Int. J. Plant Sci. 175(5):501–517. 2014.

1058-5893/2014/17505-0001$15.00 DOI: 10.1086/675977

POPULATION GENETICS METHODS APPLIED TO A SPECIES DELIMITATION PROBLEM: ENDEMIC TRUMPET DAFFODILS (*NARCISSUS* SECTION *PSEUDONARCISSI*) FROM THE SOUTHERN IBERIAN PENINSULA

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Editor: Pamela Soltis

Premise of research. Determination of species boundaries is essential for understanding and preserving biological diversity, yet it remains a difficult task for many plant lineages. Population genetics approaches explicitly taking into account the geographic context of processes driving population divergence and speciation may help to identify species boundaries in complex groups. Here, we adopt this approach to investigate genetic boundaries in an endemic group of trumpet daffodils (*Narcissus*, section *Pseudonarcissi*) whose taxonomic distinctiveness remains controversial.

Methodology. We analyzed amplified fragment length polymorphism markers from a total of 36 populations (526 individuals) spanning the group's entire distribution range in the southern Iberian Peninsula. To identify the most likely number of distinct genetic groups, model- and nonmodel-based methods (Bayesian, principal coordinates, and neighbor-joining classification) were applied. Effects of long-term historical divergence were dissected from more recent or local differentiation processes using simple and partial Mantel tests.

Pivotal results. A major genetic split, consistently supported by the three analytical methods used, differentiated all populations generally ascribed to *Narcissus bujei* in traditional taxonomic treatments from the rest, which included all populations generally designated as *Narcissus longispathus* and *Narcissus nevadensis*. The two groups exhibited contrasting levels of within-population genetic diversity and rarity. Comparative analyses of the relationship between genetic differentiation and geographic distance in these two main genetic lineages suggested that they have remained isolated through a long time period. Separate analyses of genetic and geographic patterns within each major lineage suggested contrasting evolutionary histories.

Conclusions. Genetically, geographically, and ecologically well-defined lineages of the *Pseudonarcissi* section occur throughout the southern mountains of the Iberian Peninsula, generally supporting the traditional taxonomical delimitation of this lineage and qualifying as separate units of conservation. Our findings emphasize the usefulness of molecular data and population genetics approaches in a geographic context to delineate morphologically cryptic species in complex lineages.

Keywords: amplified fragment length polymorphism (AFLP), Amaryllidaceae, conservation, geographic variation, population genetics, species delimitation.

Online enhancements: appendixes.

Introduction

Species delimitation is a fundamental issue for many fields of biology and is a first step toward understanding the evolutionary mechanisms that generate diversification. However, it remains a difficult task in many groups of organisms (Shaw 2000; Ellis et al. 2006; Hendrixson et al. 2013). In particular, determination of plant species boundaries in recently diverged lineages is a common problem for evolutionary biologists because populations may have not been isolated long enough to accumulate differences, and both morphological and genetic markers would be likely to show low levels of differentiation (Whittall et al. 2004; Shaffer and Thomson 2007; Schönswetter et al. 2009; Lega et al. 2012; Slovák et al. 2012). An alternative and powerful tool for delimiting the boundaries of closely related taxa in complex groups of plants are population genetics approaches that rely on large sets of informative and independent genetic markers (Drummond and Hamilton 2007; Joly and Bruneau 2007; Kučera et al. 2008; Duminil and di Michele 2009; Duminil et al. 2012). Because of their genome-wide sampling, high reproducibility, and ability to resolve even extremely small genetic differences, amplified fragment length polymorphisms (AFLPs) are among the most efficient markers

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Manuscript received July 2013; revised manuscript received December 2013; electronically published April 29, 2014.

for discrimination between closely related species and for studies of species boundaries (Meudt and Clarke 2007; Mereďa et al. 2008; Minder and Widmer 2008; Frey et al. 2012; Garrido et al. 2012).

Population genetics approaches to species delimitation problems generally assume that the level of differentiation between taxa will exceed that found within taxa (Drummond and Hamilton 2007). Implicit in this assumption is a defining feature of a species, namely, that their populations should be connected by gene flow (Mayr 1963). Levels of gene flow among populations are influenced by intrinsic species traits, especially those related to dispersal ability, but they are also influenced by extrinsic habitat features and other historical processes that have shaped the spatial distribution of populations in the landscape. Therefore, one important aspect that should be incorporated into population genetics studies of the processes driving patterns of populations and species divergence is the geographic context of such divergence. If the geographic context is overlooked, interpretations could fail to recognize historical population associations (Knowles and Carstens 2007) or could be unable to distinguish true discontinuities (i.e., lineage separation) from the differentiation that occurs within species as the result of other phenomena, such as clines and isolation by distance (de Queiroz 2007). One indirect way of incorporating the geographic context when assessing species boundaries is to analyze whether divergence among genetically isolated lineages occurs independently of their degree of geographic separation (Good and Wake 1992; Sites and Marshall 2003, 2004). Findings revealing that geographically distant populations of the same evolving lineage are genetically more closely related to each other than to geographically proximate populations of other lineages or that highly genetically divergent lineages are found in sympatry would be strong arguments for the recognition of truly distinct species (Minder and Widmer 2008; Koffi et al. 2010; Duminil et al. 2012).

In this study, we adopt a population genetics approach based on application of the AFLP technique to investigate the genetic boundaries and geographic patterns of genetic divergence of an endemic group of trumpet daffodils (*Narcissus* L., section *Pseudonarcissi* DC.) that are exclusively distributed throughout the Baetic Ranges of the southeastern Iberian Peninsula, one of the most prominent plant biodiversity hotspots of the Mediterranean Basin (Médail and Quézel 1997; Melendo et al. 2003; Thompson 2005). Mediterranean mountains have been the site of a significantly high number of speciation events (Martín-Bravo et al. 2010), which have resulted in a high number of endemic species and an exceptionally rich flora (Gómez-Campo 1985; Sainz-Ollero and Moreno-Saiz 2002). The genus *Narcissus* is also a remarkable example of Mediterranean lineage with a complex evolutionary history (Graham and Barrett 2004; Santos-Gally et al. 2012). The taxonomy of *Narcissus* is unsettled and problematic (Webb 1980; Blanchard 1990; Mathew 2002; Zonneveld 2008; Aedo, forthcoming); in particular, the trumpet daffodils (section *Pseudonarcissi*) are one of the most controversial groups of the genus and are a clear example of the confounding views on both the discrimination of taxa and the assignment of rank (see below). In fact, the reasons for this wide discrepancy among studies may reflect, among other processes, recent phylogenetic divergence, ongoing differentiation, high phenotypic plasticity, hybridization, or some complex combination of these (Pérez-Barrales et al. 2006; Díaz-Lifante and Andrés-Camacho 2007; Hodgins and Barrett 2007; Díaz-Lifante et al. 2009). Irrespective of the mechanisms responsible for this complexity, however, tracing species boundaries in such complex groups is a challenge unless the patterns and causes of differentiation are assessed. Because of a lack of studies of the genetic structure of this group of endemic trumpet daffodils, it remains unresolved whether truly independent genetic lineages exist or, alternatively, whether populations exemplify a genetic continuum of a single taxon in response to different environmental conditions or other biotic factors. This could be, therefore, an ideal group for studying the geographic scales at which population genetics divergence accumulates because closely related taxa are supposed to have similar ecological traits influencing gene flow, and they may also have shared common patterns of geographic migration and evolutionary history. In addition, this study may provide insights into the ecological context of past speciation events and the evolutionary forces involved in the diversification process that shape the Mediterranean flora.

To get an accurate picture of the possible different genetic lineages of this group of daffodils throughout the Baetic Ranges, multiple populations will be compared in our study, such that interspecific variation can be distinguished from intraspecific variation. To this end, we have used a broad geographic sampling of 36 natural populations covering the whole distribution area and representing as much diversity of ecological conditions as possible. We hypothesize that if there are in fact divergent genetic lineages rather than the continuum of a single taxon coexisting in the region, then (i) genetically distinct groupings should exist and (ii) geographically distant populations of the same genetic lineage would tend to be more closely related to each other than to geographically proximate populations of other genetic lineages. We will interpret our results in the context of previous taxonomical studies, discuss the potential and limitations of the AFLP technique and population genetics approaches for delimiting species, and highlight the value of detailed genetic investigations of species complexes in the western Mediterranean plant biodiversity hotspot.

