Convergent evolution of flower polymorphism in *Narcissus* (Amaryllidaceae)

Rocío Pérez, Pablo Vargas* and Juan Arroyo

Departamento de Biología Vegetal y Ecología, Universidad de Sevilla, Apartado 1095, E-41080 Seville, Spain, and *Royal Botanic Garden, CSIC, Madrid, Spain

Summary

Author for correspondence:
Juan Arroyo
Tel: +34 954557058
Fax: +34 954557059
Email: arroyo@us.es

• We present new results on morphological variation for style polymorphism and perianth features in the seven species of *Narcissus* sect. Apodanthi and in *N. pallidulus* (sect. Cyclaminei), which exhibits a wide array of flower conditions: heterostyly, style dimorphism of variable reciprocity, and style monomorphism.
• There is a significant association between perianth morphology and style polymorphism in the studied species. Among significant flower features, the two heterostylous species show pendulous flowers. Most style-dimorphic species have patent flowers, whereas monomorphic and one style-dimorphic species display erect flowers.
• Interpretation of a chloroplast (*trn*L-*trn*F) phylogeny of species representing most sections in the genus leads us to conclude that the two heterostylous species have independent origins, supporting previous hypothesis of convergence of heterostyly in *Narcissus*. Remarkable resemblance of flower features between both species indicates that pollinator activity might have driven flower convergence.
• A second chloroplast (*trn*T-*trn*L) phylogeny in sect. Apodanthi helps elucidate some evolutionary transitions in moulding heterostyly. Stylar dimorphism appears to precede distyly and style monomorphism. Population studies on style dimorphic and monomorphic species of *Narcissus* are congruent with a pollinator-mediated process also involved in a shift to monomorphism.

Key words: chloroplast sequences, evolutionary convergence, heterostyly, Mediterranean, morphometry, plant–pollinator interactions, phylogeny.

© *New Phytologist* (2003) **161**: 235–252

Introduction

Heterostyly is a genetically based, discrete style polymorphism originated independently multiple times, as indicated by its occurrence in at least 28 disparate families of angiosperms (Barrett *et al.*, 2000). This polymorphism includes, within the same population, distyly (two morphs) and tristyly (three morphs). In both cases flower morphs are characterised by a reciprocal positioning of stigmas and stamens in height (reciprocal herkogamy). In its typical form, morphs are either long-styled (L-morph), mid-styled (M-morph), or short-styled (S-morph) in tristylous species, and L- and S-styled in distylous species. Although recognition of style polymorphism and its evolutionary implications have been long discussed (Ornduff, 1992), there is not yet a general agreement about its origin and evolution. Several hypotheses on evolution of heterostyly involve different scenarios and selective forces,

which necessarily imply alternative initial and intermediate steps (Charlesworth & Charlesworth, 1979; Lloyd & Webb, 1992a,b; Richards, 1998). Experimental approaches to test predictions of evolutionary models build up a body of knowledge in evolution of flower polymorphism (Kohn & Barrett, 1992; Stone & Thomson, 1994; Lau & Bosque, 2003; Thompson *et al.*, 2003). Comparative studies in the same plant group with presumable intermediate stages of flower polymorphism are absolutely necessary to explore evolutionary pathways to heterostyly (Lloyd & Webb, 1992a).

Lloyd & Webb's model (Lloyd & Webb, 1992a,b) has generated acceptance subject to experimental, observational and comparative evaluation (Faivre, 2000; Nishihiro *et al.*, 2000; Faivre & McDade, 2001; Lau & Bosque, 2003). This model assumes that the main selective force behind heterostyly is the promotion of outcrossing through efficient pollen transfer between flower morphs. Lloyd & Webb's model (Lloyd &

Webb, 1992a) emphasizes the role of a pollination environment rather than the existence of a particular genetic incompatibility system, as considered instead by the Charlesworth & Charlesworth's model (Charlesworth & Charlesworth, 1979). Lloyd & Webb (1992a,b) hypothesized that the ancestral condition is an approach-herkogamous flower, then a style-length dimorphic flower (stylar dimorphism), and, finally, a reciprocal-herkogamous position of sex organs (strict heterostyly). Accordingly, shifts between these stages would be driven by changes to more efficient pollinators in transferring pollen between morphs. There are more cases supporting Lloyd & Webb's model (e.g. *Anchusa*, *Lithodora*, *Narcissus*) than those fitting into Charlesworth & Charlesworth's model (1979) (species in the tribe *Staticeae* of Plumbaginaceae; Lloyd & Webb, 1992a). Additionally, based on taxonomic (nonphylogenetic) information, Lloyd & Webb (1992a) assumed that most of nonheterostylous taxa within a particular heterostylous group are mostly approach herkogamous, and interpret the rarity of style dimorphism as a result of its instability for some unknown reason. However, occurrence of style dimorphism in *Narcissus* has been reported in many species (Barrett *et al.*, 1996).

Variation in flower characteristics of *Narcissus* appears to be useful for testing models of heterostyly (Lloyd *et al.*, 1990; Barrett *et al.*, 1996; Arroyo & Barrett, 2000; Arroyo, 2002). This highly diverse, Mediterranean genus includes species showing: nonherkogamous monomorphism, in which the stigma is located at the same height than anthers (e.g. *N. serotinus*); approach-herkogamous monomorphism, where stigma has a higher position than anthers (e.g. all species of sections Bulbocodii and Pseudonarcissi); style dimorphism in which populations include approach-herkogamous flowers (L-styled morphs) and reverse-herkogamous flowers (S-morph) with no anther reciprocity (e.g. some species of sections Tazetta, Jonquilla and Apodanthi); distyly, with L- and S-styled morphs and anther reciprocity (*N. albimarginatus*); and tristyly, with L-, M- and S-styled morphs and anther reciprocity (the *N. triandrus* complex). Testing evolutionary patterns behind such an array of flower polymorphism within the genus has remained elusive without any phylogenetic analysis. Hence, we have addressed phylogenetic reconstructions from neutral, chloroplast sequences (*trn*L-F, *trn*T-L) of some representative species of *Narcissus* which may help shed further light on evolution of stylar polymorphism. As an initial step, we have focused on a taxonomic group within the genus, sect. Apodanthi, which exhibits a wide range of polymorphism variation (except tristyly and approach herkogamous monomorphism) through its seven species: two monomorphic, four style dimorphic and one distylous. For the sake of comparison, *N. pallidulus* (*N. triandrus* complex), a species in sect. Cyclaminei displaying heterostyly (tristyly), has been included in the analyses.

There are three specific aims in this study. First, to ascertain the kind of stylar polymorphism in populations of a presumably natural group, section Apodanthi, which preliminary observations suggested as variable (Arroyo, 2002). Second, to investigate phylogenetic relationships and evolutionary transitions of style polymorphism between the only two known cases of heterostyly (*N. albimarginatus* and *N. triandrus* complex), and among all species in sect. Apodanthi by means of analysis of chloroplast sequences. And, third, to test whether shifts in style-polymorphism conditions are related to perianth features and pollinators, as it was explicitly suggested by Arroyo & Barrett (2000) for *N. albimarginatus*.

Materials and Methods

Study taxa

The long history of cultivation and naturalisation in *Narcissus*, coupled with extensive hybridisation, has prevented recognition of a stable taxonomic treatment (Mathew, 2002). In fact, there is no taxonomic monograph for the entire genus. We have followed the most recent, comprehensive treatment listing analytically sections and species (Dorda & Fernández-Casas, 1989). This taxonomic treatment considers six species in our study group (sect. Apodanthi): *N. rupicola*, *N. scaberulus*, *N. calcicola*, *N. cuatrecasasii*, *N. marvieri* and *N. watieri*. We include one more species (*N. albimarginatus*) described afterwards (Muller-Doblies & Muller-Doblies, 1989, see also Arroyo & Barrett, 2000).

Although sect. Apodanthi displays a wide range of flower morphology, it has been considered one of the most clearly recognised and taxonomically stable groups within the genus (Fernandes, 1967, 1975; Dorda & Fernández-Casas, 1989). Morphometrical analyses and pollination surveys were performed in sect. Apodanthi to investigate relationships between the degree of perianth variation among species and style polymorphisms, which include the occurrence of dimorphic heterostyly (distyly) in only one species of the genus (*N. albimarginatus*). One more species (*N. pallidulus*) was also included in the study to represent the other group (the *N. triandrus* complex: *N. triandrus*, *N. lusitanicus* and *N. pallidulus*) of *Narcissus* displaying heterostyly (Barrett *et al.*, 1997) and to contrast variation in perianth features and polymorphism with respect to species of sect. Apodanthi. All the species are bulbous geophytes occurring in mountains at variable elevations and blossom in late winter to spring. The geographic range of the species, their typical habitats and elevation, and population locations are shown in Table 1. A comprehensive map of the geographic range of species of sect. Apodanthi may also be found in Arroyo (2002).

Population sampling

Flower morphometry We selected two widely separated populations from each species to represent within-species variation in flower morphology (see Table 1). In each population we collected one recently opened flower per plant, typically the

Table 1 Species of *Narcissus* sampled for flower (perianth and sex organs) morphometry. (*) Populations where insect censuses were done

Species	Population	Coordinates	Habitat	Elevation	Flowers collected
N. pallidulus	1 Spain: Jaén, Segura, Navalcaballo	38°20' N 2°37' W	Pine forest on limestone	1350	122
N. pallidulus	2 Spain: Badajoz, Calera, Sierra de Tentudia	38°5' N 6°20' W	Deciduous oak forest on shale	900	63
N. albimarginatus (*)	3 Morocco: Chaouen, Jbel Bouhachem	35°15' N 5°26' W	Cedar forest on sandstone	1500	200
N. albimarginatus	4 Morocco: Chaouen, Jbel Kelti	35°21' N 5°17' W	Oak forest on limestone	1520	206
N. cuatrecasasii (*)	5 Spain: Cádiz, Grazalema, Puerto del Boyar	36°46' N 5°24' W	Rock fissures on limestone	1100	100
N. cuatrecasasii	6 Spain: Jaén, Cazorla, Las Correhuelas	37°59' N 2°54' W	Mixed pine forest on limestone	1615	100
N. calcicola	7 Portugal: Leiría, Serra de Sicó	39°55' N 8°32' W	Rock fissures on limestone	550	186
N. calcicola	8 Portugal: Leiría, Serra de Sto. Antonio	39°32' N 8°44' W	Rock fissures on limestone	714	212
N. scaberulus	9 Portugal: Viseu, Oliveira do Conde	40°26' N 7°57' W	Scrubland on granite	259	232
N. scaberulus	10 Portugal: Guarda, Ervedal	40°26' N 7°54' W	Scrubland on granite	333	109
N. rupicola	11 Spain: Jaén, Aldeaquemada	38°23' N 3°24' W	Cliff fissures on granite	1000	78
N. rupicola (*)	12 Spain: Madrid, Navacerrada, Bola del Mundo	40°47' N 3°59' W	Alpine scrubland on granite	2257	154
N. marvieri	13 Morocco: Marrakesh, Zeretken	31°27' N 7°26' W	Tree-line cedar forest on shale	2250	51
N. marvieri	14 Morocco: Taza, Tazzeka	34°5' N 4°11' W	Dense cedar forest and scrubland on shale	1837	50
N. watieri (*)	15 Morocco: Marrakesh, Oukaimeden	31°13' N 7°51' W	Soil pockets on alpine meadows on shale	2474	50
N. watieri	16 Morocco: Marrakesh, Tizi'n'Test	31°5' N 8°5' W	Holm oak forest on limestone	2050	40

earliest in the inflorescence. At least 40 flowers were immediately preserved in ethanol 70% and kept refrigerated until measurement. Given that species present vegetative multiplication through division of bulbs, an effort was made to avoid repeated sampling of ramets by collecting flowers in plants at least 2 m apart (Arroyo *et al.*, 2002). From each population we obtained additional flowers with the same procedure in order to have larger samples for estimating morph ratio in populations (see Table 1 for sample sizes).

