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ORIGINAL ARTICLE

Phylogenenetic approach of the section *Bulbocodii* D.C. of *Narcissus* based on cpDNA. A case of taxonomic inflation?

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Abstract
In this paper, we analyzed the phylogeny of the section *Bulbocodii* (genus *Narcissus*; Amarillydaceae) using the matK and trnL-F fragments of cpDNA in order to review the validity of the recognized taxa. Our results indicate that *Narcissus obesus* should be considered a valid species, and that *N. blancoi* is a distinct *taxon*. In addition, seven previously recognized species, *N. juressianus*, *N. subnivalis*, *N. graellsii*, *N. conspicuus*, *N. citrinus*, *N. nivalis*, and *N. quintanilhae*, should be assigned to an infraspecific rank under *N. bulbocodium*, as they are not valid species. In addition, we analyzed the distribution of the three morphological characters widely used in the systematics of this section and found that their variation does not agree with the phylogenetic results, rendering these characters limited taxonomical utility. This result suggests that the section *Bulbocodii* shows high morphological lability, which can explain the proliferation of nominal species.

Keywords: *Phylogeny, cpDNA, biogeography, chloroplast capture,* Narcissus

Introduction

Narcissus is a genus of bulbous monocots that belongs to the family Amaryllidaceae (Order: Asparagales). The genus is distributed throughout the Mediterranean region, from Portugal to the Middle East (Blanchard 1990). It is composed by 60–80 species (Fernandes 1975; Blanchard 1990). However, published taxonomies of this group vary extensively among authors, regarding which taxa are considered valid species. Currently, based on extensive karyological and morphological studies, two subgenera are considered: *Hermione* (Haw.) Spach (base chromosome numbers of $n = 5$, $n = 10$, $n = 11$) and *Narcissus* ($n = 7$). The subgenus *Narcissus* includes nine sections: *Narcissus*, *Tapeinanthus* Traub, *Bulbocodii* De Candolle, *Ganymedes* (Haw.) Schütt., *Jonquilla* De Candolle, *Pseudonarcissus* De Candolle, *Apodanthi* Fernandes (Fernandes 1968 & 1975), *Juncifolii* (A. Fern.) Zonneveld, and *Nevadensis* Zonneveld.

Like other sections of the subgenus *Narcissus*, section *Bulbocodii* presents the base chromosome number ($n = 7$), with *N. obesus* Salisbury being the only exception ($n = 13$). Polyploidy is a common feature in the section. Its geographical range spans through the western Mediterranean region, occurring in Portugal, Spain, Morocco, Tunisia, and Algeria (Blanchard 1990). Fernandes (1975) proposed the first phylogenetic hypothesis for this section wherein *Narcissus bulbocodium* L. is the ancestral species.

Presently, the section is composed by a high number of taxa that varies extensively among classification systems (e.g. Fernandes 1975; Blanchard 1990; Zonneveld 2008). Therefore, the taxonomy and the systematics of this group remain uncertain, and several taxonomic proposals are currently under debate. Furthermore, during the past decades, several varieties and subspecies were elevated to the species level, and new taxa were described (Barra & López González 1982, 1983, 1992; Fernández Casas 1984, 1986, 2005; Barra 2003). Aedo (2013) presents an extensive revision of the Iberian species of *Narcissus*. In the section *Bulbocodii,* he includes only three species: *N. cantabricus*, *N. bulbocodium*, and *N. hedraeanthus*,

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and only one valid subspecies *N. hedraeanthus* subsp. *luteolenthus*. Aedo considers that all other taxa do not deserve taxonomic recognition, including *N. obesus*, taxon that he considered a synonym of *N. bulbocodium*.

Graham and Barrett (2004) and, more recently, Santos-Gally et al. (2012) analyzed the phylogeny of *Narcissus*, including some species of *Bulbocodii* based on plastid DNA, revealing not only that *Narcissus cantabricus*, *N. hedraeanthus,* and the Moroccan populations of *N. bulbocodium* var. *nivalis* were closely related taxa, but also that they were a sister group of an extensive clade, which included species from the sections *Narcissus*, *Tapeinanthus*, *Ganymedes*, *Pseudonarcissus,* and *Juncifolii*. Furthermore, Graham and Barrett (2004) found one population of *N. bulbocodium* phylogenetically closer to the section *Jonquillae* than to the section *Bulbocodii,* which was later confirmed by Santos-Gally et al. (2012) with two additional populations of *N. bulbocodium*. Graham and Barrett (2004) suggested that this can be explained by introgression from an unknown species of *Jonquillae*.

Due to the unresolved systematic status of *Bulbocodii*, with phylogenetic analyses contradicting the morphological-based taxonomy, the main goal of this study is to investigate the phylogenetic relationships within the section. In particular, this study aims to: (1) understand the relationships of the section *Bulbocodii* with other *Narcissus* sections; (2) study the phylogenetic relationships within the section *Bulbocodii*; (3) evaluate the taxonomic significance of morphological characters commonly used in the systematics of this section, and (4) evaluate the systematic schemes of *Bulbocodii* and their congruence with the molecular data. In additional, we performed a preliminary approach to the phylogeography of *N. bulbocodium* in Iberia. To fulfill these aims, we analyzed the DNA sequences of two plastid regions the matK and the trnL-F genes.

Materials and methods

Total 40 populations from 16 taxa belonging to the section *Bulbocodii* were examined. At least two plants were analyzed from each sample location. One or two species from other sections were included in this study, to assess the relationship between the section *Bulbocodii* and other *Narcissus*. From the section *Pseudonarcissi*, six taxa were included, representing most of the distribution range of this section (Figure 1 and Table I).

