong to *N. bulbocodium*: one, *N.*  
380 *saltuum* Fern.Casas, was new to science and reported to occur in Northern Spain; the other,  
381 *N. tenuifolius* Salisb., although originally described by Salisbury (1796) and long regarded as  
382 a synonym of *N. bulbocodium*, is applied by Fernández Casas to plants from the southern tip

383 of Andalusia. Neither of these species overlap geographically with the populations in Cluster  
384 2 or any other sampled populations. Moreover, Fonseca *et al.* (2016) treats localized but  
385 unresolved plastid DNA variation as evidence for infraspecific taxa in *N. bulbocodium*,  
386 recognizing four subspecies and five varieties.

387

388 Three populations in Southern Morocco further highlighted the complex genetic variation  
389 found in *N. bulbocodium*. Two of these, *Our* and *Tafr*, were identified as *N. bulbocodium* in  
390 the field and this was confirmed with plastid DNA evidence, however DAPC of the  
391 microsatellites places these same populations in section *Meridionalis* with *N. cantabricus* and  
392 *N. romieuxii*. In contrast, the third population, *Tig*, was field-collected as *N. cantabricus*  
393 (Figure 2 I), but identified as *N. bulbocodium* with plastid DNA, however DAPC supported  
394 the field identification. This pattern is congruent with a hybrid origin for these populations  
395 with either *N. bulbocodium* and *N. cantabricus* (or *N. romieuxii*) as the seed parent, and  
396 shows more recent hybridisation. This pattern could also explain the differing treatments of  
397 *N. jacquemoudii* and *N. jeanmonodii* both by Marques *et al.* (2017) and Fennane (2015). In  
398 the former these are recognized species belonging to section *Bulbocodii sensu* Marques, but  
399 treated as synonyms of *N. romieuxii* by the latter author, and therefore part of section  
400 *Meridionalis*. Future sampling in the High Atlas, the type locality of both species, may help  
401 to resolve this.

402

403 A population in Spain (*JD11-8*), from which two plants were sampled, included one  
404 morphologically typical of *N. bulbocodium* and one typical of *N. cantabricus*, and while each  
405 sample grouped in its respective cluster in DAPC, both carried the same *N. bulbocodium*  
406 *matK* haplotype. This is most easily explained by gene flow through introgression. The two  
407 species rarely occur together on the Iberian Peninsula (Barra, Blanco, & Grijalbo, 2011). It is

408 possible that the rarity of hybrids between the two in the Iberian Peninsula is due to their  
409 differences in geographical range and possibly ecological preferences. However, this shows  
410 that patterns similar to that in southern Morocco can be found in the Iberian Peninsula and  
411 highlights the need to conduct studies across the whole of the natural distribution.

412

413 The evidence to accept *N. obesus* as a species (Fonseca *et al.*, 2016; Marques *et al.*, 2017),  
414 rather than a subspecies of *N. bulbocodium* (Aedo, 2013), is further supported by our results.  
415 Sub-cluster 1.2 of the DAPC corresponds to *N. obesus*, and its placement in the plastid DNA  
416 analysis is the same as that shown by Fonseca *et al.* (2016) using *matK* and *trnL-F* sequences.  
417 However, the assignment of *N. obesus* to section *Bulbocodii sensu* Marques in the DAPC, but  
418 to section *Meridionalis* in the plastid DNA analysis raises the question whether this species  
419 has also arisen through hybridisation between the two hoop-petticoat daffodil sections. The  
420 base chromosome number of *N. obesus* is  $x=13$ , while the rest of the hoop-petticoat daffodils  
421 have  $x=7$  (Fernandes, 1934; Zonneveld, 2008). This could be congruent with allotetraploid origin  
422 and subsequent chromosome losses/fusions (De Storme & Mason, 2014). Whichever of the two  
423 sections this species is correctly placed in, it is clear that it should be recognised as a species,  
424 based on chromosome number and DNA sequence, however this species cannot be reliably  
425 differentiated using morphological characters. It is pertinent to note that *N. obesus* and *N.*  
426 *bulbocodium* occur together in some locations, including population *Joa*.