Phylogenetic study Individuals and populations were selected to represent the morphological diversity of the genus, the geographical breadth of the species and flower polymorphism. The seven species of *Narcissus* sect. Apodanthi, the four of *Narcissus* sect. Cyclaminei, and seven representatives of the 10 remaining sections were sampled (a single individual per population) and sequenced for the noncoding region *trn*L(UAA)-*trn*F(GAA) (Table 2). As a result, we obtained and analyzed 27 sequences of *Narcissus*. Outgroup species (*Galanthus*, *Lapiedra*, *Pancratium*) were chosen as suggested in a previous phylogeny of Amaryllidaceae based on *rbc*L and *trn*L-*trn*F sequences (Meerow *et al.*, 1999). Additionally, a second sample focused on the seven species of *Narcissus* sect. Apodanthi was addressed to sequence and analyze the chloroplast noncoding region *trn*T(UGU)-*trn*L(UAA). *Narcissus bulbocodium* and *N. papyraceus* were used as outgroup species based on the *trn*L-F phylogeny of *Narcissus* herein presented.

Flower morphometry

Measurements Typically, a *Narcissus* species has a flower tube on the inferior ovary, to which the six stamen filaments are attached, two stamen whorls, six tepals and, above all, a corona. Each stamen whorl has three stamens reaching two respective anther levels in sect. Apodanthi and *N. triandrus* complex (Fig. 1). Preserved flowers were slit longitudinally from just above the ovary to the tube mouth. The apex of the ovary was the baseline for all measurements, which were performed by digital calipers to the nearest 0.1 mm. Measurements were (Fig. 1): flower width; tepal length; corona width; corona height; tube length; tube mouth width; style length; upper stamen height; lower stamen height; and flower angle to stalk. Stamen height was measured up to the insertion of the filament into the anther, avoiding variation caused by anther size. Anthers display up to threefold change in length during their fast ripening (R. Pérez, personal observation).

Given that all studied species have two whorls of stamens different in length, it is difficult to quantify the level of sex organ reciprocity in dimorphic species, which usually have one stamen level in distylous species. On functional grounds (i.e. proficiency of cross pollination between equivalent height of sex organs), we have computed the degree of reciprocity as the difference between means of style height in each morph and the closest stamen whorl of the alternative morph. It

New Phytologist

Table 2 Accessions for the chloroplast sequence study of Narcissus, including plant identification followed by population number, locality of wild populations, name of plant collectors and GenBank accession numbers for *trn*T-L and *trn*L-F sequences

| Taxon	Locality	GenBank accession number trnT-L	GenBank accession number trnL-F
sect. *Apodanthi* A. Fernandes			
N. calcicola 1 Mendoça	Portugal: Serra Sto. Antonio, A. Hampe & B. Garrido	Forthcoming	Forthcoming
N. calcicola 2 Mendoça	Portugal: Serra Sico, A. Hampe & B. Garrido	–	Forthcoming
N. cuatrecasasii 1 Fern. Casas *et al.*	Spain: Jaén, Sierra de Cazorla, J. Arroyo	Forthcoming	Forthcoming
N. cuatrecasasii 2 Fern. Casas *et al.*	Spain: Cádiz, Sierra de Grazalema, J. Arroyo	–	Forthcoming
N. marvieri 1 Jahand. & Maire	Morocco: Zerekten, J. Arroyo & A. Hampe	Forthcoming	Forthcoming
N. marvieri 2 Jahand. & Maire	Morocco: Tazeka, J. Arroyo & A. Terrab	–	Forthcoming
N. rupicola 1 Léon-Dufour	Spain: Cuenca, Grado de Pico, P. Vargas *et al.*	–	Forthcoming
N. rupicola 2 Léon-Dufour	Spain: Madrid, Sierra de la Cabrera, P. Vargas *et al.*	Forthcoming	Forthcoming
N. rupicola 3 Léon-Dufour	Spain: Cuenca, Grado de Pico, P. Vargas *et al.*	–	Forthcoming
N. scaberulus 1 Henriques	Portugal: Ervedal, A. Hampe & B. Garrido	Forthcoming	Forthcoming
N. scaberulus 2 Henriques	Portugal: Ervedal, A. Hampe & B. Garrido	–	Forthcoming
N. scaberulus 3 Henriques	Portugal: Oliveira do Conde, A. Hampe & B. Garrido	–	Forthcoming
N. watieri Maire	Morocco: Oukaimeden, J. Arroyo & A. Hampe	Forthcoming	Forthcoming
N. albimarginatus 1 U. Müller-Doblies & D. Müller-Doblies	Morocco: Bouhachem, J. Arroyo	Forthcoming	Forthcoming
N. albimarginatus 2 U. Müller-Doblies & D. Müller-Doblies	Morocco: Kelti, F. Ojeda	–	Forthcoming
sect. *Cyclaminei* DC.			
N. cyclamineus DC.	Spain: Pontevedra, S. Castroviejo 6162	–	Forthcoming
N. lusitanicus Dorda & Fern. Casas	Portugal: Pena Cova de Oliveira, A. Barra AB2585	–	Forthcoming
N. pallidulus 1 Graells	Spain: Madrid, Aldea del Fresno, P. Vargas & O. Gómez	–	Forthcoming
N. pallidulus 2 Graells	Spain: Jaén, Aldeaquemada, R. Pérez	–	Forthcoming
N. triandrus L.	Spain: Zamora, Mahide, B. Hernández	–	Forthcoming
sect. *Dubii* Fern. Casas			
N. tortifolius Fern. Casas	Spain: Almería, Río de Aguas, A. Barra	–	Forthcoming
sect. *Jonquillae* DC.			
N. jonquilla L.	Spain: Sevilla, Sierra Norte, R. Pérez & R. Pérez de Guzmán	–	Forthcoming
sect. *Serotini* Parl.			
N. serotinus Loefl. ex L.	Spain: Sevilla, Dos Hermanas, C. Andrés & Z. Díaz	–	Forthcoming
sect. *Tazettae* DC.			
N. tazetta L.	GenBank	–	AJ232562
sect. *Bulbocodii* DC.			
N. bulbocodium L.	Spain: Huelva, Cartaya, J. Castillo	Forthcoming	Forthcoming
sect. *Narcissus* L.			
N. poeticus L.	Spain: Lérida, Valle de Arán, X. Picó	–	Forthcoming
sect. *Pseudonarcissi* DC.			
N. pseudonarcissus L.	Spain: Ávila, Sierra de Gredos, R. Pérez & J. Arroyo	–	Forthcoming
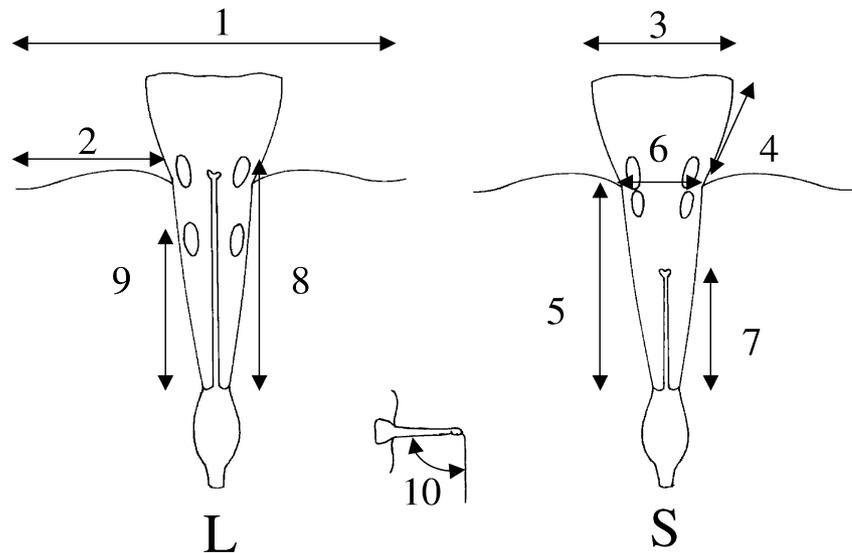
Fig. 1 Long-styled (L) and short-styled (S) flowers of a typical style-dimorphic *Narcissus* species (*N. cuatrecasasii*). Numbers correspond to the measurements taken in each flower sampled: (1) flower width; (2) tepal length; (3) corona width; (4) corona height; (5) tube length; (6) tube width; (7) style length; (8) upper stamen height; (9) lower stamen height; (10) angle of flower to stalk. Measurements were accordingly modified for tristylous, distylous and monomorphic species.

means that in one dimorphic or distylous *Narcissus* species, the long style (L-morph) has its reciprocal male organ in upper stamens of S-morph. Accordingly, S-style is the reciprocal to lower stamens of L-morph (Fig. 1). Flowers of the tristylous *Narcissus pallidulus* does not pose special problems, as this condition usually involves two stamen whorls and the comparison of reciprocal heights is straightforward, as originally described by Darwin (1877) in *Lythrum salicaria*. We averaged the absolute values of this difference across the morphs of each population in order to compare dimorphic and trimorphic species. A perfectly reciprocal population would therefore rate a value of zero. In order to control for effects of flower size in the extent of reciprocity, we also divided the reciprocity of a population by its mean flower diameter.

