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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ABSTRACT

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

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

INTRODUCTION

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

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

date, Marques *et al*. (2017), using markers from three genomes (plastid, mitochondrial, and nuclear ribosomal), found hoop-petticoat daffodils to be polyphyletic in both organellar and nuclear datasets. This led to the formal split of section *Bulbocodii* and the recognition of section *Meridionalis* I.Marques, Fuertes, Martins-Loução, Moharrek & Nieto Fel. to include some species previously in *N.* section *Bulbocodii.* These two sections are distinguished on molecular evidence and there are, as yet, no clear morphological characters to separate them.

Narcissus is most diverse in the Iberian Peninsula and Northern Africa, and current treatments of this genus in these areas are synthetic accounts. The accounts vary in their scope, breadth of new knowledge added, and the chosen breadth of species concept used to encompass morphological diversity. The most recent account of taxa occurring in the Iberian Peninsula is the treatment in *Flora iberica* (Aedo, 2013). This treatment is a detailed revision and employs broadly defined species and recognises only a few infraspecific taxa. In contrast, the treatment in *Flore Pratique du Maroc* (Fennane, 2015) is a compilation of current knowledge with a narrower species concept applied. This leads to problems in comparing different treatments of hoop-petticoat daffodils as there is only partial overlap of the taxa between the Iberian Peninsula and Morocco and the species circumscriptions, even under the same name, differ. For example, *Narcissus bulbocodium* L. is accepted in both accounts, however Aedo (2013) treats *N. bulbocodium* as a variable species including *N. obesus* Salisb. as a synonym, in contrast to the view of Webb for Flora Europaea (1978) who treats it as subsp. *obesus* (Salisb.) Maire, or Zonneveld (2008) who treats it as a species: *N. obesus*. A further issue is the treatment of *N. obesus* in Morocco. Maire (1959) treats it as *N. bulbocodium* var. *obesus* (Salisb.) Baker. Fernandes (1968) also notes its presence in Morocco, as *N. obesus*. However, Fennane (2015) treats the Moroccan *N. obesus* as a synonym of *N. tingitanus* Fern.Casas, which has now been sunk into *N. albicans* (Haw.)

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

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

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

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

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

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

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

MATERIALS AND METHODS

PLANT MATERIALS

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

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

183

184 MICROSATELLITE METHODS

185

186 Total genomic DNA was extracted using a modified CTAB protocol (Doyle & Doyle, 1987).
187 Eleven of the 15 hoop-petticoat daffodil microsatellite markers reported in Könyves *et al.*
188 (2016) were amplified following their protocol. The allele dosage of polyploids cannot
189 readily be established, therefore traditional population genetic techniques which rely on
190 correct allele frequencies (e.g. STRUCTURE, Pritchard *et al.*, 2000) cannot be applied. We
191 used a presence-absence scoring of peaks to estimate polymorphism, similar to a dominant
192 marker (e.g., amplified fragment length polymorphism [AFLP]) data set and used
193 discriminant analysis of principal components to identify clusters within the microsatellite
194 dataset (DAPC, Jombart *et al.*, 2010). To characterise the variability of the selected markers
195 the total number of alleles per marker, the number of alleles per individual, the mean number
196 of alleles per individual and the observed heterozygosity (H_o) were calculated. To assess the
197 variation between markers and within samples, and therefore the preliminary identification
198 power of the markers, an allelic diversity was calculated as the complement of Simpson's
199 diversity (D=$1 - \sum\{p_i(p_i - 1)/N(N - 1)\}$), where p_i is the frequency with which the *i*th
200 allele was detected). As some of the markers exhibited no alleles in some samples (null
201 genotypes) the proportion of null genotypes was also calculated. The presence of null
202 genotypes was confirmed by repeated PCR amplifications using both a labelled and
203 unlabelled forward primer, to rule out user error or possible adverse effect of the dye
204 labelling. The presence of null genotypes was expected due to incomplete transferability of
205 these markers in hoop-petticoat daffodils. Five samples (two samples from population *Hue*,
206 one sample each from populations *Ald*, *JTiz*, and *Sdf;* Table S1), that had failed fragment
207 analysis after successful PCR of one or more loci, were removed. In total 450 hoop-petticoat

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

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

PLASTID DNA METHODS

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

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

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

To explore the relationships between the sampled populations and other sampled *Narcissus* a statistical parsimony network was constructed from the *matK* dataset using TCS ver. 1.21 (Clement, Posada, & Crandall, 2000) under the 95% statistical parsimony criterion. Phylogenetic trees were constructed through Bayesian inference (BI) in MrBayes ver. 3.2 (Ronquist *et al.*, 2012) according to the best-fit model of evolution identified by MrModeltest ver. 2.3 (Nylander, 2004). To avoid overfitting of the model, BI analysis of the *matK* dataset was performed using only the haplotypes identified by TCS. BI analysis of the combined dataset including the *matK* haplotypes and the corresponding *ndhF* sequences was used to improve the resolution of the *matK* phylogenetic tree. The incongruence of the *matK* and *ndhF* datasets was assessed with the incongruence length difference (ILD) test in PAUP*

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

GENETIC DIVERSITY ESTIMATORS

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

RESULTS

MICROSATELLITE VARIATION

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

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

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

PLASTID DNA VARIATION

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

The hoop-petticoat daffodil sequences were grouped into 22 haplotypes, the remaining 20 haplotypes belonged to other daffodil samples in subgenus *Narcissus*. *Narcissus* section *Bulbocodii sensu* Marques and section *Meridionalis* appeared as two distantly related groups, separated by a minimum of 22bp differences. The *matK* (Figure S1), and the combined *matK* and *ndhF* BI analyses (Figure 5) recovered a topology congruent with Marques *et al*. (2017). The correspondence of the DAPC and plastid DNA results is shown in Figure 5. The correspondence of floral morphology and sectional classification is shown in Figure 4.

The DAPC results (Figure 3 A, B) showed *N. obesus* to be grouped with *N. bulbocodium* in section *Bulbocodii sensu* Marques, however, the plastid DNA analyses placed it in section *Meridionalis. Narcissus hedraeanthus* subsp. *luteolentus* was placed in the same cluster as *N. hedraeanthus* subsp. *hedraeanthus* by DAPC, but it was indistinguishable from *N. cantabricus* and *N. romieuxii* in the plastid DNA analysis. The plastid DNA analyses and DAPC differ in the placement of some samples carrying haplotypes H29, H32 and H34: the former placing them in section *Bulbocodii*, the latter in section *Meridionalis* (Figure 4). Three of these populations occur in southern Morocco and two in central Spain. The geographic distribution of the plastid DNA haplotypes and the DAPC clusters is shown in Figure 6.

DIVERSITY ESTIMATORS

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

DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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

CONCLUSION

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

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

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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. segurensis*; *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. Bilsborrow) 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

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

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

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