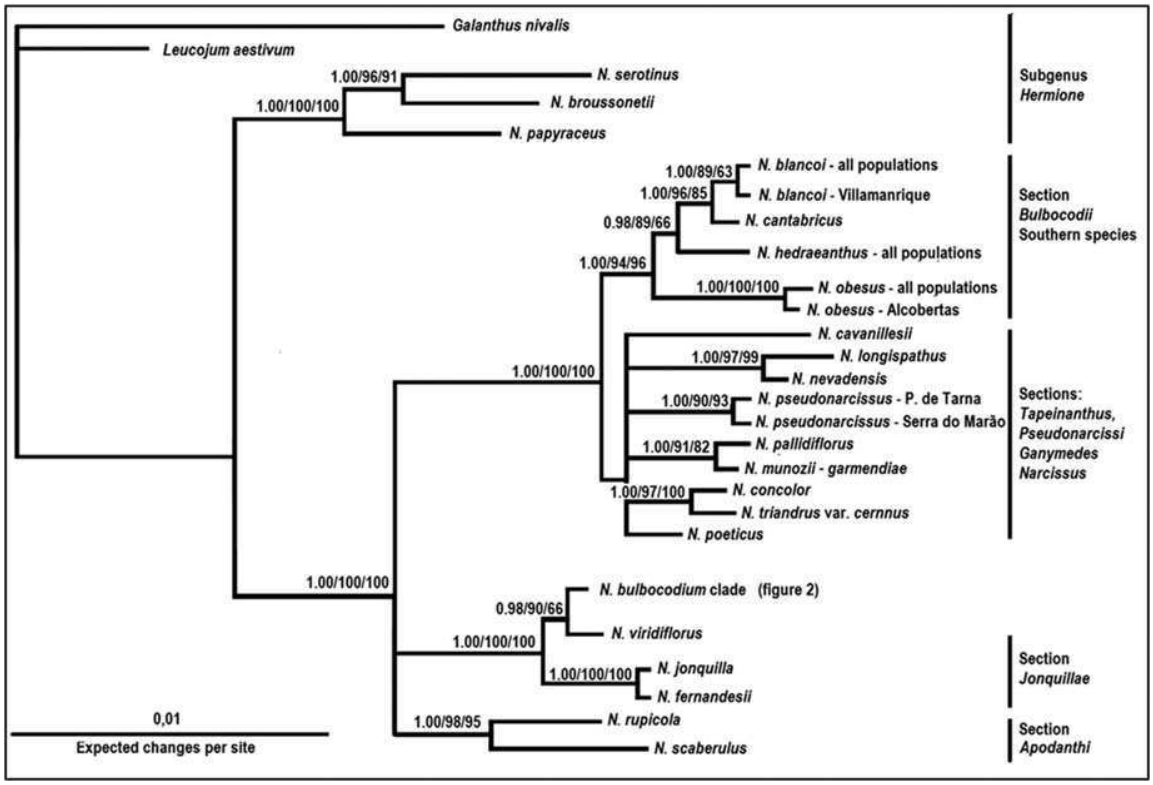

Figure 1. Bayesian phylogram based on concatenated sequences of matk and trnL-F. Branches supported by Bayesian posterior probabilities lower than 0.80 or bootstraps values smaller than 60 were collapsed. Bayesian posterior probabilities, bootstrap values for ML and for MP are separated by dashes, at left, middle, and right, respectively. The tip of each branch shows plants with the same haplotypes in matk and trnL-F. When the same haplotypes were found in the same taxa, in different populations, their locations are separated by semicolon.

Table I. Sources of plant material and vouchers. Leaves – thin < 2 mm; broad > 2 mm; size (scapus) small to medium < 30 cm; robust > 30 cm.

		Morphological characters				
Taxon	Sample locality	Leaves width	Color of flowers	Size (scapus)	Remarks	Voucher (deposited in LISI)
Section *Bulbocodii* De Candolle						
N. blancoi Barra & López	Spain; Jaen; Aldeaquemada	Thin	Whitish	Medium		377/2012
	Spain; Ciudad Real; Road Villamanrique – Venta de Los Santos	Thin	Whitish to medium yellow	Medium	Location of a *paratypus*	415/2012
	Spain; Albadalejo; Road Villapalacios, Albacete	Thin	Whitish to medium yellow	Medium	Location of *holotypus*	456/2012
N. quintanilhae (Fernandes) Fernández Casas	Portugal; Guarda; Mata de Lobos	Thin	Golden yellow	Medium	Location of a *syntypus*	382/2012
N. bulbocodium subsp. *validus* Barra	Spain; León; Besande	Most thin	Golden to medium yellow	Medium	Location of a *paratypus*	436/2012
	Spain; León; Isoba	Most thin	Golden to medium yellow	Medium		396/2012
N. nivalis Graells	Spain; Madrid; Puerto de Morcuera	Broad	Golden yellow	Dwarf		399/2012
	Portugal; Vila Real; Falperra	Broad	Golden yellow	Dwarf		402/2012
	Portugal; Guarda; Serra Estrela, East slope;	Broad	Golden yellow	Dwarf		443/2012
	Portugal; Guarda; West slope;	Broad	Golden yellow	Dwarf		444/2012
N. bulbocodium L. var. *bulbocodium*	Portugal; Setúbal; Alcáçovas	Thin	Golden yellow	Medium		472/2012
	Spain; Ciudad Real; Arroba	Thin	Golden yellow	Medium		390/2012
	Spain; Ciudad Real; Fuencaliente	Thin	Golden yellow	Medium		424/2012
	Portugal; Coimbra; Condeixa	Thin	Golden yellow	Medium		405/2012
	Portugal; Leiria; Fátima	Thin	Golden yellow	Medium		394/2012
	Spain; Ourense; Lovios	Thin	Golden yellow	Large		437/2012
	Spain; Ciudad Real; Puebla D. Rodrigo	Thin	Golden yellow	Medium		375/2012
	Spain; Badajoz; Rio Ruecas	Thin	Golden yellow	Medium		378/2012
	Portugal; Lisboa; Sintra	Thin	Golden yellow	Medium		398/2012
	Spain; Cuenca; Rio Cuervo	Thin	Pale yellow	Medium		444/2012
	Portugal; Faro; Sagres	Thin	Pale yellow	Medium		473/2012
	Spain; La Coruña; Xistral,	Thin	Medium yellow	Medium		471/2012
	Spain; Léon; Sanabria	Thin	Medium yellow	Medium		475/2012
N. bulbocodium L. var. *ectandrus* Barra & López	Spain; Cuenca; Uña, Sierra de Valmeca	Broad	Pale yellow	Dwarf	Location of a *paratypus*	492/2012
N. cantabricus DC.	Spain; Ciudad Real; Almaden	Thin	White	Small		372/2012
N. cantabricus DC. *foliosus* (Maire) A. Fernandes	Morocco; Djebel Bouhachem	Thin	White	Small		418/2012