Statistical analyses All morphometrical variables were checked for normality and homoscedasticity, and transformed when necessary. Only flower tube length across populations and species needed to be log transformed. Data were averaged for morphs and populations and compared accordingly. Student *t*-tests and one-way ANOVA were used for univariate comparisons of flower features between morphs for dimorphic and trimorphic species, respectively. In the rare cases where variances were not homogeneous (Levene's test), we applied a *t*-test with separate variance estimates. After ANOVA for difference of means, posthoc Tukey HSD test for unequal sample sizes was applied for multiple comparison between pairs. When performing multiple tests of one hypothesis, Dunn-Sidak correction of sequential Bonferroni adjustement of α levels was applied (Sokal & Rohlf, 1995). Reciprocity (between-morph stigma-anther separation) was not compared between morphs given that it was obtained as a difference between morph means, hence error estimates of the difference were not available. Multivariate analyses were performed to check the overall pattern of morphological variation of flower

perianth. An ordination by Principal Component Analysis (PCA) was performed to draw the morphological relationships between populations and species. In multivariate analysis, population means were used rather than individual data in order to avoid pseudoreplication. An analysis of the intra-population variation (Faivre & McDade, 2001) is intended to be published in a subsequent paper.

To analyse the relationships between perianth morphology and sex organ polymorphism we applied multiple regression analyses. Independent variables were the scores of the population means for each of three first PCA axes. We used these perianth pseudovariables rather than raw univariate values because axes accounted for a high variance of perianth morphology and because axes are by definition orthogonal, overcoming the serious problem of multicollinearity in multiple regression (James & McCulloch, 1990; Philippi, 1993). Two regression models were adjusted with respective dependent variables related to reciprocity of stigma-anther separation of each population: (i) the mean between morphs of absolute values for difference between style height and equivalent stamens whorl height and (ii) the values of (i) divided by flower diameter. The latter variable was considered to control for potential allometric effect of flower size on the magnitude of departures from stigma-anther reciprocity (see above). Values for monomorphic populations were scored as the highest obtained value of departure from exact reciprocity. Ordination analysis was performed with PCORD package (McCune & Mefford, 1999), whereas univariate statistics and multiple regression models were computed with STATISTICA software (Statsoft, 1997).

Pollinators

Data were taken from populations of four Apodanthi species, some in common with those of the study of flower morphometry (Table 1). We performed systematic censuses

of insects visiting the flowers and recorded positive data, providing that they enter into the tube and do contact any sex organ. Species subject to study were *N. rupicola* (time effort of 12 h, including some nocturnal periods)*, N. cuatrecasasii* (12 h), *N. albimarginatus* (9 h) and *N. watieri* (3 h). Observation timing and area depend on density of flowers in every population. In sparse patches, we made several censuses of 15 min, observing all insects visiting flowers in an area of approx. 100 m². We also made static observations in dense patches on all flowers in small areas (approx. 10 m²).

Phylogenetic studies

PCR amplification and sequencing Total genomic DNA was extracted from silica-dried material collected in the field and the living collection of the Royal Botanic Garden of Madrid, Spain. DNA extractions were performed using the DNeasy Plant Mini Kit (QIAGEN Inc., Chatsworth, CA, USA). For Polymerase Chain Reaction (PCR) amplifications, we used a Perkin-Elmer (Foster City, CA, USA) PCR System 9700 thermal cycler, primers *trn*a and *trn*b for *trn*T(UGU)-*trn*L(UAA), *trn*e and *trn*f for *trn*L(UAA)-*trn*F(GAA), and PCR conditions following Taberlet *et al.* (1991): 51°C of annealing temperature for the *trn*T-L and 50°C for the *trn*L-F plus 2-min elongation time. Amplified products were cleaned using spin filter columns (PCR Clean-up kit, MoBio Laboratories, Solana Beach, CA, USA) following the protocols provided by the manufacturer. Cleaned products were then directly sequenced using dye terminators (Big Dye Terminator vs. 2.0, Applied Biosystems, Little Chalfont, UK) and run into polyacrylamide electrophoresis gels (7%) using an Applied Biosystems Prism model 3700 automated sequencer. Primers *trn*a, *trn*b, *trn*e and *trn*f were also used for cycle sequencing of the *trn*T-*trn*L and *trn*L-*trn*F spacers under the following conditions: 95°C for 2 min followed by 25 cycles of 95°C for 10 s, 50°C for 5 s and 60°C for 4 min Sequence data were placed in a contig file and edited using the program Seqed (Applied Biosystems, Foster City, CA, USA). The limits of the *trn*T-*trn*L and *trn*L-*trn*F regions were determined by comparison with other Amaryllidaceae sequences available in the GenBank.

Sequence analyses Three different sequence matrices were used to perform the phylogenetic analyses: *trn*L-F matrix of *Narcissus*, *trn*T-L matrix of *Narcissus* sect. Apodanthi, and a combined matrix of *trn*L-F and *trn*T-L for only the species of *Narcissus* sect. Apodanthi as ingroup. Alignments of the 30 *trn*L-F and the nine *trn*T-L sequences were obtained using the program Clustal X 1.62b (http://evolution.genetics.washington.edu/phylip/software.etc1.html) with further manual adjustments. Two phylogenetic analyses were performed by using two primary approaches to tree construction: tree-searching (parsimony) and algorithmic (Neighbor-Joining). Parsimony-based analyses were conducted using Fitch parsimony (as implemented in PAUP*; Swofford,

1999) with equal weighting of all characters and of transitions:transversions. Heuristic searches were replicated 100 times with random taxon-addition sequences, Tree Bisection-Reconnection (TBR) branch swapping and with the options MULPARS and STEEPEST DESCENT in effect. Relative support for clades identified by parsimony analysis was assessed by bootstrapping with the heuristic search strategy as indicated above. In addition, phylogenetic reconstructions of sequences were also performed in PAUP* using the Kimura 2-parameter distance model (Kimura, 1980) and the Neighbor-Joining method (Saitou & Nei, 1987). Indels in the noncoding regions *trn*T-L and *trn*L-F were coded as appended characters following the logic of Kelchner (2000) and Simmons & Ochoterena (2000). As a result, additional analyses were performed when coding parsimony-informative indels affecting the *trn*L-F and *trn*T-L matrices. Character evolution of flower polymorphism was investigated using MacClade 3.5 (Maddison & Maddison, 1992). Four states of flower polymorphism (approach herkogamy, non-herkogamous monomorphism, style dimorphism and hetero-styly) were traced and mapped on the resulting *trn*L-F and *trn*T-L phylogenies of the consensus tree of all maximum-parsimony trees. We explored both accelerated transformation (ACCTRAN) and delayed transformation (DELTRAN) optimizations with equal weighting of all characters and transitions among all states equally probable.

Results

Style polymorphism

Average values of sex organ position for the seven species of sect. Apodanthi and for *N. pallidulus* are shown in Tables 3, 4 and 5, and comparative diagrams in Fig. 2. Species in section Apodanthi have a remarkable variation in style polymorphism: distyly in *N. albimarginatus*, style dimorphism in four species (*N. calcicola, N. cuatrecasasii, N. rupicola* and *N. scaberulus*), and monomorphism in *N. marvieri* and *N. watieri* (Fig. 2, Table 3). *Narcissus albimarginatus* shows two style-length morphs with approximate reciprocal positioning of anthers and style. Two patterns were found for dimorphic species: three species (*N. calcicola, N. scaberulus, N. cuatrecasasii*) present the L-morph with the stigma almost at an equivalent level of that of upper anthers, whereas in *N. rupicola* the stigma of the L-morph is positioned between the two anther levels. The latter species displayed the highest values of stigma-anther separation (Table 4), that is, the lowest reciprocity among dimorphic species. Monomorphic species present a position of sex organs similar to that in L-morph of dimorphic *N. rupicola*. (Fig. 2).

Narcissus pallidulus is the only trimorphic species in our study, with mostly reciprocal positioning of stigmas and stamen whorls. Although equivalent positions are not equal in all cases, sequence height of sex organs corresponds to typical tristylous species.

Table 3 Height of stamens and stigmas in monomorphic species of *Narcissus* sect. Apodanthi. Values are means ± 1 se in mm

Species	Population	Sample size	Style height	Upper anther height	Lower anther height	Stigma-Anther separation
N. marvieri	Zeretken	51	20.69 ± 0.30	22.55 ± 0.32	18.52 ± 0.35	1.86
N. marvieri	Tazzeka	50	10.01 ± 0.15	12.34 ± 0.18	8.65 ± 0.14	2.33
N. watieri	Oukaimeden	50	20.92 ± 0.28	22.05 ± 0.29	18.15 ± 0.23	1.13
N. watieri	Tizi'n'Test	26	17.36 ± 0.30	19.27 ± 0.40	15.95 ± 0.41	1.91

At the population level, morphs are in general unequally represented. L-morph is the most frequent (Tables 4 and 5) in all species and populations, with the exception of one population of *N. cuatrecasasii* and one of *N. rupicola*, which show similar frequency of morphs (isoplethy).

Perianth variation

Values of perianth variables are averaged over populations and morphs (Table 6). Univariate morphometrical analyses of flower tubes, tepals, coronas and flower angle of all studied species and populations revealed clearly absence of significant differences between morphs (82 between-morph comparisons; results not shown). There were only slightly significant differences in tristylous *N. pallidulus* at Cazorla for corona height (*P* = 0.01) and in distylous *N. albimarginatus* at Bouhachem for perianth width (*P* = 0.05).

Principal component analysis is shown in Fig. 3. The first, second and third axes accounted for 61.6%, 23.3% and 9.4% of the variance, although only Axes 1 and 2 have eigenvalues higher than 1.0 and are, hence, further discussed. These two axes depict quite clearly the relationships between species and populations in perianth morphology. At the negative extreme of Axis 1 we find together *N. pallidulus* and *N. albimarginatus* and, at the positive extreme of this axis, *N. marvieri*, *N. watieri* and *N. rupicola* form a second group. Axis 2 separates at its positive end the Iberian endemics *N. cuatrecasasii*, *N. calcicola* and *N. scaberulus*. Eigenvectors of perianth variables reflect trends of morphological variation. The highest values for Axis 1 are flower angle to stalk (0.4519), width of flower tube (−0.4410), and corona height (−0.4217). Largest eigenvectors for Axis 2 correspond to corona width (−0.6470), tepal length (−0.4362) and flower tube length (−0.4087). These results indicate that populations of two species (*N. pallidulus* and *N. albimarginatus*) at the left in the ordination plot are characterised by small angles (pendulous flowers), wide flower tubes and long coronas (Fig. 4). A second group of species (*N. rupicola*, *N. watieri*, *N. marvieri*) display predominantly erect flowers, narrow and very long tubes and funnel-shaped coronas. On the top of the ordination plot, three species (*N. cuatrecasasii*, *N. calcicola*, *N. scaberulus*) are characterised by patent flowers, cup-shaped coronas, small tepals and moderate flower-tube length.