Material and Methods

The Trumpet Daffodils of the Baetic Ranges

Like all members of the genus *Narcissus* L., the trumpet daffodils of the section *Pseudonarcissi* DC. that occur throughout the Baetic Ranges are long-lived perennial geophytes that have winter-growing and summer-dormant bulbs. Their natural populations are commonly associated with permanently humid habitats (including stream margins, springs, wet pastures, clearings of forests or shrublands with humid soils, and moist hillsides) and are fairly discrete and isolated due to the natural rarity and discontinuity of this type of habitat in the Mediterranean mountains. Plants often spread clonally via fision of bulbs, sometimes giving rise to clumps of genetically identical individuals. Flowering takes place in late winter–early spring. Flowering individuals produce large hermaphroditic flowers (corolla length, ~50 mm) that are usually presented solitary or more rarely in inflorescences with 2–3(4) flowers. The overall floral morphology of all the trumpet daffodils is

very similar. Flowers have wide, greenish floral tubes; large, funnel-like, bright yellow coronas; and six tepals that occasionally differ slightly in color from the corona (from creamy to pale yellow). These large flowers are supposed to be pollinated principally by solitary bees that generally forage for pollen from the anthers, which are always enclosed within the corona (see Herrera 1995 for a detailed study), although further studies of the geographic variation in the composition of the pollinator guild are required to confirm this general assumption. Fruit maturation and seed shedding take place between late May and early June. The fruit is a capsule that releases the seeds by three longitudinal splits after drying of the pericarp. The seeds are large, buoyant, and thick-coated, apparently lacking specialized dispersal mechanisms.

Taxonomic background. In the Baetic Ranges, which run from the southwestern mountains of the Andalucía region (province of Cádiz) to the eastern mountains of the Murcia region (see fig. 1*A* for location), the taxonomic literature has traditionally distinguished three main taxa belonging to section *Pseudonarcissi*: *Narcissus bujei* (Fern. Casas) Fern. Casas (the specific epithet is sometimes spelled *bugei*; Blanca et al. 1999; Navarro 2011), *Narcissus nevadensis* Pugsley, and *Narcissus longispathus* Pugsley (although the assignment of species rank has not always been maintained; Fernández-Casas 1986; Mathew 2002; Zonneveld 2008). This taxonomic treatment was adopted in the recent revision of *Narcissus* species occurring in eastern Andalucía (Navarro 2011). Slight variations in morphological floral traits (e.g., number of flowers per umbel, length of the spatha and the pedicel, and differences in color between the corona and the tepals) have been used as diagnostic characters to delimit these three taxa. However, a gradation of intermediate forms is usually found in natural populations, making it sometimes difficult to distinguish either taxon in the field. Some authors have pointed out that these three taxa show slightly different ecological preferences and largely nonoverlapping altitudinal and geographic distributions (Navarro 2011). Whereas populations of *N. longispathus* and *N. nevadensis* are confined to rare and isolated habitats, as they are strict specialists of permanently waterlogged meadows or stream margins at high elevations (900–1800 and 1400–2300 m, respectively), *N. bujei* populations are supposed to have been historically less isolated, since they are able to grow at lower altitudes (between ~600 and ~1500 m) and in a broader variety of Mediterranean habitat types, such as stream margins, poorly drained pastures, moist hillsides,

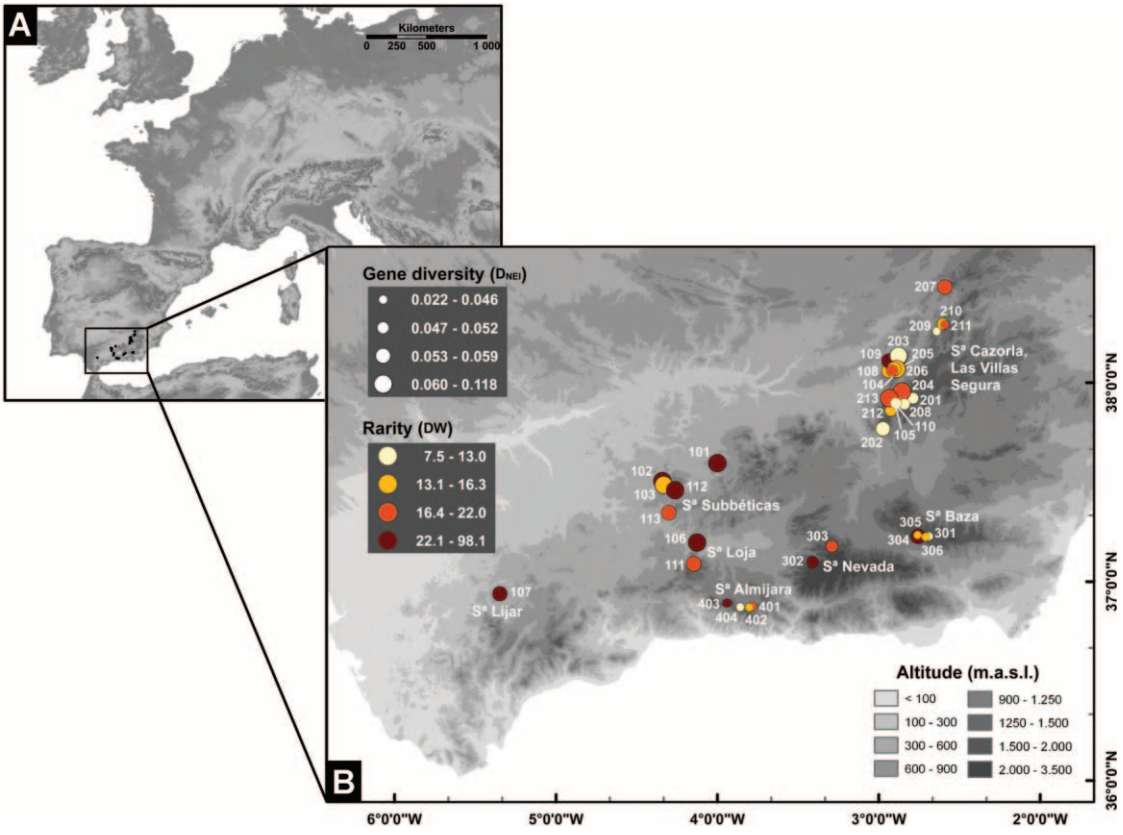

Fig. 1 Map indicating the position of the Baetic Ranges in Europe (*A*) and the exact location of the 36 *Narcissus* populations studied, showing the geographic patterns of genetic variation and divergence based on amplified fragment length polymorphism (AFLP) data throughout this area (*B*). In the latter panel, circle size represents AFLP gene diversity (D_{NEI}) within each population (the larger the circle, the more diverse the population), colors represent rarity values (frequency-downweighted marker value [DW]) for each population (the darker the color, the more rare fragments accumulated in a population), and partitions match interquartile intervals. Population numbers refer to table 1. Approximate positions of some of the most important mountain systems comprising the Baetic Ranges (specified in table 1 and discussed in the text) are also shown.

fringes of sclerophyllous forests, and clearings of deciduous shrublands.

Although only these three taxa have been generally accepted, more recently up to four different species have been described by other authors as being part of this group, namely, *Narcissus alcaracensis* Ríos, Rivera, Alcaraz, and Obón; *Narcissus segurensis* Ríos, Rivera, Alcaraz, and Obón; *Narcissus yepesii* Ríos, Rivera, Alcaraz, and Obón; and *Narcissus enemeritoi* (Sánchez-Gómez, Carrillo, Hernández, Carrión, and Güemes) Sánchez-Gómez, Carrillo, Hernández, Carrión, and Güemes (Ríos-Ruiz et al. 1999; Sánchez-Gómez et al. 2000). However, their distinction was based on even more tenuous morphological differences, and all are exclusively restricted to one or a few sparse localities on the northeastern edge of the Baetic Ranges (Sierras de Alcaraz and Segura and Sierra de Villafuerte). The validity of these additional taxa was not upheld by subsequent molecular (Medrano and Herrera 2008; Zonneveld 2008; Jiménez et al. 2009) and taxonomic (Navarro 2011; Aedo, forthcoming) work.

Most recently, a quite contrasting view was provided by the latest revision of all Iberian *Narcissus* species (Aedo, forthcoming), which concluded that all the trumpet daffodils inhabiting the Baetic Ranges should be ascribed to *Narcissus pseudonarcissus* L. subsp. *nevadensis* (Pugsley) A. Fern. The acknowledged basis for this decision was a difficulty to delimit recognizable taxonomic entities within this geographic region because of insufficient morphological differences (Aedo, forthcoming). This treatment ignores and is incongruous with the major phylogenetic reconstruction of the genus *Narcissus* based on DNA sequence data performed by Graham and Barrett (2004), in which *N. longispathus* and *N. nevadensis* are unambiguously separated from *N. pseudonarcissus* and are also well separated from each other (with a bootstrap value of 76%; see also Rønsted et al. 2008; Santos-Gally et al. 2012). Aedo (forthcoming) also negates the distinctness of *N. bujei* and *N. longispathus* on the basis of internal transcribed spacer (ITS) and inter–simple sequence repeat (ISSR) sequences reported by Jiménez et al. (2009).

Since one of the main aims of this article is to examine whether all the trumpet daffodils distributed throughout the Baetic Ranges comprise one or several species (or infraspecific taxa), we will not adhere a priori in our analyses to any of the preceding taxonomic hypotheses.