TABLE I – *continued*

Taxon	Sample locality	Morphological characters			Remarks	Voucher (deposited in LISI)
		Leaves width	Color of flowers	Size (scapus)		
N. citrinus (Baker) Fernández Casas	Spain; Astúrias; Gijon	Mostly thin	Pale yellow	Medium	Location of *syntypus*	371/2012
	Spain; Leon; Puerto de Panderrueda	Mostly thin	Golden to medium yellow	Medium	Local do *syntypus*	444/2012
N. conspicuous Haworth	Spain; Cáceres; Aldea del Cano	Thin	Golden yellow	Large	Mixed population with *N. bulbocodium* var. *bulbocodium*	371/2012
	Spain; Cáceres; Trujillo	Thin	Golden yellow	Large		407/2012
N. graellsii Webb in Graells	Spain; Madrid; Navacarros	Thin or broad	Pale yellow	Medium		477/2012
	Spain; Madrid; Guadarrama	Thin or broad	Pale yellow	Small		433/2012
N. hedraeanthus (Webb & Heldr.) Colmeiro	Spain; Jaen; Ubeda	Thin	whitish yellow	Dwarf		423/2012
	Spain; Albacete; Nerpio	Thin	whitish yellow	Dwarf		424/2012
N. juressianus Fernández Casas	Spain; Ourense; Lovios	Broad	Golden yellow	Dwarf	Location of *holotypus*	370/2012
N. obesus Salisbury	Portugal; Leiria; Alcobertas	Thin	Golden yellow	Medium		387/2012
	Portugal; Faro; Loulé	Thin	Golden yellow	Medium		384/2012
	Portugal; Lisboa; Sintra	Thin	Golden yellow	Medium		412/2012
N. romieuxii Braun-Blanquet & Maire	Morocco; Meknez	Thin	whitish to medium yellow	Medium		376/2012
N. subnivalis Fernández Casas	Spain; Cuenca; Villanueva de Alcorón	Broad	Pale yellow	Dwarf	Location of *holotypus*	444/2012
Section *Hermione*						
N. papyraceus Ker Gawler	Morocco; Ouezanne					373/2012
Section *Serotini* Parl.						
N. serotinus L.	Elvas, Portalegre, Portugal					482/2012
Section Aurelia (J. Gay) Baker						
N. broussonetii Lagasca	Morocco					Comercial source
Section *Apodanthi* Fernandes						
N. rupicola Dufour,	Spain; Madrid; Guadarrama					464/2012
N. scaberulus Henriques	Portugal, Viseu, Ervedal					452/2012
Section *Jonquillae* De Candolle						
N. fernandesii G. Pedro	Spain; Cordova; Benameji	–	–	–		463/2012
N. viridiflorus Schousboë	Spain; Cadis	–	–	–		404/2012
Section *Ganymedes* (Haw.) Schütt.						
N. concolor (Haw.) Link	Portugal; Coimbra; Lousã	–	–	–		386/2102
N. triandrus L. var. *cernus* (Salisb.) Baker	Spain; Jaen; Aldeaquemada	–	–	–		466/2012
Section *Pseudonarcissi* De Candolle						
N. nevadensis	Espanha; Granada; Sierra Nevada					393/2012

N. longispathus Pugsley	Spain; Jaén; Sierra Cazorla Spain; Ciudad Real;				414/ 2012
N. muñozzi-garmendiae Fernández Casas	Solana del Pino	–	–	–	408/2012
N. pseudonarcissus L.	Portugal; Vila Real; Marão	–	–	–	481/2012
	Spain; León	–	–	–	438/2012
N. pseudonarcissus L. subsp. *pallidiflorus* (Pugsley) A. Fernandes	Andorra	–	–	–	400/2012
Section *Narcissus*					
N. poeticus L.	Spain, Barcelona	–	–	–	430/2012
Section *Tapeinanthus*					
N. cavanillesii	Portugal, Elvas				212/2008

Most plants were classified according to the criteria proposed by Fernandes (1968). For the taxa described after 1968, the original description was used. For *Narcissus blancoi* Barra & López, we used the criteria presented by Barra and López González (1986). Samples for genetic analysis consisted of fragments of freshly cut green leaves, stored in 96% ethanol after harvesting. Whenever possible, samples were harvested in the type locality or from locations cited in the original description. In taxa characterized by slight morphological differences, this procedure reinforced the certainty that the correct taxa were sampled.

When harvesting, three morphological characteristics were recorded *in situ*: size of scapus, color of the perianth and width of the leaf cross section (Table I). Morphological data were analyzed in a qualitative way, comparing the distribution of different morphologies in the phylogenetic trees.

DNA was isolated using the MasterPure Plant Leaf DNA Purification Kit (Epicentre Biotechnologies, Inc., Madison, WI, USA). The PCR mix was prepared for a volume of 20 μl, with 10 μl of RedExtract-N-Amp PCR reaction mix (Sigma-Aldrich, St. Louis, MO, USA), 0.8 μl of each primer (10 M), 4.4 μl of sigma-water, and 4 μl of template DNA.

The matK region was amplified using two pairs of primers: −19F (Kocyan et al. 2004) paired with matfr (5′–GATCCTGTACGGTTGAGACCA–3′); and matrf (5′–ATCTTTTGGAACTTTTCTTG-AACG–3′) paired with 2R (Johnson & Soltis 1995). The primers matrf and matfr were designed for this study, and trnL-F region was amplified using the primers trnc and trnf (Taberlet et al. 1991).

The same PCR protocol was used to all fragments, with adjustments of elongation time. PCRs included an initial denaturation of 2 min at 94°C, followed by 35 cycles of 1- min denaturation (94°C), 1-min annealing (50°C), 1 -min elongation at 72°C, and final extension at 72°C (7 min). For the primers 19F and 2R, the elongation time was 2 min. PCR products were purified with the Microclean kit (Microzone, Ltd, Haywards Heath, UK) and sequenced using the amplification primers (STABVIDA, Lisboa, Portugal).