The interrelationship between perianth variables and stylar polymorphism is evaluated by means of multiple regression analyses. We calculated two measurements of reciprocity in the position of sex organs, considering or not flower size effects. Both regression equations fitted quite similarly with scores of populations along PCA axes on perianth variables. As a result, we may conclude that there is a significant relationship between perianth morphology and sex organ position across populations and species, with 72–80% of the variance being explained by the regression models (Table 7). Positioning of reciprocal stigma and anthers (heterostyly) corresponds with the group of pendulous flowers. Style dimorphic species (*N. calcicola*, *N. cuatrecasasii*, *N. scaberulus*, see Table 4) with medium values of reciprocity have patent flowers, among other features (see above). Monomorphism and less reciprocal style dimorphism in *N. rupicola* (Table 4) correspond to the erect flowered group.

Pollinators

As typically occurs in early blooming species, it was difficult to observe pollinators in four species of *Narcissus* sect. Apodanthi (0–4.67 insect visits per hour). Twelve hours of observations in *N. cuatrecasasii* (Jaén, Cazorla range) revealed rarity of pollinators, although medium sized solitary bees (*Anthophora* sp.) were consistently spotted (10 visits) probing nectar, and small unidentified bees (8 visits) reaching pollen of the upper anthers. In the same area, *Anthophora* bees visited flowers of *Helleborus foetidus*, but always in different bouts. Casual observations made on populations at the opposite extreme of the geographic range of *N. cuatrecasasii* (Cádiz, Grazalema range) allow recognising *Anthophora* as the most common visitor. By censuses in *N. rupicola* at the core of its geographic range (Avila, Puerto de Mijares; Madrid, Puerto de Navacerrada), we observed only two visits by the moth *Macroglossum stellatarum*, one by the hoverfly *Eristalis tenax* and one by an unidentified small bee. Flies and small bees tried to enter the flower tube, but they only contacted upper anthers and L-stigma because of its narrowness and short tongues. Infrequent pollinator visits is not the result of low number of insects, as they visited profusely another co-occurring flowers of some genera (*Gagea, Ranunculus, Saxifraga, Armeria, Cytisus, Erysimum*). Some nocturnal censuses revealed

Table 4 Height of stamens and stigmas in dimorphic species of *Narcissus* sect Apodanthi. L, long-styled morph; S, short-styled morph. Values are means ± 1 se in mm. For estimating stigma-anther separation we consider only the equivalent whorl to the stigma of the alternate morphs to be the reciprocal (L stigma vs upper stamen whorl of S flower, S stigma vs lower stamen whorl of L flower, see Fig. 1). Appropriate *t*-tests are given for the differences between means of these lengths (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

Species	Population	Sample size L, S	Style height L	Style height S	Upper anther height L	Upper anther height S	Lower anther height L	Lower anther height S	Stigma-Anther separation L	Stigma-Anther separation S	Morph ratio (1)
N. albimarginatus	Bouhachem	69, 58	24.40 ± 3.8***	9.77 ± 1.70	15.97 ± 2.14***	24.54 ± 2.89	9.96 ± 1.78***	21.80 ± 2.55	0.14	0.19	0.65 : 0.35
N. albimarginatus	Kelti	53, 45	24.37 ± 0.32***	9.36 ± 0.19	14.89 ± 0.28***	21.67 ± 0.36	8.49 ± 0.23***	18.81 ± 0.41	2.70	0.87	0.66 : 0.34
N. cuatrecasasii	Grazalema	66, 34	15.08 ± 0.20***	8.01 ± 0.17	13.79 ± 0.18 ns	14.36 ± 0.26	9.05 ± 0.16***	12.03 ± 0.27	0.72	1.04	0.65 : 0.35
N. cuatrecasasii	Cazorla	42, 58	14.60 ± 0.20***	6.90 ± 0.12	13.18 ± 0.18 *	13.95 ± 0.15	8.25 ± 0.14***	10.74 ± 0.11	0.65	1.62	0.42 : 0.58
N. calcicola	Sicó	50, 43	16.36 ± 0.34***	8.19 ± 0.18	14.46 ± 0.18 **	15.25 ± 0.29	9.25 ± 0.21***	11.32 ± 0.18	1.12	1.38	0.69 : 0.31
N. calcicola	Sto. Antonio	51, 44	16.49 ± 0.29***	8.06 ± 0.27	15.30 ± 0.23 ns	15.37 ± 0.22	9.44 ± 0.21***	11.68 ± 0.23	1.11	1.06	0.76 : 0.24
N. scaberulus	Oliveira	51, 31	15.12 ± 0.24***	7.72 ± 0.17	14.56 ± 0.15 ns	15.05 ± 0.27	9.21 ± 0.16***	11.50 ± 0.24	0.07	1.49	0.86 : 0.14
N. scaberulus	Ervedal	50, 14	14.77 ± 0.29***	7.78 ± 0.28	14.85 ± 0.21 ns	14.99 ± 0.65	9.86 ± 0.15***	11.71 ± 0.37	0.22	2.08	0.77 : 0.23
N. rupicola	Aldeaquemada	42, 36	14.94 ± 0.50***	10.43 ± 0.22	16.08 ± 0.27***	18.19 ± 0.21	11.09 ± 0.25***	13.98 ± 0.21	3.25	0.66	0.54 : 0.46
N. rupicola	Bola del Mundo	53, 35	13.19 ± 0.24***	8.16 ± 0.14	15.45 ± 0.23 ns	16.31 ± 0.29	10.56 ± 0.18***	12.66 ± 0.25	3.12	2.40	0.72 : 0.28

(1) Sample size used for morph ratio is the total number of flowers collected (see Table 1).

occurrence of some moths around the flowers of *N. rupicola*, but they were not observed to come into contact with flowers. In *N. watieri*, insect visits were much more abundant. After a 3-hour census, we observed long-tongued visitors (diurnal moths: six visits; pierid butterflies: three visits) and short-tongued hoverflies (five visits). Despite considerable time (9 h) spent on *N. albimarginatus*, we were not able to spot any pollinator.

Chloroplast sequence variation

Length of *trn*L-F sequences in *Narcissus* ranges between 337 bp (population 1 of *Narcissus pallidulus*) and 365 bp (population 2 of *Narcissus marvieri*). This sequence variation is caused by seven indels of 1–19 bp. Number of variable/parsimony-informative characters is of 17/11 across species of *Narcissus*. The highest pairwise divergence (Kimura-2-parameter model) of species sequences (3.4%) was found between accessions of *N. bulbocodium* and *N. cyclamineus*. Sequence divergence neither was found between species of sect. Apodanthi (excluding *N. calcicola* and *N. scaberulus*) nor was observed between two of the three species of the *N. triandrus* complex (*N. pallidulus* and *N. lusitanicus*). Kimura-2-parameter distances between accessions of the same species did not rendered either divergence.

In *Narcissus* sect. Apodanthi, length variation of *trn*T-L sequences ranges between 827 bp (*N. marvieri*) and 966 bp (*N. calcicola*). Ten gaps in the *trn*T-L sequence matrix from 1 bp to 130 bp are responsible for great sequence variation. An unusual indel of 130 bp is shared by *N. rupicola*, *N. marvieri* and *N. watieri*. The number of variable/parsimony-informative characters is 19/11 in *Narcissus* sect. Apodanthi. The highest pairwise divergence (Kimura-2-parameter model) between sequence species (1.4%) was found between accessions of *N. calcicola* and *N. cuatrecasasii*, whereas the lowest (0.21%) was between *N. calcicola* and *N. scaberulus*.

Phylogenetic relationships

Maximum parsimony (MP) using the 27 *trn*L-F sequences of *Narcissus* and *Galanthus*, *Lapiedra* and *Leucojum* as the outgroup, yielded 1529 trees of 78 steps (CI: 0.95 and RI: 0.93, including uninformative characters) (results not shown). A congruent, somewhat more resolved topology was retrieved in the semistrict consensus tree of 13 225 trees (101 steps, CI: 0.85, and RI: 0.85) when coding the seven indels as additional characters; however, new branches do not display significant values (bootstrap below 50%) (Fig. 5). From both analyses (with and without indel coding) a basal polytomy is depicted in the semistrict consensus tree, of which two well-supported clades are formed by the accessions of five species of sect. Apodanthi (clade 1) and six species including the three of the *N. triandrus* complex (clade 2). Relatively long branches in the Neighbor-Joining (NJ) trees using both alignments

Table 5 Height of stamens and stigmas in trimorphic *Narcissus pallidulus*. For calculation of stigma-anther separation see Table 4. L, long-styled morph; M, mid-styled morph; S, short-styled morph. Values are means ± 1 se in mm. Appropriate posthoc comparisons of means (Tukey HSD test for unequal sample sizes) after one-way ANOVAS are given (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

Species	Population	Stigma height L	M	S	Upper anther height L	M	S	Lower anther height L	M	S	Morph ratio L:M:S
N. pallidulus	Segura	24.64 ± 0.34	14.65 ± 0.31	8.68 ± 0.28	21.06 ± 0.39	21.69 ± 0.25	22.30 ± 0.50	10.54 ± 0.18	10.07 ± 0.18	13.99 ± 0.41	0.40 : 0.42 : 0.18
L		–	***	***	–	ns	ns	–	ns	*	
M		–	–	***	–	–	ns	–	–	ns	
S		–	–	–	–	–	–	–	–	–	
Stigma-Anther Separation L								1.86			
Stigma-Anther Separation M						2.95			1.39		
Stigma-Anther Separation S							2.34			0.66	
N. pallidulus	Calera	25.89 ± 0.52	15.15 ± 0.35	9.03 ± 0.30	20.46 ± 0.49	20.77 ± 0.39	22.05 ± 0.42	10.82 ± 0.28	10.24 ± 0.40	14.07 ± 0.44	0.42 : 0.41 : 0.17
L		–	***	***	–	ns	ns	–	ns	**	
M		–	–	***	–	–	ns	–	–	**	
S		–	–	–	–	–	–	–	–	–	
Stigma-Anther Separation L								1.79			
Stigma-Anther Separation M						5.12			1.21		
Stigma-Anther Separation S							3.84			1.08	

Fig. 2 Sex organ position of species of *Narcissus* sect. Apodanthi and *N. pallidulus* (sect. Cyclaminei). Numbers in parentheses correspond to populations in Table 1. For simplicity, only two out of three stamens in each whorl are represented. Drawings are based on mean values (mm) included in Tables 3–5.