Sample Collection

A total of 538 individuals from 36 localities (hereafter called populations) covering the whole distribution area of this group of trumpet daffodils throughout the Baetic Ranges and representing as much ecological diversity as possible were included in this study (for further details, see table 1; fig. 1). Information on the distribution and location of all the natural populations sampled for this study was gathered from published literature and by consulting the chorological information of the Spanish Plants Information System (available at the Anthos program website: http://www.anthos.es/). Field collections were conducted from 2004 to 2008. Mean geographic distance between the 36 populations sampled was 99.1 km (range, 0.82–301.8 km; fig. 1). The altitudinal range of the sampled sites varied between 540 and 2065 m asl and spanned the whole altitudinal range of this group of *Narcissus* (table 1). The number of individuals sampled per population varied from 10 to 18 (mean ± SD, 14.9 ± 1.5; table 1). To avoid sampling one genetic individual more than once, each sampled plant was usually separated from the next by at least 0.5 m, and sampled plants were as widely spaced as the spatial extent of the population allowed. One young, intact leaf of each individual was collected, separately placed in a paper envelope, immediately dried in ziplock plastic bags containing abundant silica gel, and stored at room temperature until DNA extraction.

DNA Extraction and AFLP Fingerprinting

Similar amounts of dried leaf tissue (~20–25 mg) of each sampled individual were homogenized to a fine powder using a Retsch MM 200 mixer mill. Total genomic DNA was extracted from ground leaf material using the DNeasy Plant Mini Kit (Qiagen), in accordance with the manufacturer's instructions. The quality and quantity of the extracted DNA was checked on 1% TBE agarose gels.

AFLP analyses were performed by adopting the protocols established by Vos et al. (1995) with minor modifications, including the use of the restriction endonucleases *Pst*I and *Mse*I (New England Biolabs, Ipswich, MA), the use of nonradioactive fluorescent dye–labeled primers (Applied Biosystems, Foster City, CA) for selective amplification, and multiplex analysis using an automated DNA sequencer (ABI Prism 3130xl; Applied Biosystems). Full details of the AFLP protocol used in this study are included in appendix B (appendixes A and B are available online).

An initial screening of selective primers was performed using 12 primer combinations. The four selected primer combinations that yielded appropriate numbers of clear peaks (fluorescent dye in brackets) were *Pst*I(FAM)-AA/*Mse*I-CGC, *Pst*I(VIC)-AT/*Mse*I-CGT, *Pst*I(NED)-AC/*Mse*I-CAC, and *Pst*I(PET)-AG/*Mse*I-CTT. The pair of restriction enzymes used here (*Pst*I/*Mse*I) generates markers that are more extensively distributed over the genome and is more efficient at detecting polymorphism and interspecific differentiation than the most commonly used pair, *Eco*RI/*Mse*I (Powell et al. 1997; Vuylsteke et al. 2000; Scotti-Saintagne et al. 2004). Electropherograms were visualized, aligned with an internal size standard (Gene Scan 500 LIZ), and manually analyzed using the program GeneMapper (ver. 3.7; Applied Biosystems). For each individual, all fragments detected in the range of 180–500 bp were manually scored as present (1) or absent (0).

Special care was taken to ensure reproducibility of AFLP analyses. For each primer combination, 16 negative amplification controls without template DNA were run. To check the consistency of individual results, 32 individuals (6.0%) were independently replicated for all four primer combinations starting from different steps in the AFLP protocol: extraction, restriction, and ligation or preselective amplification. Negative controls and replicates were distributed over different runs of the sequencer and over different 96-well plates. Fragments appearing in more than two negative controls, nonreliable bands (i.e., fragments that were clearly not reproducible; specifically, those for which the error rate was >10%), and individuals with odd or low-quality profiles (a total of 12 individuals;

Table 1

Location Details and Amplified Fragment Length Polymorphism (AFLP)–Based Genetic Diversity Estimates for the 36 *Narcissus* Populations Included in This Study

Population code	Locality name	Location: Mountain system ("sierras")	Location: Province	Geographic coordinates (lat./long.)	Elevation (m asl)	N[a]	NFPI	D_{NEI}	DW
101	Ahillos	Sur de Jaén	Jaén	37.599/−4.022	1200	16	51.6	.096	51.9
102	Cabra-1	Subbéticas de Córdoba	Córdoba	37.505/−4.368	962	17 (2)	48.1	.083	27.2
103	Cabra-2	Subbéticas de Córdoba	Córdoba	37.486/−4.360	974	16 (4)	39.7	.087	15.9
104	Raso de la Escalera	Cazorla–Segura–Las Villas	Jaén	38.067/−2.915	1380	16	42.8	.047	18.7
105	Arroyo de la Garganta	Cazorla–Segura–Las Villas	Jaén	37.905/−2.892	1440	12	42.6	.047	12.8
106	El Hachuelo	Loja	Granada	37.198/−4.144	841	14	56.7	.118	98.1
107	Líjar	Líjar	Cádiz	36.922/−5.379	693	15 (1)	49.1	.059	96.3
108	Collado del Lobo	Cazorla–Segura–Las Villas	Jaén	38.067/−2.934	1219	13	50.0	.057	15.7
109	Muela Alta	Cazorla–Segura–Las Villas	Jaén	38.116/−2.934	1345	16	44.0	.053	22.1
110	Cabeza del Tejo	Cazorla–Segura–Las Villas	Jaén	37.908/−2.880	1650	10	45.7	.051	10.7
111	Charco Negro	Loja	Granada	37.091/−4.164	1480	16 (1)	38.3	.054	21.3
112	El Palancar	Subbéticas de Córdoba	Córdoba	37.461/−4.287	541	16 (1)	45.1	.068	30.5
113	Rute	Subbéticas de Córdoba	Córdoba	37.346/−4.326	1100	15	42.5	.058	20.3
201	La Cabrilla	Cazorla–Segura–Las Villas	Jaén	37.930/−2.781	1650	13	46.5	.047	7.7
202	La Canal	Cazorla–Segura–Las Villas	Jaén	37.775/−2.973	1360	15	44.3	.055	9.3
203	Carrales	Cazorla–Segura–Las Villas	Jaén	38.143/−2.873	1130	15 (1)	53.2	.071	13.0
204	Cuevas Bermejas	Cazorla–Segura–Las Villas	Jaén	37.964/−2.851	1185	17 (1)	51.8	.063	22.0
205	Collado Perenoso	Cazorla–Segura–Las Villas	Jaén	38.077/−2.885	1380	14	54.5	.081	13.3
206	Cortijo de la Traviesa	Cazorla–Segura–Las Villas	Jaén	38.076/−2.903	1330	15	48.1	.057	13.9
207	Dehesa de Bayona	Cazorla–Segura–Las Villas	Jaén	38.487/−2.576	1308	15	50.3	.054	18.3
208	Guadalentín (cercado)	Cazorla–Segura–Las Villas	Jaén	37.900/−2.836	1320	18	42.9	.049	12.5
209	Fuente de la Jordana	Cazorla–Segura–Las Villas	Jaén	38.265/−2.630	1337	15	43.1	.034	7.5
210	Navalcaballo	Cazorla–Segura–Las Villas	Jaén	38.302/−2.590	1340	14	43.1	.046	14.5
211	Arroyo del Tejuelo	Cazorla–Segura–Las Villas	Jaén	38.299/−2.577	1350	15	42.7	.044	16.4
212	Tornillos de Gualay	Cazorla–Segura–Las Villas	Jaén	37.868/−2.924	1410	13	46.6	.052	14.1
213	Arroyo del Valle	Cazorla–Segura–Las Villas	Jaén	37.930/−2.933	690	15	53.5	.069	18.2
301	Barranco del Aguadero	Baza	Granada	37.234/−2.683	1880	16	43.5	.022	8.1
302	Prados del Aire	Nevada	Granada	37.105/−3.416	2065	14	42.8	.047	26.1
303	Barranco de los Tejos	Nevada	Granada	37.184/−3.295	2020	15	45.0	.046	19.6
304	Nacimiento del Morás	Baza	Granada	37.234/−2.751	1854	16	47.7	.053	37.2
305	Cortijo del Sotillo	Baza	Granada	37.241/−2.755	1821	15 (1)	42.9	.040	15.1
306	Nacimiento del Uclías	Baza	Granada	37.231/−2.705	1868	16	45.1	.033	14.7
401	Arroyo de las Golondrinas	Almijara	Granada	36.879/−3.791	1118	15	59.7	.044	20.7
402	Cortijo de las Monjas	Almijara	Granada	36.875/−3.811	1122	15	60.5	.046	16.3
403	Haza del Aguadero	Almijara	Granada	36.896/−3.952	1372	15	61.7	.046	22.6
404	Arroyo de la Venta	Almijara	Granada	36.876/−3.869	1032	15	41.9	.034	8.0

Note. Population code, locality name, location (mountain system and administrative province), geographic coordinates, elevation above sea level, and number of individuals included in AFLP analyses (N) are indicated for each population. Values for the mean number of fragments per individual (NFPI), within-population Nei's gene diversity (D_{NEI}), and rarity of markers (expressed as the frequency-downweighted marker value [DW]) are also shown.

[a] Shown in parentheses are the number of individuals with anomalous or low-quality AFLP profiles that were discarded from the final data set.

table 1) were provisionally discarded from the data set for error checking and further analysis. Samples where anomalies persisted after repetition of analyses were definitively discarded. Genotyping error rates were estimated as the ratio of the total number of loci with discordant scores (all individuals combined) to the product of the number of individuals by the total number of scored loci (Bonin et al. 2004), and they are included in appendix A (table A1).