DNA sequences were edited using BioEdit v. 7.0.1 (Hall 1999) and aligned using ClustalW (1.81) (Thompson et al 1997). Reconstruction of phylogenetic relationships was conducted using maximum parsimony (MP), Bayesian inference (BI), and maximum likelihood (ML) methods in each DNA marker separately, and in all markers combined. *Leucojum aestivum* L. and *Galanthus nivalis* L. were used as outgroups (Accession numbers for *Galanthus nivalis*, trn L-F: AY357136.1; matk: AY101335.1. For *Leucojum aestivum*, trn AY357135.1; matk FN663901.1). The best-fit model of nucleotide

evolution was obtained using jmodeltest 3.7 (Posada 2008) with the Akaike information criterion (Guindon & Gascuel 2003).

The MP analysis was conducted in PAUP 4.0b10 (Swofford 2003), using a heuristic search with random stepwise addition (1000 replicates) and tree-bisection-reconnection branch swapping. All characters and character-state changes were equally weighted, and gaps were coded as missing values. Bootstrap analysis (1000 replicates) was used to assess the relative robustness of branches of the MP trees (Felsenstein 1985). The incongruent length difference (ILD) was performed in PAUP. The BI was performed using MCMC as implemented in Mr. Bayes 3.1 (Ronquist & Huelsenbeck 2003), with two independent runs of four Metropolis-coupled chains of 2 million generations each, to estimate the posterior probability distribution. Topologies were sampled every 100 generations, and a majority-rule consensus tree was estimated after discarding the first 2000 generations sampled. Sequences were partitioned by region (matk and trnL-F). The ML analyses were conducted in RAxML BlackBox (Stamatakis et al. 2008). Branch support was tested by 100 rapid bootstrap replicates, and the general time-reversible substitution matrix was enforced in all analyses. Concatenated sequences that have equal matk and trnL-F haplotypes were included only once in the phylogenetic analysis.

Preliminary results revealed that some *Bulbocodii* species from Iberia were more closely related to *N. viridiflorus* (section *Jonquillae*) than to the other species of its section. This relationship was already observed in two previous studies (Graham & Barrett 2004; Santos-Gally et al. 2012). Therefore, we included *N. viridiflorus* and these Iberian *Bulbocodii* species in a phylogeographic analysis to reconstruct haplotype relationships and to investigate the evolutionary history of these taxa in Iberia. Parsimony network analyses were conducted with the software TCS 1.18 (Clement et al. 2000).

Results

Amplification of the matK and trnL-F yielded fragments of 1813 bp and 932 bp. The ILD test did not reveal significant differences between the two markers (ILD = 0.02, $P < 0.05$) (Swofford 2003) and the two fragments were concatenated, resulting in a 2745 bp alignment, with 153 bp parsimony informative sites, which was used in all subsequent analyses. Assessment of the most suitable nucleotide substitution model retrieved the TIM1 + I + G for matk and TPM1uf + G for trnL-F.

Overall, the BI approach resulted in a better resolved and strongly supported phylogenetic tree (Figures 1 and 2).

The reconstruction of the phylogenetic relationships of the section *Bulbocodii*, using the concatenated fragment, revealed that the section is not monophyletic (Figure 1). *Narcissus obesus*, *N. hedraeanthus*, *N. blancoi*, *N. cantabricus,* and *N. romieuxii* are grouped in a single clade, closely related with the species of the sections *Narcissus*, *Tapeinanthus*, *Ganymedes*, and *Pseudonarcissi*. The individuals of *N. romieuxii*, which is thought to be an allotetraploid species originated by the cross between *N. cantabricus* and *N. bulbocodium* (Fernandes 1959), shared the matK and trnL-F haplotype with individuals from two populations of *N. cantabricus* (Figure 1).

A second major clade, hereafter referred to as *N. bulbocodium* clade (that includes the type species, *N. bulbocodium*), was more closely related with section *Jonquillae* (Figure 1). This clade also contained *N. conspicuus*, *N. jurassianus*, *N. quintanilhae*, *N. graellsii, N. citrinus*, *N. subnivalis,* and *N. nivalis* (Figure 2).

A closer look into the phylogenetic relationships within the *N. bulbocodium* clade revealed the presence of two subclades (Figure 2). One subclade contained individuals from central Iberia (the Central Iberian Clade) and the other individuals from the littoral region of the Iberian (the Littoral Clade).

At least one of the specimens of *N. subnivalis*, *N. bulbocodium* var. *ectandrus*, *N. jurassianus,* and *N. graellsii* shared haplotypes with the nearest geographical populations of *N. bulbocodium* var. *bulbocodium*. For instance, one specimen of *N. subnivalis* and another of *N. bulbocodium* var. *ectandrus* shared the matk and trnL-F haplotypes with two plants from the geographically closest population of *N. bulbocodium* var. *bulbocodium* (Rio Cuervo, Spain) (Figure 2). The other specimens of *N. subnivalis* and *N. bulbocodium* var. *ectandrus* showed minor differences (one indel and one substitution in trnL-F, respectively). The two sampled individuals from *N. jurassianus* (collected in the holotype's sample location) were also genetically identical to individuals of *N. bulbocodium* var. *bulbocodium* from Lovios (Spain). Two populations of *N. graellsii* shared matk and trnL-F haplotypes with the nearest populations of both *N. nivalis* (from Puerto de Morcuera, Spain) and *N. bulbocodium* var. *bulbocodium* (from Arroba, Spain) (Figure 2).

Four of the nine species included in the *N. bulbocodium* clade were polyphyletic, as individuals appeared both in the central Iberian and in the littoral subclades, whereas *N. jurassianus*, *N. graellsii*, *N. quintanilhae,* and *N. subnivalis* occurred only in one of the subclades (Figure 2).