(results not shown) were congruent with the major, well-supported clades of the MP trees. Apart from three groups of species retrieved from the parsimony-based analysis, a forth grouping formed by the accessions of *N. scaberulus* and *N. calcicola* is recognised in the NJ tree (results not shown). Low molecular variation across *trn*L-F sequences and tree resolution prevent from inferring whether species of sect. Apodanthi form a monophyletic group. Our *trn*L-F phylogeny (Fig. 5) indicates that heterostylous species (*N. albimarginatus*, *N. lusitanicus*, *N. pallidulus* and *N. triandrus*) form part of two independent clades (clade 1 and clade 2). Optimization of character states for flower polymorphism

Table 6 Perianth variables of species of *Narcissus* sect. Apodanthi and *N. pallidulus* (see Fig. 1 for variable names)

Species	Flower width	Tepal length	Corona width	Corona height	Tube length	Tube width	Flower angle
N. pallidulus	30.48 ± 0.23	14.78 ± 0.13	8.68 ± 0.10	8.35 ± 0.09	13.55 ± 0.09	4.54 ± 0.05	46.38 ± 1.63
N. albimarginatus	38.28 ± 0.32	18.67 ± 0.17	7.40 ± 0.09	7.11 ± 0.07	12.57 ± 0.14	4.74 ± 0.04	54.15 ± 2.29
N. cuatrecasasii	24.55 ± 0.19	10.57 ± 0.09	4.20 ± 0.04	6.81 ± 0.08	13.92 ± 0.08	4.64 ± 0.04	102.65 ± 1.65
N. calcicola	18.75 ± 0.14	7.95 ± 0.10	7.33 ± 0.07	5.66 ± 0.05	13.92 ± 0.11	4.20 ± 0.06	111.41 ± 1.30
N. scaberulus	14.23 ± 0.14	5.87 ± 0.08	5.91 ± 0.07	4.19 ± 0.05	14.49 ± 0.10	3.63 ± 0.05	120.17 ± 1.67
N. rupicola	23.19 ± 0.21	11.01 ± 0.11	8.87 ± 0.13	5.19 ± 0.07	18.99 ± 0.19	3.58 ± 0.04	159.97 ± 1.20
N. marvieri	21.27 ± 0.25	9.75 ± 0.16	9.74 ± 0.16	6.01 ± 0.12	19.12 ± 0.53	3.32 ± 0.06	146.83 ± 2.04
N. watieri	23.19 ± 0.35	10.70 ± 0.21	7.99 ± 0.19	3.74 ± 0.09	22.72 ± 0.22	3.24 ± 0.05	168.22 ± 1.61

For all species, data are averaged over populations and, in polymorphic species, data are averaged over morphs. Values are means ± 1 se in mm. See text for statistical differences.
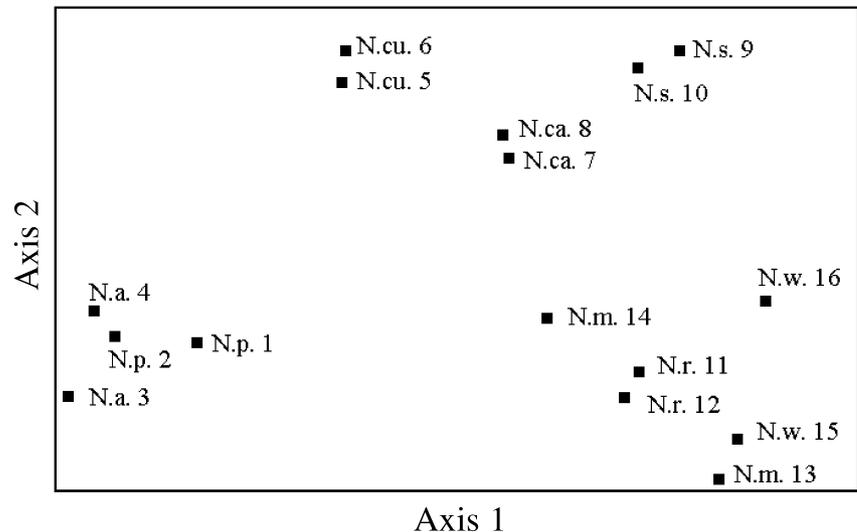
Fig. 3 Principal component analysis (PCA) of perianth variables of *Narcissus* sect. Apodanthi and *N. pallidulus*. Species names are abbreviated by their initials and numbers correspond to populations in Table 1.

using MacClade reconstructions reveals occurrence of heterostyly at least twice as a result of two independent events (results not shown). Low number of nucleotide substitutions in *trn*L-F sequences within species of clade 2 also prevent from inferring whether heterostyly in the *N. triandrus* complex is the result of one or additional evolutionary processes.

MP analyses of *trn*T-L sequences of sect. Apodanthi and two outgroup species yielded congruent results in both analyses with and without coding indels. A most resolved semistrict consensus tree was retrieved when performing equal weighting of the 10 indels in which a basal-most position of *N. papyraceus* is followed by a clade of *Narcissus* sect. *Apodanthi* species resolved biphyletically into two subclades (Fig. 6). Two outgroup species are, however, an insufficient number to infer monophyly of sect. Apodanthi. Close relationship between *N. scaberulus* and *N. calcicola* is evidenced by a well-defined *trn*T-L subclade (100% bootstrap). A second subclade consists of the distylous species *N. albimarginatus* together with *N. cuatrecasasii, N. rupicola, N. watieri* and *N. marvieri*

(98% bootstrap). Within this subclade, basal position of *N. albimarginatus, N. cuatrecasasii* and a pectinate branch of three species (76% bootstrap) is observed, in which *N. rupicola* is sister to *N. waitieri* and *N. marvieri* (77% bootstrap). Character reconstruction of flower polymorphism in sect. Apodanthi was traced onto the *trn*T-L tree (Fig. 6). MacClade reconstructions help interpret that distyly may be a relatively primitive character in sect. Apodanthi, whereas style monomorphism is most derived.

Discussion

Our results clearly show that *Narcissus* sect. Apodanthi is very variable both in terms of stylar conditions and in terms of perianth features. Moreover, there is a consistent pattern of concordance between perianth features herein described and style polymorphism formerly outlined (Arroyo, 2002). Interestingly, morphological correlates of distyly only found in sect. Apodanthi are also described in the other reported case of heterostyly in *Narcissus* (Barrett *et al.*, 1997).
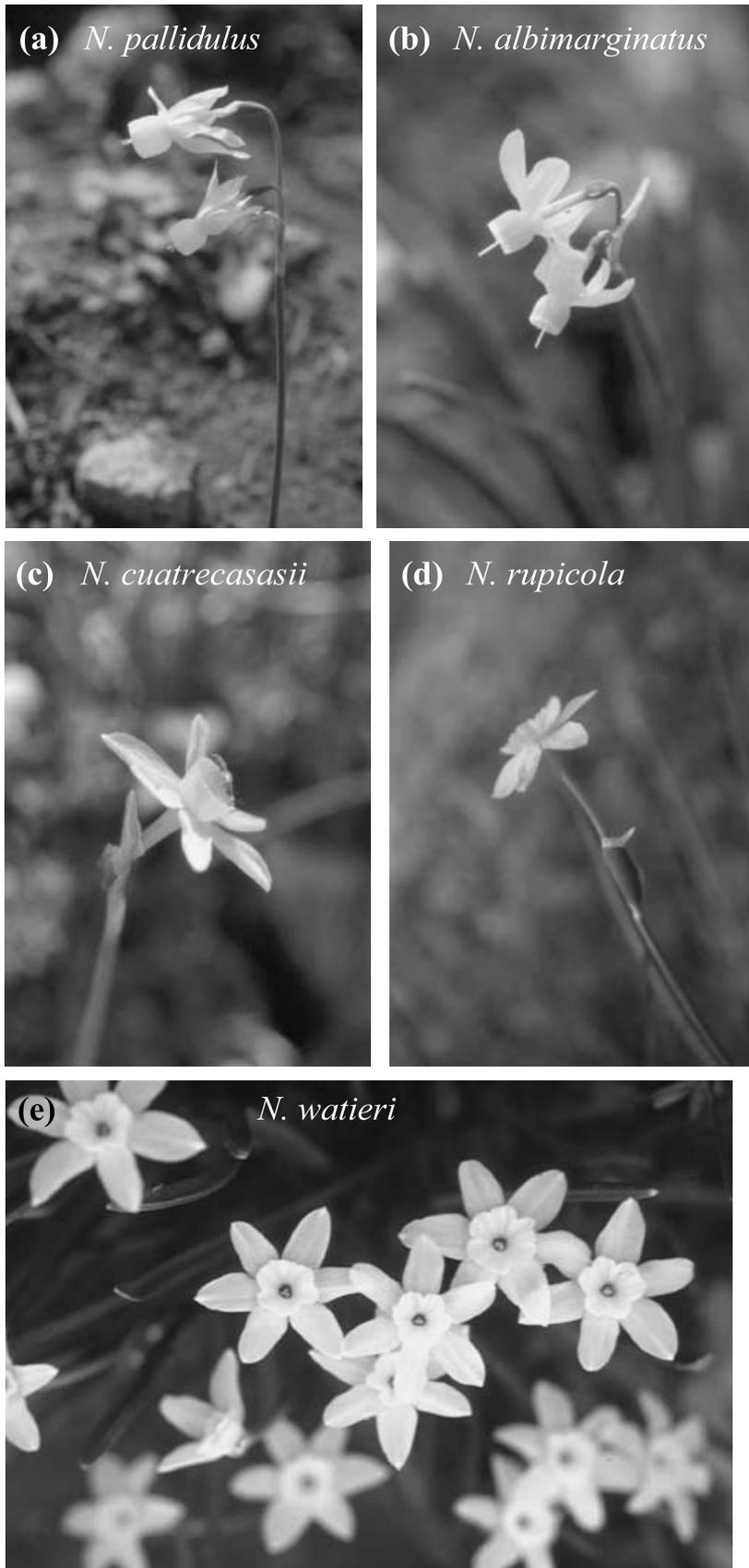
Fig. 4 Photographs of five *Narcissus* species displaying contrasted flower morphologies: pendulous flowers (a and b), patent to erect flowers (c, d and e), cup-shaped coronas (a, b and c) and funnel-shaped coronal (d and e). (a) *N. pallidulus*; (b) *N. albimarginatus*; (c) *N. cuatrecasasii*; (d) *N. rupicola*; (e) *N. watieri*.