427

428 *Narcissus hedraeanthus* is endemic to Spain, and limited to a small area between Albacete,  
429 Ciudad Real, Jaen and Granada (Barra & López González, 1986; Aedo, 2013). While  
430 morphologically distinct, the stem is at an angle of 45 degrees or less to the ground opposed  
431 to the upright stem found in all other species (Blanchard, 1990), its taxonomic position has  
432 been often debated. It was previously treated as a subspecies or variety of *N. bulbocodium*  
433 (Baker, 1888; Richter, 1890), a species (Fernandes, 1963), or a subspecies of *N. cantabricus*

434 (Fernández Casas, 1982). Fonseca *et al.* (2016) and Marques *et al.* (2017) have shown that *N.*  
435 *hedraeanthus* is closely related to *N. cantabricus* and belongs to section *Meridionalis* and this  
436 is confirmed by our findings.

437

438 At subspecies rank the taxon *luteolentus* has been placed in either *N. hedraeanthus* (Aedo,  
439 2013) or *N. cantabricus* (Barra & López González, 1982); but also at species rank as *N.*  
440 *blancoi* (Barra Lázaro & López González, 1992). Based on plastid DNA data, Fonseca *et al.*  
441 (2016) accepts *N. cantabricus* subsp. *luteolentus*. We collected fifteen samples from four  
442 populations. Of these, three populations were identified as subsp. *luteolentus* (*Hue*; *JD11-14*;  
443 *JD11-17*, N=14), while the remaining population (*JD12-8*, N=1) was identified as subsp.  
444 *hedraeanthus* based on morphology. The DAPC assigned all *N. hedraeanthus* samples to  
445 Cluster 4, together with a sample of *N. × cazorlanus*, a known hybrid of *N. hedraeanthus* and  
446 *N. triandrus*. The plastid DNA haplotype of subsp. *hedraeanthus* was unique (H8), however,  
447 the samples from the subsp. *luteolentus* populations shared haplotype H1 with *N. cantabricus*  
448 and *N. romieuxii*. The possibility of *N. hedraeanthus* subsp. *luteolentus* being a unique form  
449 of *N. cantabricus* was debated by Fernández Casas (1984), and Barra & López González  
450 (1986). Our data support treatment of *luteolentus* as a potential hybrid between *N.*  
451 *hedraeanthus* and *N. cantabricus* (which would be indicated as the nothospecies *N. ×*  
452 *blancoi*).

453

## 454 CONCLUSION

455

456 Combining microsatellite data with plastid DNA data has highlighted incongruence between  
457 patterns of relationship recovered from nuclear and organellar genomes that is indicative of  
458 hybridisation at many levels within *Narcissus* evolution. There remains a clear signal that

459 there are two lineages of hoop-petticoat daffodils, consistent with those recently identified by  
460 Marques *et al.* (2017) using ITS and organellar DNA. However, there is also evidence of  
461 ongoing hybridisation between these two sections. The haplotype diversity recovered in these  
462 two sections is similar, and this is congruent with evidence of evolutionary age, *N.* section  
463 *Bulbocodii sensu* Marques was estimated at 3.43 Myr and *N.* section *Meridionalis* excluding  
464 *N. obesus* (which has distinct haplotypes), 3.37 Myr (Marques *et al.*, 2017). There remains  
465 the conundrum of taxa that can be recognised morphologically such as *N. cantabricus* and *N.*  
466 *romieuxii*, but are genetically indistinguishable and, in contrast, taxa which have been  
467 synonymised based on morphology but have distinct genetic profiles, such as *N. obesus*, and  
468 subspecies *luteolentus*. Here we advise extreme caution in using plastid genome data alone to  
469 name new *Narcissus* taxa and argue strongly for a multi-evidence approach.

470

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479

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- 634

635 Figure Legends

636

637 **Figure 1.** Examples of floral variation within and between *Narcissus* sections and  
638 subsections *sensu* Marques *et al.* (2017). *Narcissus* subsection *Pseudonarcissi*: A) *N. nobilis*,  
639 B) *N. cyclamineus*, C) *N. moschatus*, D) *N. bujei*; *Ganymedes*: E) *N. triandrus*; *Nevadensis*:  
640 F) *N. segurenensis*; *Juncifolii*: G) *N. assoanus*; *Braxireon*: H) *N. cavanillesii*; *Meridionalis*: I)  
641 *N. cantabricus*; *Apodanthi*: J) *N. rupicola*; *Bulbocodii sensu* Marques: K) *N. bulbocodium*;  
642 *Jonquillae*: L) *N. jonquilla*, M) *N. viridiflorus*; *Dubii*: N) *N. tortifolius*; *Angustifolii*: O) *N.*  
643 *elegans*; *Tazettae*: P) *N. tazetta*, Q) *N. papyraceus*; *Aurelia*: R) *N. broussonetti*; *Narcissus*  
644 subsection *Narcissus*: S) *N. poeticus*; *Serotini*: T) *N. serotinus* Scale bar = 5 cm. Copyright  
645 the authors except for B (J. Bilborrow) and O (T. Sanders).