The two specimens of *N. quintanilhae* showed different haplotypes in matk (two substitutions), but they occur together in the tree, supported by good

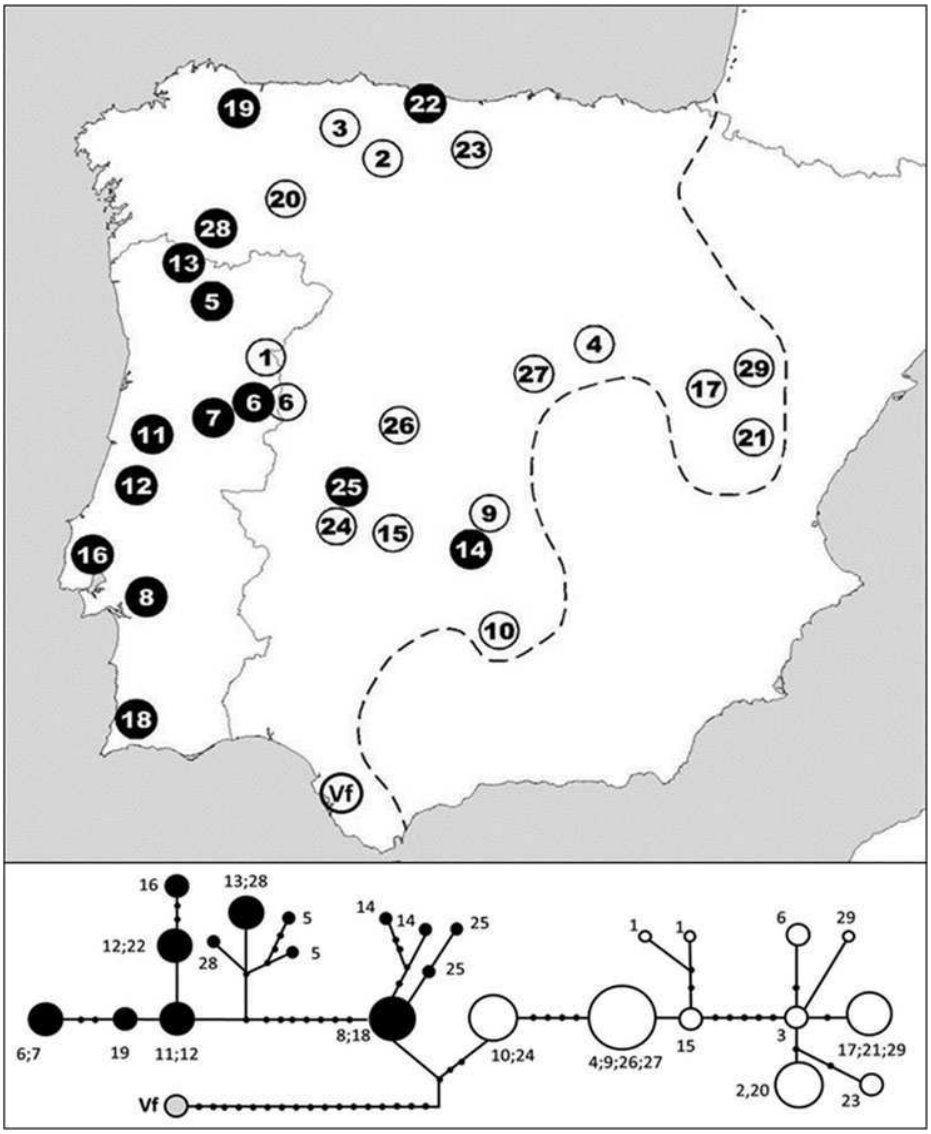

Figure 2. *Bulbocodium* clade. Continuation of the phylogram from Figure 1.

bootstraps and posterior probabilities. The genetic differences between them and *N. bulbocodium* var. *bulbocodium* from Rio Ruecas (Spain) or from *N. nivalis* from Serra da Estrela (Portugal) were very small, differing only by three nucleotide substitutions.

All sequences have been deposited in GenBank (available at www.ncbi.nlm.nih.gov/) (Accession numbers: KC145283–KC145380; KC238472–KC238568).

The superimposition of the distribution of the three morphological characters in the inferred phylogenetic tree of the *N. bulbocodium* clade showed that there was no correspondence between the lineages and the presence/absence of these characters. The same analysis was conducted for the remaining species of the *Bulbocodii* section and, although less conclusive, showed that the two different colors of the perianth (white and yellow) also occurred in the two distinct lineages (Figure 3).

The reconstruction of the haplotype relationships using a network approach identified two main lineages corresponding to individuals from the central and the western regions of the Iberian Peninsula (Figure 4), supporting the results obtained in the phylogenetic analyses. This network also showed that the haplotypes of *N. bulbocodium* that were closer to *N. viridiflorus* are present in southern Iberia.

Discussion

Phylogenetic trees showed that the section *Bulbocodii* is not monophyletic, as found in previous studies, with species divided between two well-defined, albeit