Table 7 Results of multiple regression analyses of perianth features as resumed by PCA axes on stigma-anther reciprocity considering the possible effect of flower size (see details about measurements in the text)

	Dependent variables (sex organ reciprocity)	
Independent variables (perianth)	Size uncorrected stigma-anther separation	Size corrected stigma-anther separation
Intercept	0.0771***	1.7497***
PCA axis I	0.0126***	0.1700*
PCA axis II	−0.0135**	−0.4616***
PCA axis III	−0.0145*	ns
R^2	0.804	0.724
$F_{3,12}$	16.452***	10.512**
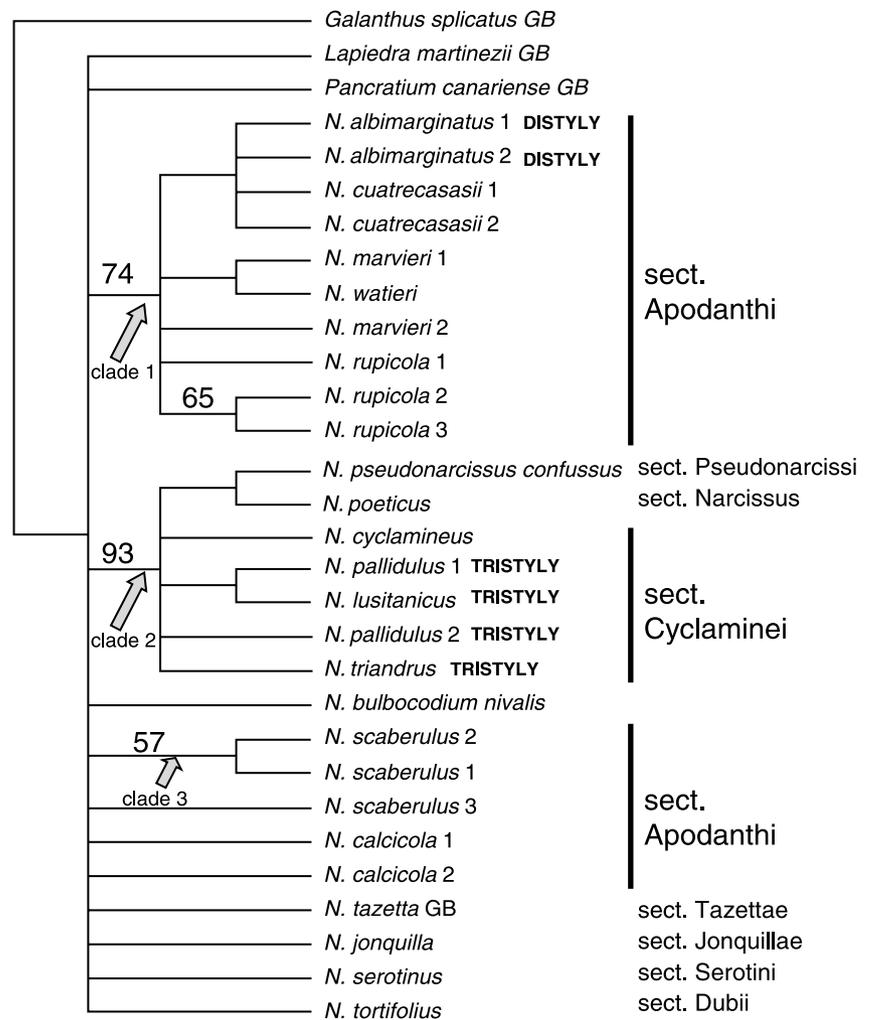
Fig. 5 Semistrict consensus tree of 13 225 most-parsimonious trees of 101 steps from the analysis of the *trn*L-F sequences (with indel coding) of *Narcissus* and outgroup genera (CI: 0.85; RI: 0.85, including uninformative characters). *Galanthus*, *Lapiedra* and *Pancratium* served as the outgroup. Bootstrap values above 50% are shown above tree branches. The three main clades are discussed in the text. Numbers/ letters after species names refer to numbering in Table 2 as GeneBank (GB) accessions. Heterostylous species are marked in bold. Sectional classification of *Narcissus* is marked on the right margin.

Flower morphology

Variation in sex organ position Description of distyly for the first time in *Narcissus* based on a limited sample (Arroyo & Barrett, 2000) is herein confirmed with extensive data from a second population of *N. albimarginatus* (Pérez *et al.*, unpub-

lished). The two populations studied of *N. pallidulus* have a typical tristylous condition, as it was previously documented in detail (Barrett *et al.*, 1997, as *N. triandrus* s.l.), and served efficiently to contrast distyly. In these two heterostylous species sex organs show approximately reciprocal position. Perfect reciprocity is seldom accomplished by typical heterostylous
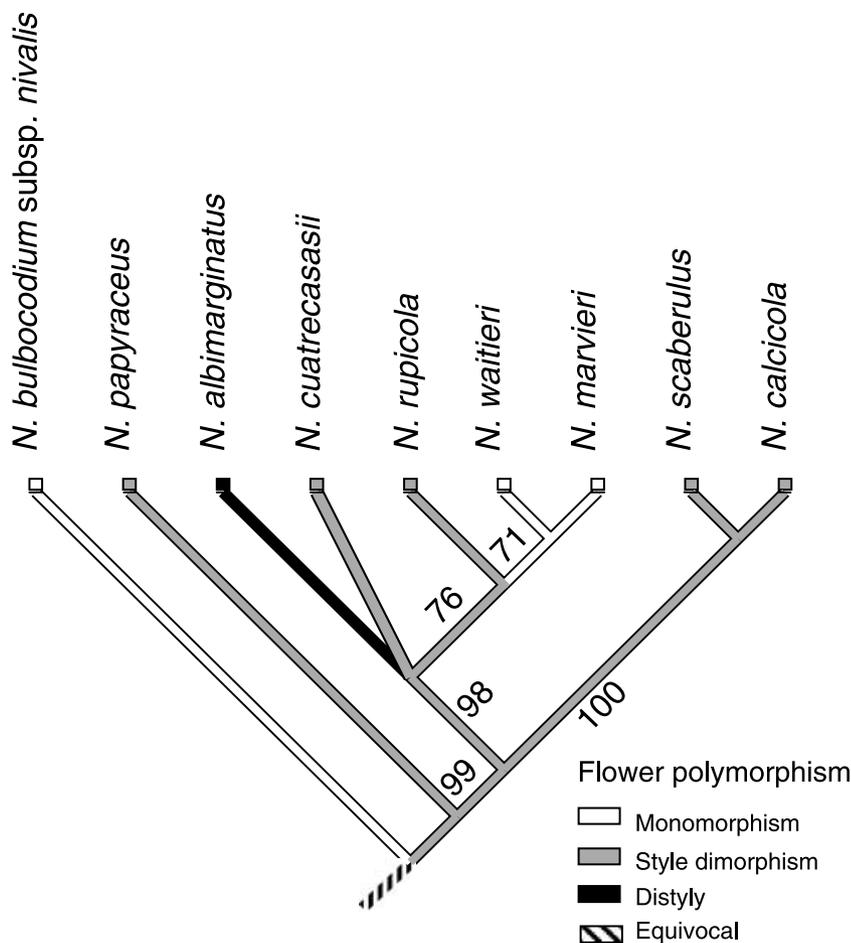
Fig. 6 Semistrict consensus of the three most-parsimonious trees of 90 steps from analysis of *trn*T-L sequences (with indel coding) of *Narcissus* sect. *Apodanthi* and outgroup species (CI: 0.96; RI: 0.92, including uninformative characters), onto which style polymorphism has been mapped after implementing ACCTRAN optimization of MacClade (Maddison & Maddison, 1992). *Narcissus bulbocodium* ssp. *nivalis* and *N. papyraceus* were used as the outgroup. Bootstrap values above 50% are also shown beside tree branches. See Table 2 for accession numbers.

species (Lloyd *et al.*, 1990; Faivre & McDade, 2001), although it is required to have at least a reciprocity in the sequence of presentation of sex organs to pollinators (Lloyd & Webb, 1992a,b).

The majority of species in sect. Apodanthi (*N. cuatrecasasii, N. calcicola, N. scaberulus, N. rupicola*) has style dimorphism and deviated reciprocity. However, this deviation is variable in this species group. We had the opportunity to check sex organ reciprocity in a continuous range of variation in the whole species sample. Among the dimorphic species, *N. cuatrecasasii* yielded the lowest anther-stigma separation and *N. rupicola* the highest. That is, the lowest and the highest deviation from reciprocity, respectively. Style dimorphism has been shown to be very frequent in *Narcissus* (Barrett *et al.*, 1996) and remains one of the unsolved challenges to the Lloyd & Webb's (1992a,b) model for the evolution of heterostyly. The reported variability in style dimorphism may explain why this condition is maintained in *Narcissus*. At least in some cases there may be enough disassortative mating as to maintain style morphs even without a reciprocal positioning.

Only two species show monomorphism in sect. Apodanthi, the Moroccan endemics *N. marvieri* and *N. watieri*. Interest-

ingly, the position of the sex organs is very similar to that of the L-morph found in the Iberian *N. rupicola* (Fig. 2). Obviously, we cannot rule out existence of dimorphism in undiscovered populations of both species, as both dimorphic and monomorphic populations occur sometimes within the same species (*N. papyraceus*: Arroyo *et al.*, 2002; *N. tazetta*: Arroyo & Dafni, 1995; *N. dubius*: Baker *et al.*, 2000a; *N. jonquilla*: J. Arroyo, unpublished).

We have found an L-biased morph ratio in most populations of dimorphic and distylous species, as described in a former species survey (Barrett *et al.*, 1996). Given the occurrence of intramorph compatibility in most of these species (Dulberger, 1964; Barrett *et al.*, 1996; Sage *et al.*, 1999; Baker *et al.*, 2000b; Arroyo *et al.*, 2002), it is interpreted that L-biased morph ratios are a consequence of differential level of assortative-disassortative mating between morphs in dimorphic species. These ratios also depend on population size (Arroyo *et al.*, 2002; Baker *et al.*, 2000a) and the level of sex organ reciprocity. All these factors might explain why widely distributed species (*N. cuatrecasasii, N. rupicola*) display strong differences in morph ratio between populations (Table 4), which larger surveys have made clearer (Arroyo, 2002; R. Pérez, unpublished).

Morph ratio in distylous *N. albimarginatus* is also L-biased in the two populations surveyed, which contrasts with the former isoplethy reported by Arroyo & Barrett (2000). It is likely a higher level of assortative mating for the L-morph than formerly suggested. A breeding system program across species in sect. Apodanthi is under study and may shed further light on this respect. At least *N. scaberulus*, *N. calcicola* and *N. cuatrecasasii* seem to present self-incompatibility and intramorph compatibility (unpublished data).