646 **Figure 2.** Examples of morphological variation of hoop-petticoat daffodils: A, B) *N.*  
647 *romieuxii*; C) *N. hedraeanthus* subsp. *hedraeanthus*; D) *N. hedraeanthus* subsp. *luteolentus*;  
648 E, F) *N. obesus*; G, H, I) *N. cantabricus*; J, K, L) *N. bulbocodium*. Scale bar = 5 cm.

649 **Figure 3.** Results of the DAPC. A) Individual membership probabilities of each of four  
650 genetic clusters ( $k=4$ ) of 450 individuals; B) Results of DAPC of Cluster 1 samples, showing  
651 membership probabilities of either of two genetic clusters ( $k=2$ ); C) Results of DAPC of  
652 Cluster 3 samples, showing membership probabilities of each of three genetic clusters ( $k=3$ ).  
653 Population order follows the natural distribution from north to south (top to bottom).

654 **Figure 4.** Haplotype network of *matK* sequences. A) Coloured circles represent the observed  
655 haplotypes. B) **Error! Reference source not found.** Recoloured according to the DAPC  
656 cluster and sub-cluster assignments. Labels show identifications made in the field or *ex situ*.  
657 Species names in black represent hoop-petticoat taxa, grey labels represent other taxa. All  
658 section *Bulbocodii sensu* Marques haplotypes refer to *N. bulbocodium*, apart from the ones  
659 with labels. The size of the circle is proportional to the haplotype frequency. Open circles

660 indicate inferred haplotypes, dashes indicate indel positions. The length of connecting lines  
661 does not have meaning.

662 **Figure 5.** Bayesian inference majority rule consensus tree of the combined *matK* and *ndhF*  
663 dataset. Posterior probabilities are shown at nodes. Scale bar shows the number of  
664 substitutions per site. *N. c.* = *N. cantabricus*, *N. r.* = *N. romieuxii*. Haplotype colours and  
665 names correspond to Figure 4 A. Labels correspond to Figure 1 and Figure 2.

666 **Figure 6.** Geographic distribution of A) the hoop-petticoat daffodil *matK* haplotypes  
667 [numbers and colours correspond to Figure 4 A] and B) distribution of the DAPC clusters  
668 [colours correspond to Figure 3, labels show population codes]. Areas within the dotted lines  
669 indicate regions in which these species are found but from which we did not have opportunity  
670 to sample.

671 **Figure S1.** Bayesian inference tree of the *matK* dataset. Posterior probabilities are shown at  
672 nodes. Scale bar shows the number of substitutions per site. Haplotype colours and names  
673 correspond to Figure 4 A. *N. c.* = *N. cantabricus*, *N. r.* = *N. romieuxii*.  
674



675 Table captions

676

677 **Table 1.** Details of the PCR cycling conditions for the plastid DNA markers.

678

679 **Table 2.** Summary statistics of the 11 amplified microsatellites based on 450–455 hoop-  
680 petticoat daffodil samples.  $H_o$  = observed heterozygosity; s.e. = standard error. N indicates  
681 number of samples across the table.

682

683 **Table 3.** Genetic diversity estimators for each taxon.  $N_H$  = the number of different haplotypes  
684  $N_C$  = the number of different clusters, A = the total number of different alleles across all loci;  
685  $A_p$  = the number of private alleles across all loci; R = the genotypic richness. N without  
686 subscript indicates number of samples across the table.

687

688 **Table S1.** Geographic location and voucher information of hoop-petticoat daffodil samples.

689 N = number of sampled individuals included in the plastid DNA (cpDNA) and microsatellite  
690 (SSRs) analyses.

691

692 **Table S2.** Geographic location or source, and voucher information of *Narcissus* samples. N =  
693 number of individuals included in the chloroplast DNA analysis; N/K = not known; N/A =  
694 not applicable.