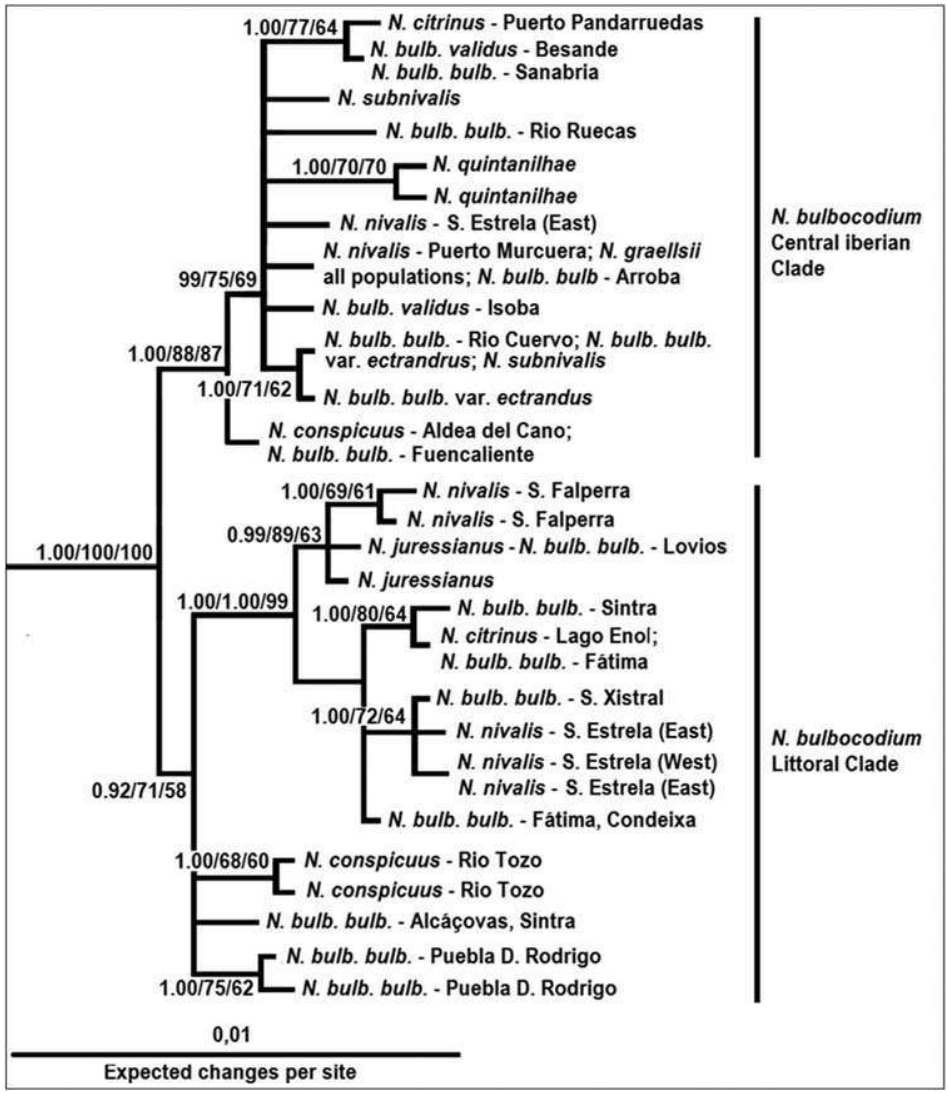

Figure 3. Distribution of the three morphological characters in simplified phylograms (based on Figures 1, 2 and Table I), in *Narcissus bulbocodium* clade (left) and southern *Bulbocodii* species (right). In each clade, branches with the same morphological characters were joined.

not closely related clades, at least in what concerns chloroplastic inheritance. One clade appears to be mainly composed by species that occur from the South of the Iberian Peninsula to northern Africa (Blanchard 1990): *N. obesus*, *N. cantabricus*, *N. romieauxii*, *N. blancoi*, and *N. hedraeanthus*. The other clade includes *N. bulbocodium* and other species that occur in the central and northern Iberia. These results contradict the phylogenetic hypothesis of Fernandes (1975), which stated that *N. obesus*, *N. cantabricus*, and *N. hedraeanthus* evolved directly from *N. bulbocodium*.

It is worth noting that *N. romieauxii* shared the same haplotypes with *N. cantabricus*. This is consistent with the hypothesis of Fernandes (1959), in which *N. romieauxii* would be an allotetraploid, with *N. cantabricus* as one of the parental species. The Spanish population of *N. cantabricus* subsp. *cantabricus*, the Moroccan populations of *N. cantabricus* subsp. *foliosus* and *N. romieauxii* share the trnL-F and matk haplotypes. Our results showed that the Iberian populations of *N. bulbocodium* are more closely related to the section *Jonquillae* than the remaining members of its section. This phylogenetic relationship, found in three populations in previous studies (Graham & Barrett 2004; Santos-Gally et al. 2012), is widespread throughout the Iberian Peninsula, being found in 29 populations.

It is highly unlikely that the nuclear genome of *N. bulbocodium* is derived or even related to *N. viridiflorus*, as the latter has chromosomes that are more than twice as large. Indeed, *N. viridiflorus* has 28 chromosomes (Fernandes 1968), and Zonneveld (2008) showed that it has $2n=4x=28$ chromosomes and 63.2 pg of nuclear DNA, resulting in $Cx = 15.8$ pg. The diploid *N. bulbocodium* has