Perianth features and pollinators Only two perianth features displayed significant differences between morphs within populations of the only two heterostylous species studied: *N. pallidulus* (corona height) and *N. albimarginatus* (flower width). These differences have been seldom interpreted as a mechanism to compensate asymmetric pollen flow (Ganders, 1979). Alternatively, there is reliable evidence that reflects allometric effects associated with different patterns of flower development of morphs (Dulberger, 1992; Richards & Barrett, 1992; Faivre, 2000).

Morphometrical analysis of perianth features clearly groups together heterostylous species (*N. pallidulus* and *N. albimarginatus*), despite they belong both to different taxonomic sections (Dorda & Fernández-Casas, 1989; Muller-Doblies & Muller-Doblies, 1989) and unrelated lineages (Fig. 5). These species look indeed similar (Fig. 4). Morphological resemblance was already discussed by Arroyo & Barrett (2000), who pointed out a role played by pollinators on driving flower similarity. PCA and the multiple regression analyses resulted in significant perianth correlates of style polymorphism (Table 7). This relationship is unaffected by flower size, because a regression model accounting for allometric effects displayed similar results (Table 7). All associated traits are of importance for pollinator activity, from attraction (flower size) to insect handling of flowers (flower tube and angle, corona size and shape). Heterostyly in *Narcissus* is associated with pendulous flowers, short and wide perianth tubes and large, cup-shaped coronas. The two heterostylous species share additional traits, such as similar fragrances, at least to humans, reflexed tepals (Fig. 4), and flower number per inflorescence (1–3), being the latter trait however, very variable in *Narcissus* (Worley *et al.*, 2000; see also Fernandes, 1975, for a evolutionary hypothesis). The remaining Apodanthi species have a contrasting flower morphology, albeit variable. Interestingly, two sets of morphological features parallel two stylar conditions: style dimorphism (*N. calcicola*, *N. scaberulus*, *N. cuatrecasasii*) with patent flowers, variable perianth-tube length and cup-shaped coronas; and monomorphism (*N. watieri*, *N. marvieri*) with erect flowers, narrow and long perianth tubes and funnel shaped coronas. Both morphological types present patent tepals. *Narcissus rupicola* displays an intermediate situation, in which perianth features are similar to those of monomorphic species, and but sex organ position to that of style dimorphic species.

Large bees actively work in pendulous and patent flowers. In fact, *Anthophora* and *Bombus* species have been reported as pollinators of the heterostylous species *N. triandrus* and *N. pallidulus* (Barrett *et al.*, 1997; J. Arroyo, personal observations), where they feed on both nectar and pollen whilst handling large cup-shaped coronas. Similarly, within the variable genus *Ixia* (Iridaceae), species of cup-shaped flowers are pollinated by *Anthophora* species (Goldblatt *et al.*, 2000). Unfortunately, we do not have reliable data on pollinators of *N. albimarginatus*. Taking into account relative flower similarity between *N. pallidulus* and *N. cuatrecasasii* (Fig. 4) with respect to *N. albimarginatus*, we hypothesize that *Anthophora* spp. could act as pollinators in the tree species (Barrett *et al.*, 1997, the present study). These solitary endothermic bees are able to fly even under relatively cold weather conditions (Batra, 1994; Stone, 1994) and are able to face harsh winter conditions when *N. albimarginatus* blooms. It has been shown that bees are comparatively more proficient for disassortative pollen transfer, a necessary condition for proper functioning of reciprocal herkogamy (Stone, 1996). *Anthophora* bees have been found to pollinate the heterostylous *Jasminum fruticans*, of similar flower size, shape and colour in Iberia (Guitián *et al.*, 1998). One of the most striking similarities between the two heterostylous species concerns pendulous flowers. Flower angle is variable in *Narcissus* (Blanchard, 1990), even within a single species. This feature limits pollinator types handling flowers and, in fact, it has been shown to vary in populations with different pollinators (Arroyo & Dafni, 1995).

We hypothesize that low reciprocal style dimorphic (*N. rupicola*) and monomorphic species of *Narcissus*, which have the narrowest and longest flower tubes, are mostly pollinated by long-tongued pollinators, as it has been found in other similar species (Arroyo *et al.*, 2002; Thompson *et al.*, 2003). Despite scarcity of actual observations of insects visiting flowers, there is some evidence that they do occur. In the population from Madrid of *N. rupicola*, we found a 94% of fruit set in the subsequent fruiting season. It is probably a result of night pollination by moths. The only white-flowered species, *N. watieri*, shows a variety of pollinators, including butterflies. It is remarkable the similarity of monomorphic species and the L-morph of *N. rupicola* (Fig. 2). The promotion of assortative mating between L plants by long-tongued lepidopterans (Stone, 1996) may fix the L morph. A similar process of pollinator-mediated shift has been suggested to promote L monomorphism in populations of *N. tazetta* (Arroyo & Dafni, 1995), *N. papyraceus* (Arroyo *et al.*, 2002), and perhaps *N. dubius* (Baker *et al.*, 2000a), which are white-flowered species with similar flower traits and array of pollinators.

Evolution of flower polymorphisms in *Narcissus*

Phylogenetic analyses of 27 *trn*L-F sequences representing much of the diversity of *Narcissus* yielded low resolution (Fig. 5) because of a limited number of variable/parsimony-

informative characters (17/11). Some conclusions are, however, inferred from analysis of phylogenetic relationships: the three species of the *N. triandrus* complex, coupled with three more species of sect. Cyclaminei, Pseudonarcissi and Narcissus (clade 2) form a natural group; a well-defined, independent lineage includes five of the seven species of sect. Apodanthi (clade 1); the remaining two species of sect. Apodanthi (*N. scaberulus*, *N. calcicola*) and the representatives of sections Tazetta, Jonquilla, Serotini and Dubii are unresolved at a basal-most position. These results indicate that sect. Cyclaminei, Pseudonarcissi and Narcissus are closely related based on this chloroplast marker. Circumscription of sect. Apodanthi, including *N. scaberulus* and *N. calcicola* together with *N. watieri*, *N. marvieri*, *N. rupicola*, *N. albimarginatus*, and *N. cuatrecasasii*, needs further investigation.

Phylogenies based on molecular markers have been rarely used to reconstruct character evolution of flower features related to heterostyly (but see Graham & Barrett, 1995, and Kohn *et al.*, 1996 for Pontederiaceae). Evolutionary pathways of reproductive features concerning gynodioecy in *Lycium* (Miller & Venable, 2003) and selfing in *Collinsia* (Armbruster *et al.*, 2002) have been already investigated in detail by using nuclear sequences. In our study, phylogenetic reconstructions of chloroplast sequences suggest that heterostyly has taken place at least twice in the course of the evolution of *Narcissus*. Two well-defined lineages including heterostylous species and high sequence divergence between them (average of 1.96% in pairwise distance estimates) lead us to interpret two independent evolutionary histories in acquisition of distyly (*N. albimarginatus*) and tristyly (*N. triandrus* complex). Although limited resolution is depicted in the clade 2 of the *trn*L-F phylogeny (Fig. 5), low molecular divergence (< 0.31%) between species of the *N. triandrus* complex (*N. lusitanicus, N. pallidulus, N. triandrus*) indicates close relatedness. The occurrence of two independent lineages with heterostylous species supports a prediction (Arroyo & Barrett, 2000) of independent acquisition of heterostyly in *Narcissus*. The role of pollinators in acquisition of heterostyly remains an open question. However, a significant flower similarity of both heterostylous species supports this hypothesis. Multiple origins of heterostyly within a single genus is not surprising, because independent origins have been already documented at the familial level (Barrett, 1992). To our knowledge this is the first report of convergence of heterostyly at the generic level. Unfortunately, basal polytomies in the *trn*L-F phylogeny prevents from inferring whether ancestral condition to heterostyly is style dimorphism, as proposed by Lloyd & Webb (1992a,b).

The *trn*T-L phylogeny of section Apodanthi shows a clade resolution (Fig. 6), in which an evolutionary pattern of style polymorphism can be inferred. First, split of an early lineage of *N. scaberulus-N. calcicola* endemic to Portugal and the remaining species of sect. Apodanthi is suggested. These two Portuguese species have a style dimorphism condition in common, with some reciprocity in lower anthers and a similar perianth. Therefore, stylar dimorphism appears to be an ancestral condition within sect. Apodanthi, as predicted by Lloyd & Webb's (1992a,b) model of heterostyly. Although the parsimony-based (cladistic) reconstruction gives limited resolution (Fig. 6), to pinpoint a sister species to the heterostylous *N. albimarginatus*, a distance-based phylogeny (NJ) clearly joins *N. albimarginatus* and *N. cuatrecasii*, result in congruence with similarity of perianth morphology and stylar condition between both species. The remaning three Apodanthi species form a well-defined lineage with a pectinate topology, giving strong support for an evolutionary pattern in which acquisition of nonherkogamous monomorphism (*N. marvieri* and *N. watieri*) may have occurred from an ancestor with a style-dimorphic condition (as in *N. rupicola*). The loss of stylar polymorphism to monomorphism has been shown to occur in other taxonomic groups (Kohn *et al.*, 1996). With the available phylogenetic information, it is not possible to determine shifts from style dimorphism to monomorphism in other sections in *Narcissus*. However this may be tendency at least in some cases. Similarity between L-morph of *N. rupicola* and flowers of the two monomorphic species, together with the frequent loss of the S-morph in other dimorphic species of sect. Tazetta (Arroyo & Dafni, 1995; Arroyo *et al.*, 2002) support this view. A similar pollinator array in *N. marvieri* to that in sect. Tazetta suggests that this shift may be mediated by insects. Style polymorphism shift from style dimorphism to monomorphism and the role of pollinators should be explored in other sections of *Narcissus*.

Phylogenetic reconstructions, coupled with patterns of flower similarity both in stylar conditions and perianth features across unrelated lineages, support the role of pollinators in moulding style polymorphim in *Narcissus*. Our results lead us to conclude that multiple convergence events have occurred in the course of evolution of *Narcissus*, particularly to build up heterostyly (distyly and tristyly) and monomorphism through intermediate, insect-mediated stages, as proposed by the Lloyd & Webb's (1992a) model.

References

Armbruster WS, Mulder CPH, Baldwin BG, Kalisz S, Wessa B, Nute H. 2002. Comparative analysis of late floral development and mating-system evolution in tribe Collinsieae (Scrophulariaceae s.l.). *American Journal of Botany* **89**: 37–49.

Arroyo J. 2002. *Narcissus*, la evolución de los polimorfismos florales y la conservación más allá de las 'listas rojas'. *Revista Chilena de Historia Natural* **75**: 39–55.