Data Analyses

Genotype-based inferences about delimitation of genetic groups. Because alternative approaches to analyzing AFLP data may lead to different conclusions (Burnier et al. 2009; Lihová et al. 2010; Espíndola and Álvarez 2011), to identify the most likely number of distinct genetic groups present in our data set we decided to apply three different methods based on distinct model assumptions and methodologies (Gao et al. 2007; Jombart et al. 2009). By explicitly adopting this combined approach, it will be possible to evaluate the robustness of conclusions to different analytical methods.

First, to estimate the distribution of individuals among natural genetic groups (K), the AFLP fragment presence/absence matrix for the complete data set was analyzed by Bayesian model–based clustering methods as implemented in the software STRUCTURE (ver. 2.3; Falush et al. 2007). The primary assumptions of the model are Hardy-Weinberg equilibrium

within populations (or metapopulations) and linkage equilibrium between loci, although the latter assumption has been relaxed (Falush et al. 2003). This program seeks to cluster individuals without regard to their population of origin on the basis of rough conformity to Hardy-Weinberg genetic expectations. We chose settings adequate for dominant data as implemented in STRUCTURE version 2.3.3. Specifically, we used the admixture model with recessive alleles and correlated allele frequencies among the populations. To determine the most likely number of natural genetic groups (K), we conditioned our data on different values of K ranging from 1 to 10. To test the stability of the results, 30 runs were performed for each K. The length of the burn-in period was set to 50,000, and the Markov chain Monte Carlo chains after burn-in were run for additional 100,000 replicates. Computations were carried out using both the Galician Supercomputing Center and the freely accessible Bioportal (University of Oslo, Norway, website: http://www.bioportal.uio.no/). The web-based program Structure Harvester (Earl and von Holdt 2012; available at http://taylor0.biology.ucla.edu/struct_harvest/) was used to summarize the output files generated by STRUCTURE. This tool produces an output consisting of a series of files including the means of the estimated posterior log probability of the data over the run replicates for each K value (denoted as mean ln $P(K)$) and the ad hoc statistic ΔK described by Evanno et al. (2005), which identifies the highest rate of change in the log-likelihood between successive Ks (see Evanno et al. 2005 for details). Similarity among results of different runs for the same K was also calculated according to Nordborg et al. (2005) using the R script (R Development Core Team 2011) Structure-sum-2009 (http://uit.no/ansatte/organisasjon/ansatte/person?p_document_id=41186&p_dimension_id=). The best value of K that captured the main structure in the data was defined by the model in which simultaneously the increase in likelihood of the data started to stabilize, there was a peak in ΔK, and results were consistent over multiple runs.

In situations of complex and hierarchical data sets, the first optimal K solution obtained by the preceding approach will represent correctly only the uppermost level of the hierarchical genetic substructure (Evanno et al. 2005). Therefore, we implemented this method in a hierarchical framework to fully identify the complete substructure in our data set. Specifically, we repeated the STRUCTURE analyses (estimation of the number of natural genetic groups and assignment of the individuals to the groups) on each of the K groups inferred in the previous step using the same parameters. In all cases, the replicate runs of the best K were merged with CLUMPP (ver. 1.1.1; Jakobsson and Rosenberg 2007) using the Greedy algorithm and 5000 replicates. Relative cluster membership coefficients of all individuals were then averaged for each population. Spatial genetic structures were plotted on geographic maps using ArcMap (ver. 10.0; Esri, Redlands, CA), with populations represented as pie charts showing the number of individuals assigned to each cluster.

The second method used to explore the internal structure of the presence/absence matrix for the entire data set was principal coordinates analysis (PCoA; Krzanowski 1990). PCoA uses multivariate statistics to depict the genetic structure and is free from all the population genetics assumptions underlying STRUCTURE, which could be more useful when population structure conforms to a simple isolation-by-distance model (Engelhardt and Stephens 2010). Genetic distances between all possible pairwise comparisons of individuals within and among populations were estimated using standard Jaccard similarity index transformed into a distance matrix as D = sqrt(1 − Jaccard similarity). The Jaccard similarity index is defined as the fraction of fragments shared by two individuals (Kosman and Leonard 2005). It considers only shared presence as contributing to the similarity of individuals and disregards shared absence, which is more prone to homoplasy (Gaudeul et al. 2012). PCoA based on the Jaccard distance matrix was computed and visualized using the R CRAN package ade4 (Dray et al. 2007; R Development Core Team 2011).

A third method was used to visualize the relationships among all individuals and populations studied, based on the construction of a phenogram by applying the neighbor-joining (NJ) method to the matrix of Nei and Li (1979) genetic distances. Pairwise genetic distances among AFLP phenotypes were calculated as the complementary value of Nei and Li's (1979) similarity coefficient as implemented in PAUP* (ver. 4.0b10; Swofford 2002). The same program was used to construct unrooted NJ phenograms (Saitou and Nei 1987) from the distance matrix. Support for each node was tested by 2000 bootstrap replicates. The majority rule consensus tree from the 2000 bootstrapped trees was constructed using PAUP and visualized with Geneious Pro bioinformatics software (ver. 5.5; Biomatters, Auckland, New Zealand).

Analyses of molecular variance (AMOVAs; Excoffier et al. 1992) were finally used to compare variability within and among populations and to test clustering results. All the AMOVAs were computed in Arlequin (ver. 3.5; Excoffier et al. 1992; Excoffier and Lischer 2010), and the significance of the results was obtained from 5000 permutations.

Within-population and among-group genetic diversity and divergence. For each population or population grouping arising from clustering analyses, we estimated genetic diversity by calculating the average number of fragments per individual and the average proportion of pairwise differences between individuals (using the corresponding R functions in the AFLPdat script; Ehrich 2006). The latter estimate corresponds to Nei's gene diversity calculated from marker frequencies (Nei 1987): $D_{\mathrm{NEI}} = N/(N-1) \times [1 - (\mathrm{freq}(1)^2 + \mathrm{freq}(0)^2)]$, where N is the sample size and freq(1) and freq(0) are the frequencies of the marker presence and absence, respectively, calculated for each marker and then averaged (Ehrich 2006). To assess the genetic divergence among populations, the frequency-downweighted marker value (DW) was calculated according to Schönswetter and Tribsch (2005) using the corresponding function in AFLPdat. For each individual, the occurrence of a marker was divided by the total number of occurrences of that marker in the data set. All markers were summed for each individual and then averaged over the population, to diminish the effect of differences in sample size following Ehrich et al. (2007). The value of DW is expected to be high in long-term isolated populations, where rare markers are assumed to accumulate due to mutations, whereas newly established populations are expected to exhibit low values.

Geographic patterns of genetic differentiation. To determine the proportion of total variance of genetic distances between populations that could be attributed to long-term his-

torical divergence or more recent and local isolation-by-distance processes controlling for differences in geographic distances, simple and partial Mantel permutation tests were employed (Sacks et al. 2004; Telles and Diniz-Filho 2005; Duvernell et al. 2008). In all these analyses, the dependent matrix was the one of genetic distances (expressed as F_{st}), while the independent (predictor) matrices corresponded to geographic distances (km) and "grouping." The pairwise grouping matrices consisted of 0s and 1s indicating, respectively, that the two given populations are in the same or different genetic groups. Because the partial Mantel correlation coefficients between genetic distances and grouping controlled for the effect of geographic distance, their significance would support the hypothesis that genetic groups accounted for genetic distances better than geographic distances alone. Note, however, that elevated type I error could be an important concern when using partial Mantel tests, in particular with spatially autocorrelated data (Raufaste and Rousset 2001; Rousset 2002; Guillot and Rousset 2013), and that although these tests seem to perform more appropriately to resolve questions that involve distance matrices (Legendre and Fortin 2010), their results should be interpreted with caution. To obtain estimates of the genetic differentiation between populations, we computed pairwise F_{st} using the software Arlequin (ver. 3.5). The geographic distance matrix was computed using Geographic Distance Matrix Generator (ver. 1.2.3; Ersts 2012) on the basis of distances between the geographic coordinates for each collected population. Mantel tests were carried out using IBD (ver. 1.52; Bohonak 2002) with 10,000 random permutations. Separate comparisons were performed for the entire set of 36 populations included in our study as well as for the two subsets of populations obtained from our first STRUCTURE analyses.

Results

AFLP Data Summary

The total number of markers, size ranges, and mean genotyping error rates for each primer combination are given in appendix A (table A1). The four primer combinations applied to the 526 samples generated a total of 510 AFLP fragments ranging between 180 and 499 bp. Different primer pairs amplified a variable number of markers, from 106 to 139, with an average of 127.5 fragments per primer combination. Replicated samples indicated a high reproducibility (mean error rate ± SD for the whole set of four combinations, 2.17% ± 0.34%). The average number of AFLP markers per individual varied among populations from 38.3 to 61.7 (mean ± SD, 47.5 ± 9.7; table 1). There was extensive polymorphism (none of the 510 AFLP fragments was monomorphic), and all the samples analyzed had distinct multilocus AFLP fingerprints.