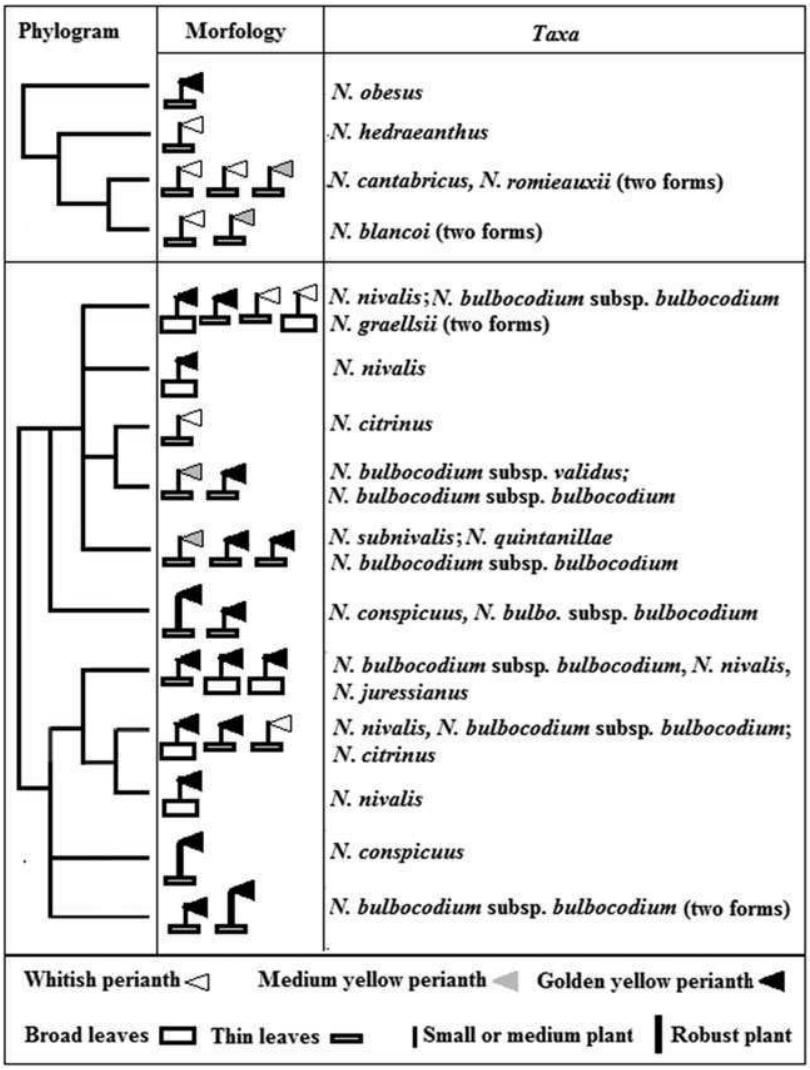

Figure 4. Locations of sampled populations: (upper) and haplotype network (down) of *Narcissus bulbocodium* clade and *N. viridiflorus*. Black circles, littoral subclade; white circles, Central Iberian subclade. Dashed line indicates the geographical range of *Narcissus bulbocodium* (adapted from Franco & Afonso 1994; Barra 2003). Dots between circles indicate mutational steps. subclade: 1, *N. citrinus*, Gijon; 2, *N. bulbocodium* var. *bulbocodium*, Sierra do Xistral; 3, *N. juressianus*, Lovios; 4, *N. bulbocodium var. bulbocodium*, Lovios; 5, *N. nivalis*, Serra da Falperra; 6, *N. nivalis*, Serra Estrela East slope; 7, *N. nivalis*, Serra Estrela West slope; 8, *N. bulbocodium var. bulbocodium*, Condeixa; 9, *N. bulbocodium var. bulbocodium*, Fátima; 10, *N. conspicuus*, Trujillo; 11, *N. bulbocodium var. bulbocodium*, Puebla D. Rodrigo; 12, *N. bulbocodium var. bulbocodium*, Sintra; 13, *N. bulbocodium var. bulbocodium*, Alcáçovas; 14, *N. bulbocodium* var. *bulbocodium*, Sagres. Iberian subclade: 6, *N. nivalis*, Serra Estrela East slope; 15, *N. bulbocodium validus*, Isoba; 16, *N. citrinus*, Puerto de Panderruedas; 17, *N. bulbocodium subsp. validus*, Besande; 18, *N. bulbocodium* var. bulbocodium, Sanabria; 19, *N. bulbocodium var.nivalis*, Puerto de Morcuera; 20, *N. quintanilhae*, Mata de Lobos; 21, *N. graellsii*, Guadarrama; 22, *N. graellsii*, Navacarros; 23, *N. subnivalis*, Villanueva de Alcorón; 24, *N. bulbocodium var. bulbocodium*, Rio Cuervo; 25, *N. bulbocodium var. ectandrus*, Uña; 26, *N. bulbocodium var. bulbocodium*, Arroba; 27, *N. conspicuus*, Aldea del Cano; 28, *N. bulbocodium var. bulbocodium*, Rio Ruecas; 29, *N. bulbocodium var. bulbocodium*, Fuencaliente. vf – *N. viridiflorus*.

$2n = 2x = 14$ and 14.2 pg resulting in Cx = 7.1 pg. Thus, the introgression of *N. viridiflorus* in *N. bulbocodium* genome seems to be limited to the chloroplast genome, and our results should be interpreted as a case of "chloroplast capture" as defined by Rieseberg and Soltis (1991).

There is an alternative explanation for the pattern detected. We can state that *N viridiflorus* is the sister taxa of N. *bulbocodium*, originated from a common ancestral in allopatry. However, we found this alternative very unlikely because both species are morphologically very distinct. *N. bulbocodium* is so similar to other *Bulbocodii* taxa that some authors consider that is conspecific with *N. obesus*. *N. viridiflorus* is quite distinct from any other species. Furthermore, Cx value (15.8 pg) of *N. viridiflorus* is very similar to values found in other *Jonquillae* species (Zonneveld 2008), whereas Cx value (7.1) is very

similar to other *Bulbocodii* species. Finally, it seems very unlikely that *N. bulbocodium* has evolved from *Jonquillae*, producing a morphology and a karyology so similar to *Bulbocodii*.

The division of the *N. bulbocodium* clade into the two main subclades found in the phylogenetic trees is consistent with the haplotype network, supporting this split and giving some clues on the phylogeography of this clade.

Predictions from coalescent theory assume that older alleles should prevail in populations and be characterized by a higher number of descending lineages and a geographically wider distribution (e.g. Posada & Crandall 2001). However, in interspecific phylogenies, these parameters depend much more on sample design and differences in the history of the taxa (Bradbury & Holloway 1988). In interspecific haplotype networks, when different species are present, it is assumed that older haplotypes occur in positions closest to the connection between species. This approach was used to identify the older haplotypes, and the geographical origin of different lineages of plants (e.g. Hedenäs 2009).

Using the same approach, our haplotype network showed that the most similar haplotypes between *N. bulbocodium* clade and *N. viridiflorus* were found in Southern Iberia (Figure 4), suggesting that *N. bulbocodium* clade differentiated in Southern Iberia. This result is consistent with the actual geographical range of *N. viridiflorus*, which is restricted to the extreme south of Spain and Morocco (Blanchard 1990), but alternative explanations could be pointed out and we stress that this approach should be tested with other data. The validity of *N. blancoi* as a valid species has been a controversial issue. Barra and López González (1982) first described this taxon as *N. cantabricus* subsp. *luteolenthus*, and later reclassified it as *N. blancoi* (Barra & López González 1992). However, Fernández Casas (1984) and Espinosa Jimenez and Fernández López (1985) synonymized it with *N. hedraeanthus*. Our results showed that *N. blancoi* is genetically distinct from *N. hedraeanthus*, and its nearest species is *N. cantabricus*, as suggested by Barra and López González (1992). We cannot clarify whether this taxon deserves the specific or infraspecific rank, but we emphasize that the genetic distinction between *N. blancoi* and *N. cantabricus* is slight (two nucleotide substitutions). This is smaller than the differences found between most populations of *N. bulbocodium*, suggesting that an infraespecific rank is appropriate.