Arroyo J, Barrett SCH. 2000. Discovery of distyly in *Narcissus* (Amaryllidaceae). *American Journal of Botany* **87**: 748–751.

Arroyo J, Barrett SCH, Hidalgo R, Cole WW. 2002. Evolutionary maintenance of stygma-height dimorphism in *Narcissus papyracens* (Amaryllidaceae). *American Journal of Botany* **89**: 1242–1249.

Arroyo J, Dafni A. 1995. Variations in habitat, season, flower traits and pollinators in dimorphic *Narcissus tazetta* L. (Amaryllidaceae) in Israel. *New Phytologist* **129**: 135–146.

Baker AM, Thompson JD, Barrett SCH. 2000a. Evolution and maintenance of stigma-height dimorphism in *Narcissus*. I. Floral variation and style-morph ratios. *Heredity* **84**: 502–513.

Baker AM, Thompson JD, Barrett SCH. 2000b. Evolution and maintenance of stigma-height dimorphism in *Narcissus*. II. Fitness comparisons between style morphs. *Heredity* **84**: 514–524.

Barrett SCH. 1992. *Evolution and function of heterostyly*. Berlin, Germany: Springer-Verlag.

Barrett SCH, Cole WW, Arroyo J, Cruzan MB, Lloyd DG. 1997. Sexual polymorphism in *Narcissus triandrus* (Amaryllidaceae): Is this species tristylous?. *Heredity* **78**: 135–145.

Barrett SCH, Jesson LK, Baker AM. 2000. The evolution and function of stylar polymorphisms in flowering plants. *Annals of Botany* **85**: 253–265.

Barrett SCH, Lloyd DG, Arroyo J. 1996. Stylar polymorphisms and the evolution of heterostyly in *Narcissus* (Amaryllidaceae). In: Lloyd DG, Barrett SCH, eds. *Floral biology, studies on floral evolution in animal-pollinated plants*. New York, USA: Chapman & Hall, 339–376.

Batra SWT. 1994. *Anthophora pilipes villosula* SM (Hymenoptera, Anthophoridae), a manageable japanese bee that visits bluberries and apples during cool, rainy spring weather. *Proceedings of the Entomological Society of Washington* **96**: 98–119.

Blanchard JW. 1990. *Narcissus, a guide to wild daffodils*. Surrey, UK: Alpine Garden Society.

Charlesworth D, Charlesworth B. 1979. A model for the evolution of distyly. *American Naturalist* **114**: 467–498.

Darwin C. 1877. *The Different forms of flowers on plants of the same species*. London, UK: John Murray.

Dorda E, Fernández-Casas J. 1989. Estudios morfológicos en el género *Narcissus* L. Anatomía de hoja y escapo III. *Fontqueria* **27**: 103–162.

Dulberger R. 1964. Flower dimorphism and self-incompatibility in *Narcissus tazetta* L. *Evolution* **18**: 361–363.

Dulberger R. 1992. Floral polymorphisms and their functional significance in the heterostylous syndrome. In: Barrett SCH, ed. *Evolution and function of heterostyly*. Berlin, Germany: Springer-Verlag, 41–84.

Faivre AE. 2000. Ontogenetic differences in heterostylous plants and implications for development from herkogamous ancestor. *Evolution* **54**: 847–858.

Faivre AE, McDade LA. 2001. Population level variation in the expression of heterostyly in three species of Rubiaceae: does reciprocal placement of anthers and stigmas characterize heterostyly? *American Journal of Botany* **188**: 841–853.

Fernandes A. 1967. Contribution à la connaisance de la biosystematique de quelques espècies de genre *Narcissus*. *Portugaliae Acta Biologica (B)* **9**: 1–44.

Fernandes A. 1975. L'Evolution chez le genre *Narcissus* L. *Anales del Instituto Botánico Cavanilles* **32**: 843–872.

Ganders FR. 1979. The biology of heterostyly. *New Zealand Journal of Botany* **17**: 607–635.

Goldblatt P, Bernhardt P, Manning JC. 2000. Adaptative radiation of pollination mechanisms in *Ixia* (Iridaceae: Crocoideae). *Annals of the Missouri Botanical Garden* **87**: 564–577.

Graham SW, Barrett SCH. 1995. Phylogenetic systematics of Pontederiales: Implications for breeding-system evolution. In: Rudall PJ, Cribb PJ, Cutler DF, Humphries CJ, eds. *Monocotyledons: systematics and evolution*. Kew, UK: Royal Botanic Gardens, 415–451.

Guitián J, Guitián P, Medrano M. 1998. Floral biology of the distylous Mediterranean shrub *Jasminum fruticans* (Oleaceae). *Nordic Journal of Botany* **18**: 195–201.

James FC, McCulloch CE. 1990. Multivariate-analysis in ecology and systematics – Panacea or Pandora Box. *Annual Review of Ecology and Systematics* **21**: 129–166.

Kelchner SA. 2000. The evolution of non-coding chloroplast DNA and its application in plant systematics. *Annals of the Missouri Botanical Garden* **87**: 482–498.

Kimura M. 1980. A simple method for estimating evolutionary rate of base substitution through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* **16**: 11–120.

Kohn JR, Barrett SCH. 1992. Experimental studies on the functional significance of heterostyly. *Evolution* **46**: 43–55.

Kohn JR, Graham SW, Morton B, Doyle JJ, Barrett SCH. 1996. Reconstruction of the evolution of reproductive characters in Pontederiaceae using phylogenetic evidence from chloroplast DNA restriction-site variation. *Evolution* **50**: 1454–1469.

Lau P, Bosque C. 2003. Pollen flow in the distylous *Palicourea fendleri* (Rubiaceae): an experimental test of the disassortative pollen flow hypothesis. *Oecologia* **135**: 593–600.

Lloyd DG, Webb CJ. 1992a. The evolution of heterostyly. In: Barrett SCH, ed. *Evolution and function of heterostyly*. Berlin, Germany: Springer-Verlag, 151–178.

Lloyd DG, Webb CJ. 1992b. The selection of heterostyly. In: Barrett SCH, ed. *Evolution and function of heterostyly*. Berlin, Germany: Springer-Verlag, 179–207.

Lloyd DG, Webb CJ, Dulberger R. 1990. Heterostyly in species of *Narcissus* (Amaryllidaceae), *Hugonia* (Linaceae) and other disputed cases. *Plant Systematics and Evolution* **172**: 215–227.

Maddison WP, Maddison DR. 1992. *MacClade, Version 3: Analysis of Phylogeny and Character Evolution*. Sunderland, MA, USA: Sinauer Associates, Inc.

Mathew B. 2002. Clasification of the genus *Narcissus*. In: Hanks GR, ed. *Narcissus and Daffodil*. London, UK: Taylor & Francis, 30–52.

McCune B, Mefford MJ. 1999. *PC-ORD. Multivariate Analysis of Ecological Data, Version 4*. Gleneden Beach, OR, USA: MjM Software Design.

Meerow AW, Fay MF, Guy CL, Li Q-B, Zaman FQ, Chase M. 1999. Systematics of Amaryllidaceae based on cladistic analysis of plastid *rbc*L and *trn*L-F sequence data. *American Journal of Botany* **86**: 1325–1345.

Miller JS, Venable DL. 2003. Floral morphometrics and the evolution of sexual dimorphism in *Lycium* (Solanaceae). *Evolution* **57**: 74–86.

Muller-Doblies D, Muller-Doblies U. 1989. *Narcissus albimarginatus*, plate 1986. In: *The flowering plants of Africa, Vol. 50 Part 2*. Cape Town, South Africa: Creda Press.

Nishihiro J, Washitani I, Thomson JD, Thomson BA. 2000. Patterns and consequences of stigma height variation in a natural population of distylous plant, *Primula sieboldii*. *Functional Ecology* **14**: 502–512.

Ornduff R. 1992. Historical perspective on heterostyly. In: Barrett SCH, ed. *Evolution and function of heterostyly*. Berlin, Germany: Springer-Verlag, 31–39.

Philippi TE. 1993. Multiple regression: Herbivory. In: Scheiner SM, Gurevitch J, eds. *Design and analysis of ecological experiments*. New York, USA: Chapman & Hall, 183–210.

Richards AJ. 1998. Lethal linkage and its role in the evolution of plant breeding systems. In: Owens SJ, Rudall PJ, eds. *Reproductive biology*. Kew, UK: Royal Botanic Gardens.

Richards JH, Barrett SCH. 1992. The development of heterostyly. In: Barrett SCH, ed. *Evolution and function of heterostyly*. Berlin, Germany: Springer-Verlag, 85–124.

Sage TL, Strumas F, Cole WW, Barrett SCH. 1999. Differential ovule development following self- and cross-pollination: the basis of self-sterility in *Narcissus triandrus* (Amaryllidaceae). *American Journal of Botany* **86**: 855–870.

Saitou N, Nei M. 1987. The Neighbor-Joining method: a new method for reconstructing phylogentic trees. *Molecular Biology Evolution* **4**: 406–425.

Simmons MP, Ochoterena H. 2000. Gaps as character in sequence-based phylogenetic analysis. *Systematic Biology* **49**: 369–381.

Sokal RR, Rohlf FJ. 1995. *Biometry, 3rd ed*. New York, NY, USA: Freeman.

StatSoft. 1997. *STATISTICA for Windows, Version 5.1*. Tulsa, OK, USA: StatSoft Inc.

Stone GN. 1994. Patterns of evolution of warm-up rates and body temperatures in flight in solitary bees of the genus *Anthophora*. *Functional Ecology* **8**: 324–335.

Stone JL. 1996. Components of pollination effectiveness in *Psychotria surrensis*, a tropical distylous shrub. *Oecologia* **107**: 504–512.

Stone JL, Thomson JD. 1994. The evolution of distyly: pollen transfer in artificial flowers. *Evolution* **48**: 1595–1606.

Swofford DL. 1999. *PAUP: Phylogenetic Analysis Using Parsimony, Version 4.0*. Champaign, IL, USA: Illinois Natural History Survey.

Taberlet P, Gielly L, Pautou G, Bouvet J. 1991. Universal primers for amplification of three non-coding regions of choroplast DNA. *Plant Molecular Biology* **17**: 1105–1109.

Thompson JD, Barrett SCH, Baker AM. 2003. Frequency-dependent variation in reproductive success in *Narcissus*: implications for the maintance of stigma-height dimorphism. *Proceedings of the Royal Society of London B* **270**: 949–953.

Worley AC, Baker AM, Thompson JD, Barrett SCH. 2000. Floral display in *Narcissus*: variation in flower size and number at the species, population, and individual levels. *International Journal of Plant Science* **161**: 69–79.