Delimitation of Genetic Groups

Bayesian analysis of the complete data set (526 individuals, 510 AFLP fragments) using STRUCTURE revealed an optimum number of $K = 2$ to capture the most variation in genetic structure (fig. 2*A*). The three criteria used to choose the best value of K were fully congruent: likelihood values for each K reached a plateau at $K = 2$, the rate of change of the log-likelihood function between successive Ks (Evanno's ΔK) exhibited a maximum value at $K = 2$ (fig. 2*A*), and similarity coefficients among replicate runs were also highest at $K = 2$ (mean ± SD, 0.998 ± 0.0002). The first division of the data set reflects an important divergence that segregates unambiguously one group comprised of 183 individuals and 13 populations (populations 101–113; depicted in blue in fig. 2*B* and hereafter referred to as the blue group), which included most of the populations ascribed to *Narcissus bujei* in classical taxonomic treatments, from the rest of the 343 individuals and 23 populations (populations 201–404; depicted in green in fig. 2*B* and hereafter referred to as the green group). These two genetic groups were highly homogeneous internally, with almost no uncertain assignments at the population or individual level. Excepting the southwesternmost population (107), which exhibited considerable admixture (77%), the rest of the populations were exclusively made up of one of these two gene pools (proportion of membership ≥96%). Similarly, the vast majority of individuals (~95%) were assigned to one of these two genetic groups with a probability ≥0.9, and only individuals from the southwesternmost population (107) showed intermediate assignment probabilities (range, 0.69–0.91). These two main genetic groups occurred sympatrically at the northeastern extreme of the Baetic Ranges (Sierras de Cazorla–Segura–Las Villas; see populations 104, 108, 109, 203, 205, and 206 in fig. 2*B*), yet they remained clearly distinct there, as reflected by the fact that admixed individuals or populations were not more frequent there than elsewhere (fig. 2*B*).

Separate Bayesian analyses for the two main genetic groups revealed further genetic substructuring within each (fig. 2*C*–2*F*). Within the green group, $K = 3$ was chosen as the most appropriate number of genetic subgroups according to our three criteria (fig. 2*C*; similarity coefficient, 0.996 ± 0.0004). These three genetic subgroups reflected different geographic lineages (fig. 2*D*) and were also able to discriminate individuals according to previously recognized taxonomic units, namely, (*a*) one northern group composed of all 192 individuals and 13 populations sampled within Sierras de Cazorla–Segura–Las Villas, which have been traditionally recognized as *Narcissus longispathus* (populations 201–213; depicted in light green in fig. 2*D* and hereafter referred to as green_N); (*b*) one central group containing all 91 individuals and six populations sampled at the Sierra Nevada and Sierra de Baza ranges, commonly named *Narcissus nevadensis* (populations 301–306; depicted in pale green in fig. 2*D* and hereafter referred to as green_C); and (*c*) one southern group with the 60 individuals sampled at the four localities of the Sierras de Almijara ranges, whose taxonomic affiliation is unknown because they do not seem to have been studied previously (populations 401–404; depicted in dark green in fig. 2*D* and hereafter referred to as green_S). Only minor admixture was detected between these three gene pools, suggestive of very limited gene flow between these three separate mountain regions.

By our three criteria, the most likely number of subclusters within the blue group was 4 (fig. 2*E*; similarity coefficient, 0.96 ± 0.13). This subdivision, however, was less clear than that for the green group, as reflected by the very low values of the ΔK parameter, its multimodality (with peaks at $K = 2$ and 4), and the strong decrease in log-likelihood values for each K (fig. 2*E*). For $K = 4$, the blue group was divided into three geographically structured subgroups of populations,

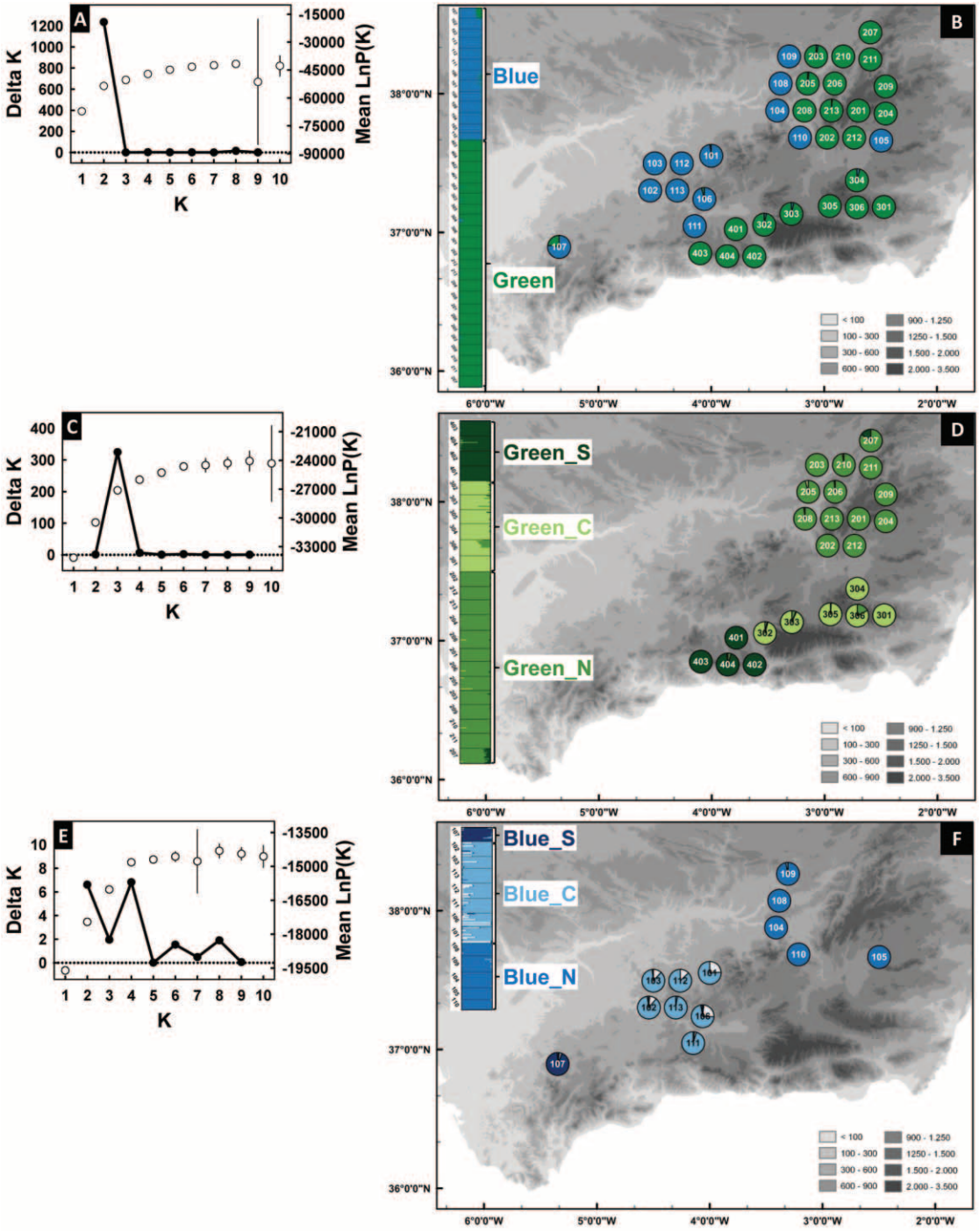

Fig. 2 Results of Bayesian clustering analyses with the software STRUCTURE performed on the entire amplified fragment length polymorphism (AFLP) data set (526 individuals, 510 AFLP fragments; *A*, *B*), the green group (343 individuals, 394 AFLP fragments; *C*, *D*), and the blue group (183 individuals, 425 AFLP fragments; *E*, *F*). *A*, *C*, *E*, Estimation of the most probable number of groups of populations (K) for $K = 1$–10 and 30 STRUCTURE simulations each: posterior probabilities of the data ln $P(K)$ (open circles) and values of Evanno's ΔK (filled circles). *B*, *D*, *F*, Maps of the distribution of the different genetic groups obtained as the optimal solution. Pie charts represent the proportions of the different genetic pools detected in each population. Note that pie charts were drawn as near as possible to the site each population was located, but to improve visualization a few populations were slightly displaced. Bar graphs in each panel show the posterior assignment probability (horizontal axis) of individual genotypes (vertical axis) to each gene pool (in different shades of blue or green). Population numbers correspond to population codes in table 1.