The three locations sampled of *N. obesus* form a monophyletic taxon, and it does not have a close relationship with *N. bulbocodium*. Therefore, it should not be classified as *N. bulbocodium* subsp. *obesus* (Salisb.) Maire but as *N. obesus* Salisbury.

The reconstruction of the phylogenetic relationships within the *N. bulbocodium* clade revealed that the specific rank should not be applied to *N. conspicuus*, *N. citrinus*, and *N. nivalis* because these taxa are polyphyletic and also due to their genetic similarity with individuals of *N. bulbocodium* subsp. *bulbocodium*, with some individuals sharing the matk and trnL-F haplotypes. Therefore, using the criteria reviewed by Christensen (1987), *N. citrinus* should be considered a subspecies of *N. bulbocodium* because it is allopatric with the other morphological forms of this species (Barra 2003; Blanchard 1990). *Narcissus bulbocodium* subsp. *validus* also occurs in allopatry with the other forms of *N. bulbocodium* (Barra 2003), and thus the subspecific rank should be maintained. On the contrary, *N. conspicuus* and *N. nivalis* should be considered as varieties because they are sympatric with other forms of *N. bulbocodium,* occurring in distinct populations as well in mixed populations (see Table I; Rivas Ponce et al. 1985; Barra 2003). However, we must point that there are no consensual criteria for the use of subspecific ranks in the botanical literature (Hamilton & Reichard 1992).

Our molecular data do not support the existence of *N. juressianus* as a distinct species. This taxon shares haplotypes with the geographically nearest population of *N. bulbocodium* var. *bulbocodium* (Lovios, Spain). Furthermore, the morphological distinction between *N. juressianus* and *N. bulbocodium* var. *nivalis* is not striking. In the species description, the author recognized this similarity stating "*N. nivalis graells non dissimilis, sed paulo maior*" (not different from *N. nivalis*, but a little bigger), but he did not provide biometric comparisons (Fernández Casas 1986). Therefore, *N. juressianus* should be considered a synonym of *N. bulbocodium* var. *nivalis*.

Similarly, although *N. graellsii* is easily distinguished from other forms of *N. bulbocodium* due to the whitish perianth, all the individuals analyzed shared the same haplotypes with the geographic nearest population of both *N. bulbocodium* var. *bulbocodium* and *N. bulbocodium* var. *nivalis*. *Narcissus graellsii* is a tetraploid, and its populations occur in sympatry with tetraploid populations of *N. bulbocodium* var. *nivalis* (Bobadilla et al. 1981; Barra & López González 1984; Zonneveld 2008), suggesting that ploidy levels (are not) do not seem to be an effective reproductive barrier between both taxa. These data indicate that it should be assigned to an infraspecific rank within *N. bulbocodium*.

Another previously recognized species, *N. subnivalis*, and the variety *N. bulbocodium* subsp. *bulbocodium* var. *ectandrus* were very similar from a genetic point of view from the nearest population of *N. bulbocodium* var. *bulbocodium* (rio Cuervo, Spain). Considering that morphological

distinction between these two taxa is not obvious (see: Barra & López González 1983; Fernández Casas 1986), they should be considered as an infraspecific rank, under *N. bulbocodium*. As *N. bulbocodium* subsp. *bulbocodium* var. *ectandrus* was described first it has precedence, and this taxon should be classified under this name.

Narcissus quintanilhae (Fernandes) Fernández Casas is a triploid or a hexaploid taxon, first described by Fernandes (1987) as a subspecies: *N. bulbocodium* subsp. *quintanilhae*. In 2005, Fernández Casas proposed the change to the specific rank. Both authors highlighted the habitat differences from the diploid forms of *N. bulbocodium*, but gave little indications about morphological differences. Fernandes (1987) showed that *N. quintanilhae* produce viable pollen with 7, 14, and 21 chromosomes and suggested that it was able to produce diploid, triploid, tetraploid, pentaploid, and hexaploid offspring. This indicates that triploidy does not provide genetic isolation from other forms of *N. bulbocodium*. These findings coupled with the very small genetic differentiation found in the present results, where *N. quintanilhae* falls within the *N. bulbocodium* clade, suggest that this taxon should be assigned infraspecific rank within *N. bulbocodium*, as first indicated by Fernandes (1987). Palermo et al (2010) used the same criteria to assign subspecific rank for *Plantago brutia* Ten. within *P. media* L.

The superimposition of the three morphological markers to the phylogenetic trees revealed that closely related populations exhibited very different morphologies, suggesting that the section *Bulbocodii* has strong phenotypic lability and that morphological variation can arise without phylogenetic significance. This variability appears to have led to an unjustified proliferation of nominal species in this section, without molecular support. Polyploidy is very prominent in *N. bulbocodium*, ranging from diploidy to octoploidy, and this could explain, at least partially, the phenotypic variability, but other factors are certainly involved. For instance, the presence of broad leaves in mountain forms of *N. bulbocodium* do not results from ploidy because they occur in diploid and tetraploid forms (Bobadilla et al.1981; Barra & López González 1984; Zonneveld 2008).

In summary, for the taxa analyzed, the systematic scheme that best fits the data presented here is:

Narcissus bulbocodium Linnaeus
- subsp. *bulbocodium*
  - var. *bulbocodium*
  - var. *ectandrus* Barra & López
  - var. *conspicuus* (Haworth) Baker
  - var. *nivalis* (Graells) Baker
  - var. *graellsii* (P.B. Webb) Baker
- subsp. *citrinus* (Baker) Fernández Casas
- subsp. *quintanilhae* A. Fernandes
- subsp. *validus* Barra

Narcissus cantabricus De Candolle
- subsp. *cantabricus*
  - var. *cantabricus*
  - var. *foliosus* (Maire) A. Fernandes
- subsp. *luteolentus* Barra & López

N. romieuxii ssp. *romieuxii* Braun Blanquet & Maire

Narcissus hedraeanthus (P.B. Webb & Heldreich) Colmeiro

Narcissus obesus Salisbury

Concerning the validity of the section *Bulbocodii*, it is premature to draw strong conclusions. Only a wider approach with a wider coverage of genus *Narcissus* and the use of nuclear markers may assess the entire significance of the dyphyletic pattern recovered with plastid DNA.

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