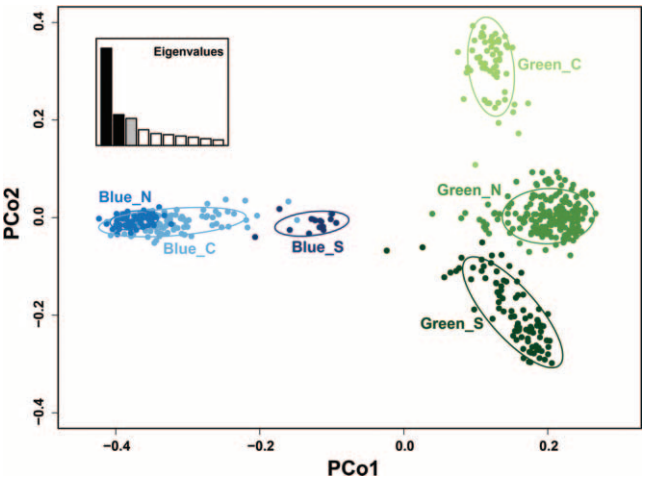

Fig. 3 Results of the principal coordinates analysis based on a matrix of Jaccard distances for the entire data set (526 individuals, 510 amplified fragment length polymorphism fragments). The different colors in the bidimensional plot correspond to the genetic groups identified by Bayesian clustering and match those in fig. 2*D* and 2*F*. Bivariate ellipses reflect both the variance among individuals in each group and the covariance on the two axes. Eigenvalues for the first 10 ordination axes are shown in the bar plot. The first and the second ordination axes (black bars) explain 43.4% and 13.7% of the total variation in the data matrix, respectively.

namely, (*a*) one northern group comprising populations 104, 105, 108, 109, and 110 from the Sierras de Cazorla–Segura–Las Villas (referred to as blue_N in fig. 2*F* and hereafter); (*b*) one central group including populations 101, 102, 103, 106, 111, 112, and 113, located in and around the Sierras Subbéticas (referred to as blue_C in fig. 2*F* and hereafter); and (*c*) the southwesternmost population 107 (referred to as blue_S in fig. 2*F* and hereafter). Although very little admixture was detected again in the northern and southwestern subgroups, two of the four gene pools appeared substantially admixed in the geographically intermediate group of populations (fig. 2*F*).

PCoA based on Jaccard distance was in close agreement with Bayesian results (fig. 3). The two most divergent groupings of individuals in the PCoA ordination space were completely separated along the first axis (which explained 43.4% of total genetic variation) and corresponded with the two main genetic groups identified by the Bayesian analysis (designated the blue and green groups). The second axis (13.7%) mostly allowed the delineation of a substructure within the green group similar to the one obtained by subsequent Bayesian analyses. However, the substructure according to the blue group fitted less well in the PCoA (fig. 3).

The unrooted NJ tree based on the complete data set (fig. 4) resolved two distinct, well-supported major clades with high bootstrap support (98.3% BS), which matched the blue and green groups identified by both Bayesian and PCoA. However, within the green clade only the node containing the southernmost subgroup (green_S subgroup, populations 401–404; fig. 4) had a high BS (92.5%). Likewise, within the blue clade the relationship between the subgroups identified by STRUCTURE had weak bootstrap support (<50%). In general, however, the tree structure reflected well the different geographic areas and mountain ranges where the populations were sampled.

AMOVA results (app. A; table A2) confirmed on statistical grounds the differentiation between the clusters identified by Bayesian analyses. The variation between the two main groups of populations and individuals as defined by the first Bayesian analyses accounted for 38.2% of the overall genetic variation. The subgroups of populations within each of these two genetic groups still yielded high and significant percentages of the among-group variance, although greater population divergence was found within the green group (F_{st} = 0.50) than the blue group (F_{st} = 0.35).

Within-Population and Among-Group Genetic Diversity and Divergence

Genetic diversity and divergence within populations varied widely across the 36 localities studied (see table 1; fig. 1*B*), with Nei's gene diversity ranging from 0.022 in population 301 to 0.118 in population 104 and with rarity of AFLP markers (DW values) ranging from 7.5 (population 209) to 98.1 (population 106). When the different genetic groups arising from Bayesian analyses were compared, populations of the blue group were significantly more diverse and also contained more rare fragments, on average, than green populations (mean, D_{NEI} = 0.066 vs. 0.049 and DW = 33.9 vs. 16.0, respectively, for the blue and green groups; Mann-Whitney tests; table 2). Different patterns emerged for the geographic distribution of genetic diversity within each of these two main population groups. Within the green group, the northern subgroup of populations (green_N) were significantly more diverse

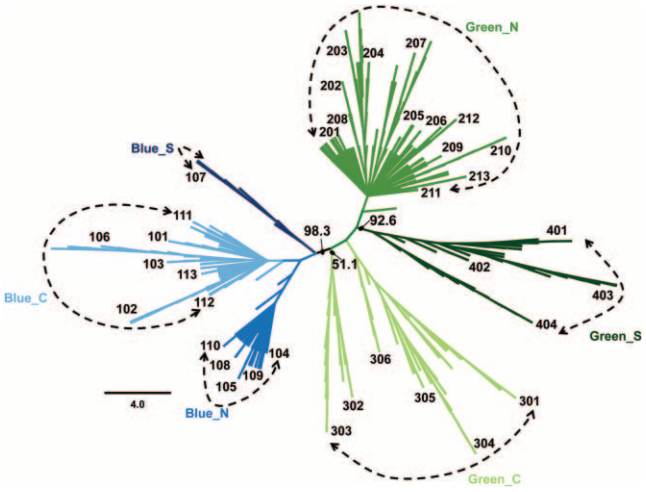

Fig. 4 Unrooted neighbor-joining tree illustrating the genetic relationship among the 526 sampled individuals, based on Nei and Li's (1979) genetic distances calculated from the 510 amplified fragment length polymorphism markers. Tree branch lengths are proportional to genetic distances (see scale bar). Numbers on the branches indicate bootstrap support (BS) >50%. BS was not shown for terminal nodes within populations. To improve legibility, numbers on terminal labels refer to population codes (see table 1). The different colors correspond to the genetic groups identified by Bayesian clustering and match those used in fig. 2*D* and 2*F*.

Table 2

Amplified Fragment Length Polymorphism Diversity and Divergence Values, Expressed as Within-Population Nei's Gene Diversity (D_{NEI}) and Rarity of Markers (Frequency-Downweighted Marker Value [DW]), for the Different Genetic Groups and Subgroups Identified by Bayesian Analyses

	Group			Green subgroups				Blue subgroups			
	Green	Blue	Test[a]	Green_S	Green_C	Green_N	Test[b]	Blue_S	Blue_C	Blue_N	Test[b]
N_{pop}	23	13		4	6	13		1	7	5	
N_{ind}	343	183		60	91	192		14	102	67	
D_{NEI}:											
Mean[c]	.049	.066	2.81**	.055[a]	.052[a]	.072[b]	8.06*	.059[a]	.078[b]	.051[a]	7.25*
Range	.047–.118	.022–.081		.044–.059	.029–.068	.044–.104		...	.054–.118	.047–.057	
DW:											
Mean	16.05	33.97	2.45*	16.9	20.1	13.9	3.29[ns]	96.3	37.9	16.0	2.88[ns]
Range	7.5–37.2	10.7–98.1		8.0–22.6	8.1–37.2	7.5–22.0		...	15.9–98.1	10.6–22.1	

Note. N_{pop} = number of populations; N_{ind} = number of individuals; ns = nonsignificant ($P > 0.05$).

[a] Mann-Whitney rank-sum test.

[b] Kruskall-Wallis test.

[c] Values labeled with the same letter are not significantly different ($P > 0.05$) by Tukey's HSD test.

* $P < 0.05$.

** $P < 0.01$.

than the central (green_C) and southern (green_S) subgroups, which did not differ significantly between them (table 2). However, within the blue group, genetic diversity decreased significantly from central populations (blue_ C) toward both the southern and northern extremes (blue_S and blue_N subgroups; table 2). No significant differences or geographic pattern in amount of genetic divergence (DW values) were found among population subgroups within either the green or the blue group (table 2).

Geographic Patterns of Genetic Differentiation

Plots of pairwise genetic distance (F_{st}) versus geographic distance for the entire set of 36 populations and the two subsets comprising the green and blue groups are shown in figure 5. Simple Mantel tests indicated that, irrespective of whether the whole set of populations or the two subsets were considered, the genetic distance matrix was highly correlated with either the geographic distance or the grouping matrices taken singly ($P < 0.0001$ in all cases; table 3). Whereas across the entire set of populations only ~15% of total variance in pairwise genetic distance was explained by geographic distance, the latter accounted for a considerably greater fraction of variance in genetic distance within either the green or blue groups considered separately (53% and 72%, respectively; table 3). This result clearly indicates that geographic distance played a prominent role in explaining genetic distance between populations only after the latter were split into the two distinct groups revealed by the Bayesian clustering.

Not unexpectedly, pairwise genetic distances between populations belonging to different groups were greater than those between populations in the same genetic group (fig. 5). More interestingly, for both the whole set of populations and the green group, partial Mantel tests showed that the grouping matrix remained a significant predictor of genetic distance after having statistically accounted for the effect of geographic distance. This result indicates that populations of the same genetic group were more genetically similar to each other than would have been predicted on the basis of consideration of geographic distance alone, as denoted by the two clearly distinct intragroup and intergroup regression lines in figure 5*A* and 5*B*. In contrast, partial Mantel tests for the blue group showed that any single predictor matrix was able to explain only a small portion of genetic variation after statistically accounting for the effect of the other predictor matrix (table 3).

Taken together, the preceding results suggest that the first subdivision of our data set into green and blue groups, as well as the subdivision within the green subgroup, could be most parsimoniously explained in terms of long-term historical processes rather than microevolutionary processes resulting from isolation by distance. In contrast, isolation-by-distance phenomena are the most parsimonious explanation to account for genetic differentiation within the blue group, as clearly indicated by the fact that the vast majority of genetic differences between populations was accounted for by their geographic separation alone ($r_M = 0.85$; table 3; see also fig. 5*C*).

Discussion

The present study of the endemic group of trumpet daffodils (*Narcissus* L., section *Pseudonarcissi* DC.) inhabiting the Baetic Ranges in the southern Iberian Peninsula strongly supports the recognition of genetically distinct lineages exhibiting a sharp geographic pattern that apparently have remained isolated from each other for a long time. Our investigation was based on a large number of highly variable genetic markers, adopted different analytical approaches, and exhaustively sampled populations within the entire extant distribution of the group, all of which considerably strengthen the conclusions of the study. The Baetic Ranges are emerging as an extraordinary reservoir of plant genetic diversity, involving both narrowly endemic and geographically widespread taxa (Herrera and Bazaga 2008*a*, 2008*b*; Fuertes-Aguilar et al. 2011; Castilla et al. 2012), a pattern also confirmed here for the genus *Narcissus* (see also Barrett et al. 2004; Medrano and Herrera 2008;

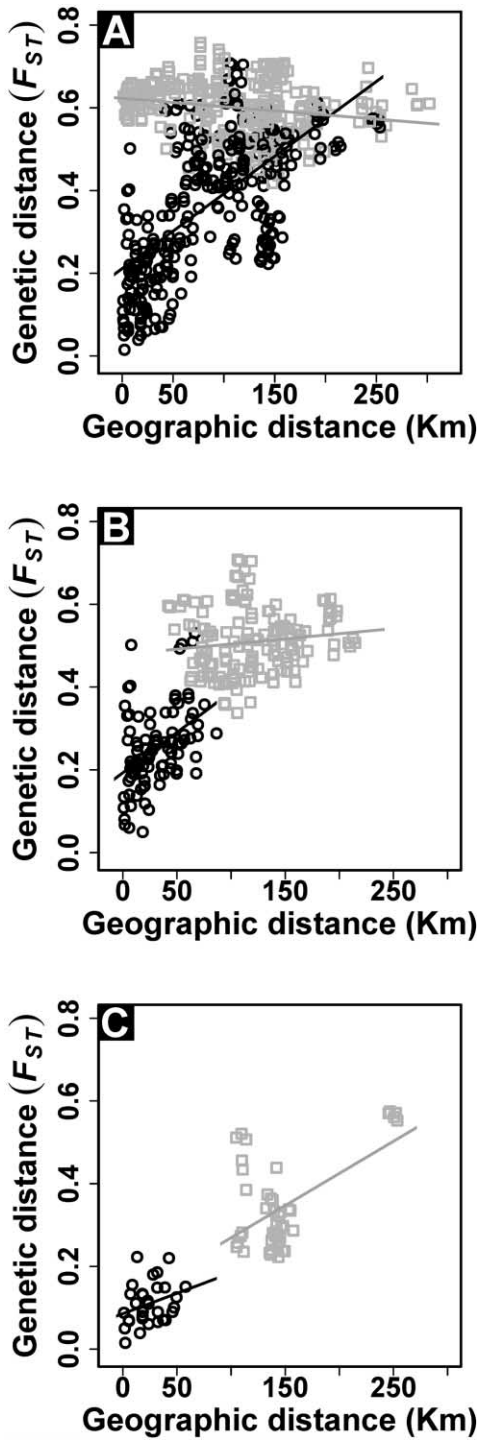

Fig. 5 Relationships between pairwise genetic distance (F_{st}) and geographic distance for all populations included in the study (*A*), the 23 populations in the green group (*B*), and the 13 populations in the blue group (*C*). In each graph, relationships involving populations in the same genetic group (as depicted in the three panels of fig. 2) are shown as open circles, and those involving populations assigned to different genetic groups are shown as gray squares. Regression lines are also shown separately for between-group (gray line) and within-group (black line) relationships.

Jiménez et al. 2009). The trumpet daffodils dealt with in this study are closely related to the widely distributed, highly polymorphic *Narcissus pseudonarcissus* (Graham and Barrett 2004), the most commonly cultivated trumpet daffodil of horticulture. Information on the large amounts of naturally occurring genetic diversity presented here is also of value for the conservation of gene pools of these close relatives of cultivated daffodils.

Some limitations of AFLP markers, including lack of homology of comigrating bands (homoplasy), possible nonindependence of fragments, and low per-marker information content, may restrict their utility in phylogenetics or systematics, particularly when long divergence times between lineages are involved (deep phylogenetics; Koopman 2005; Althoff et al. 2007; Simmons et al. 2007; but see García-Pereira et al. 2010, 2011; Caballero et al. 2013). In contrast, their usefulness in the delimitation of recently diverged taxa (shallow phylogenetics), identification of genetically homogeneous lineages within species complexes, and analyses of adaptive population divergence has been frequently documented (Scotti-Saintagne et al. 2004; Meudt and Clarke 2007; Shaffer and Thomson 2007; Kropf et al. 2009; Reeves and Richards 2011). In particular, our main findings corroborate earlier studies illustrating the practical value of combining AFLP markers and a population genetics approach for distinguishing distinct metapopulations and for taxonomic delimitation in recent plant radiations, closely related taxa, and/or morphologically cryptic species (Martínez-Ortega et al. 2004; Joly and Bruneau 2007; Koopman et al. 2008; Duminil et al. 2012; Garrido et al. 2012; Lega et al. 2012; Slovák et al. 2012) and also emphasize that a pertinent sampling strategy is crucial for a representative analysis of species boundaries (Taberlet et al. 1998). It must be kept in mind, however, that definitive species recognition will depend on fulfilling other key requirements, including the identification of diagnostic criteria based on suitable morphological or functional features or the phylogenetic criterion of monophyly. In this respect, it is important to acknowledge that conclusions regarding species limits or the number of distinct species in a given group are inherently determined by the species concept used and the different features that are considered important for species delimitation in the specific framework associated with that particular concept. Discussion on the vast literature of contemporary species concepts is beyond the scope of this article except to explicitly mention that we basically adhere to the widely adopted unified species concept (de Queiroz 2005, 2007), which considers the existence of separately evolving metapopulation lineages as the only necessary defining property of the species category (de Queiroz 2007). However, the key question of what degree of "separation" is required to define two distinct segments in one lineage remains. De Queiroz (2005, 2007) suggests that other secondary properties acquired by lineages during the course of divergence (such as reproductive isolation, morphological diagnosability, ecological distinctiveness, or gene-tree monophyly) could be considered relevant to species delimitation to the extent that they provide evidence of lineage separation. As shown by our results, evidence obtained from analyses of the geographic context of such divergence could also be helpful to solve this question. Further investigations using a multifaceted approach, including the study of the contrasting habitat-type associations

Table 3

Simple and Partial Mantel Tests Testing for Associations between Pairwise Matrices of Genetic Distances between Populations (Measured as F_{st}, the Dependent Matrix in All Analyses) and the Two Independent Matrices Depicting Geographic Distance (GEO; Analysis 1) and "Grouping" (Matrix of 0s and 1s, Indicating Whether a Given Population Pair Belonged to the Same or Different Group, Respectively; Analysis 2)

	Complete data set (36 populations)		Green group (23 populations)		Blue group (13 populations)	
Independent matrices	r_M	% of variance explained (r^2)	r_M	% of variance explained (r^2)	r_M	% of variance explained (r^2)
Analysis 1:						
GEO	.39***	15.5	.73***	52.8	.85***	71.8
GEO (grouping)	.37***	13.7	.21*	4.3	.53***	27.9
Analysis 2:						
Grouping	.67***	44.9	.83***	68.5	.79***	61.7
Grouping (GEO)	.66***	43.7	.60***	36.1	.14^{ns}	2.1

Note. In each analysis, the independent matrix of interest was included in computations as either the single predictor or partialed on the other independent matrix. Separate tests were performed on the complete data set and on the green and blue subgroups defined by Bayesian clustering (see fig. 1 for more details). r_M = (partial) Mantel correlation coefficient; ns = nonsignificant ($P > 0.05$).

* $P < 0.05$.

*** $P < 0.0001$.

exhibited by the different genetic lineages evidenced here and the use of DNA sequence data, will be required to corroborate our findings. In the meantime, however, our results shed new light on the substantial taxonomic problem of distinguishing differentiated entities in this controversial group of wild daffodils and contribute to a better understanding of both the role of geography in driving population divergence and the many pathways involved in processes of speciation in western Mediterranean mountain plants, as discussed below.

Results of this study have direct implications for the taxonomic treatment of southern Spanish trumpet daffodils. In the light of the results presented here, the traditional recognition of *Narcissus bujei*, *Narcissus longispathus*, and *Narcissus nevadensis* as distinct species (such as in Fernández-Casas 1986; Zonneveld 2008; Navarro 2011) appears to be a more appropriate treatment of this section than the recent proposal by Aedo (forthcoming), whereby all the trumpet daffodils from the Baetic Ranges are ascribed to a single taxonomic entity (named *Narcissus pseudonarcissus* subsp. *nevadensis*). Our data do not support the recognition of the more recently described species (Ríos-Ruiz et al. 1999) from the northeastern extreme of the Baetic Ranges (including *Narcissus segurensis* or *Narcissus yepesii*). The recognition of *N. bujei* as a genetically differentiated taxonomic unit within the *Pseudonarcissi* section is particularly well supported here by multiple lines of evidence. First, in all analyses the clearest and most reproducible genetic break among all the populations studied clearly separated one group of 13 populations (the blue group), which included all those previously assigned to *N. bujei* (Blanca et al. 1999), from the remaining populations (the green group). This split into two major groups was strongly supported by the results of AMOVAs. Second, in accordance with the lower altitudinal preferences suggested for *N. bujei* (Navarro 2011), populations of the blue group occurred at significantly lower elevations (mean ± SD, 1140 ± 327 m asl) than did those in the green group (mean ± SD, 1432 ± 347 m asl; fig. 2*B*). Third, these two STRUCTURE groups exhibited strikingly different levels of within-population genetic diversity and rarity values. Whereas populations of the blue group were more diverse and contained more rare fragments on average, populations of the green group were significantly less diverse and less rare (table 2). Higher genetic variability and more rare fragments in the blue group may reflect the fact that these populations grow on widely available drier habitats at lower altitudes, thus forming populations that are larger and more continuous than those from the green group, which are associated with highly fragmented, humid habitats. Fourth, geographically distant populations of each of these two main groups were genetically more closely related to each other than to geographically closer populations of the other group (fig. 5*A*), as denoted by the medians of between-group F_{st} values (0.61) being significantly greater than the medians of within-group F_{st} distributions (0.40). In addition, the virtual absence of admixed individuals in the mountain range where the green and blue groups occur in sympatry (Cazorla–Segura–Las Villas) suggests that despite instances of hybridization being known for this genus (Blanchard 1990; Díaz-Lifante and Andrés-Camacho 2007; Marques et al. 2007, 2010; Díaz-Lifante et al. 2009) and the occasional co-occurrence of plants from both groups in adjacent habitats within this area, some type of reproductive isolation between them may be operating. Fifth, the comparison of the relationship between genetic and geographic distances revealed substantial differences between the within- and between-group regression lines (fig. 5*A*). Our data therefore corroborate the findings of Jiménez et al. (2009), inferred from ribosomal DNA (ITS) and ISSR sequencing, suggesting that *N. bujei* was highly divergent from the remaining species of *Narcissus* inhabiting the Baetic Ranges as well as other phylogenies that relate the green and blue groups to very different clades of the *Pseudonarcissi* section (Graham and Barrett 2004; Rønsted et al. 2008; Santos-Gally et al. 2012), which may indicate that they originated in two different colonization events. Our results are also in accordance with the marked differentiation in genome sizes between *N. bujei* and other closely related species reported by Zonneveld (2008) and support as well the morphological distinctiveness criteria proposed by this author for *N. bujei*, which include solitary and

bright yellow flowers, spirally twisted tepals and leaves, and a black spot on the anthers (Zonneveld 2010).

In contrast to the blue group, where genetic differences between recognizable subgroups exemplify a geographically defined continuum and can be parsimoniously explained in terms of isolation-by-distance alone (figs. 3, 5*C*), genetic differences between the three subgroups within the green group are better explained in term of long-term differentiation and geographic isolation (fig. 5*B*). One of these subgroups (green_N) comprises populations traditionally ascribed to *N. longispathus* from the Cazorla–Segura–Las Villas massif (Blanca et al. 1999; Barrett et al. 2004; Medrano and Herrera 2008), and another (green_C) is made up of populations historically referred to as *N. nevadensis* from the Baza and Sierra Nevada mountain systems (Blanca et al. 1999; Navarro 2011). Our results therefore demonstrate that the names *N. longispathus* and *N. nevadensis* actually designate genetically distinct, geographically discontinuous groups of populations. The third subgroup (green_S) comprises four populations from the Almijara Mountains that have been sometimes included within *N. nevadensis* (Navarro 2011). Our results indicate, however, that they are genetically well differentiated from both green_N and green_C, and its separation as a differentiated entity has a very high bootstrap support (fig. 4). Further studies are still needed to evaluate the taxonomic status of these poorly known populations from the Almijara Mountains in the context of the whole group of endemic trumpet daffodils from the Baetic Ranges. Particularly helpful would be investigations aimed at identifying diagnostic morphological traits to distinguish the genetically differentiated groups identified here as well as new data from plastid and nuclear genes that enable the reconstruction of the evolutionary and genealogical relationships among them and the assessment of their monophyly.

Mediterranean mountains are characterized by their complex geological history and intricate geomorphology (Woodward 2009). These factors have significantly enhanced the diversification and speciation of plants in the region (Martín-Bravo et al. 2010), which has resulted in an exceptionally rich flora comprising a large proportion of narrowly endemic taxa (Gómez-Campo 1985; Sainz-Ollero and Moreno-Saiz 2002). Another factor contributing to the outstanding diversity of Mediterranean floras is environmental heterogeneity, which has promoted ecological specialization and the natural fragmentation and isolation of conspecific populations (Thompson 2005; Hughes et al. 2006; Frajman and Oxelman 2007; Martín-Bravo et al. 2010). The diversifying role of plant specialization on specific substrates and its consequences for ecological speciation in the Baetic Ranges has often been emphasized in relation to dolomitic outcrops (Mota et al. 2002, 2008), but the contribution of other similarly fragmented, infrequent habitat types to the high endemism rates characterizing the region has been infrequently considered (Medrano et al. 2006; Medrano and Herrera 2008). All populations considered in the present study comprising the green group occurred in permanently waterlogged meadows (associated with permanent springs or small mountain streams) that were surrounded by xerophytic vegetation. The discontinuity, isolation, and rarity of these humid microhabitats in the region should have historically hindered gene flow between populations, leading to their extensive genetic differentiation.

Although other approaches have been suggested to identify biologically relevant "units of conservation" (Fraser and Bernatchez 2001), taxonomically based criteria are still useful for setting conservation priorities in groups where insufficient biological information precludes the application of better elaborated procedures (Dimmick et al. 1999; Padial and de la Riva 2006). *Narcissus bujei*, *N. longispathus*, and *N. nevadensis*, the three previously named taxa to which most of the samples included in this study should probably be ascribed, are currently considered as endangered species, requiring management and protection measures to counteract threats arising from habitat alteration (Blanca et al. 1999; Cabezudo et al. 2005). Insufficient biological information on these taxa (e.g., on possible mechanisms of reproductive isolation) precludes elucidating whether their conservation status is a reflection of "taxonomic inflation," the spurious increase in number of species as a consequence of lower-rank taxa (typically subspecies) being raised to species as a result of changes in the species concept (typically a shift from the biological to the phylogenetic species concept; Isaac et al. 2004). Results of genetic analyses presented in this article, however, show that irrespective of their formal taxonomic treatment, the trumpet daffodils of the Baetic Ranges comprise several well-defined groups and subgroups of populations, each of which qualify as a separate unit of conservation on the basis of their genetic individuality. Genetic diversity has received much less attention in biodiversity assessments than species diversity (Laikre et al. 2010), despite frequent demonstrations of its importance for the persistence of populations (Frankham 1995). In complex mountain landscapes with highly fragmented plant populations, species richness alone may be a poor surrogate for overall biodiversity, as shown by Taberlet et al. (2012) for the high-mountain flora of the Alps and the Carpathians. If similar circumstances apply to the southern Spanish Baetic Ranges, then direct genetic analyses such as those conducted in the present study may eventually prove more useful for the identification of suitable conservation units than achieving a precise delineation of the taxonomic ranks associated with each of the genetically different groups of populations.

Acknowledgments

We thank José Antonio Algarra, Abelardo Aparicio, Miguel Arrufat, Alfredo Benavente, José Antonio Díaz, Víctor Fernández Pasquier, Rafael González Albaladejo, Juan Francisco Jiménez, Mario Ruiz, Enrique Triano, and Antonio Velázquez for invaluable help in locating *Narcissus* populations and assistance in sample collection. Pilar Bazaga conducted the first amplified fragment length polymorphism (AFLP) screening tests and provided helpful advice on the AFLP technique; Rocío Requerey assisted with the laboratory work. We are also grateful to the Bioportal (University of Oslo) and the Centro de Supercomputación de Galicia (CESGA; XUNTA de Galicia–CSIC) services for computational resources and facilities, especially to Aurelio Rodríguez (CESGA) for programming advice. The comments of two anonymous reviewers and the associate editor greatly improved the manuscript. Consejería de Medio Ambiente (Junta de Andalucía) provided permission to collect samples. We are indebted to the staff of the Sierras de Cazorla–

Segura–Las Villas and Sierras Subbéticas Natural Parks for providing logistic facilities during fieldwork. Initial financial support for this work was provided by grant P06-RNM-01627 (Consejería de Innovación, Ciencia y Empresa, Junta de Andalucía). Support during manuscript preparation was provided by grants P09-RNM-4517 (Consejería de Innovación, Ciencia y Empresa, Junta de Andalucía) and CGL2010-15964 (Ministerio de Ciencia e Innovación, Gobierno de España).

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