# New evidence for the Darwinian hypothesis of heterostyly: breeding systems and pollinators in *Narcissus* sect. Apodanthi

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## Summary

• Here we analysed the role played by breeding systems and pollinators in the evolution of heterostyly by testing whether evolution towards heterostyly is associated with style polymorphism and changes in pollinator proficiency or breeding system variation (Darwinian hypothesis).
• We studied pollinators, pollen-transfer efficiency, and incompatibility systems in all seven species of *Narcissus* sect. Apodanthi for which we also obtained chloroplast DNA (cpDNA) sequences from three spacers to infer phylogenetic relationships.
• Five species are self-incompatible and within-morph cross-compatible. Heterostylous (*Narcissus albimarginatus*) and style-dimorphic (*Narcissus cuatrecasasii*) species that have a high degree of reciprocity in stigma and anther height are primarily pollinated by solitary bees. The style-monomorphic species (*Narcissus watieri*) and the style-dimorphic species with the least stigma–anther reciprocity (*Narcissus rupicola*) are both self-compatible and pollinated by butterflies, moths and hover flies.
• Phylogenetic reconstruction of character transitions indicates that the shift from style dimorphism to distyly is associated with a shift to bee pollination. Pollination by lepidopterans and flies is associated with stable style dimorphism and monomorphism. Evolution and maintenance of style polymorphisms in this group of species are independent of incompatibility systems. Taken together, our results strongly support the pollinator-based model for evolution of heterostyly and style length polymorphisms in general.

**Key words:** incompatibility system, lepidopterans, phylogenetic reconstruction, solitary bees, speciation, style dimorphism, syrphids, Trichoptera.

## Introduction

Heterostyly is a genetically based flower polymorphism in which two (distyly) or three (tristyly) morphs present stigmas and anthers in a reciprocal position within a single population. Darwin (1877) considered that heterostylous plants are self- and morph-incompatible, in such a way that only pollination between sex organs of reciprocal height, i.e. disassortative mating, is possible. It has been traditionally considered that the incompatibility system and the sex polymorphism are tightly linked (De Nettancourt, 1977), an assumption that has greatly conditioned models for the evolution of heterostyly. Ever since the original description and first evolutionary interpretation (Darwin, 1877; reviewed in Ornduff, 1992), several hypotheses about the evolutionary mechanisms favouring heterostyly have been proposed. The most important are those by Charlesworth & Charlesworth (1979) and Lloyd & Webb (1992a,b). The former considers a genetic mechanism to have primacy whereas the later emphasizes ecological factors. Accordingly, the sequence of evolutionary events is different in each model. Charlesworth & Charlesworth (1979) consider the avoidance of selfing and inbreeding depression as key factors in the evolution of

heterostyly. In their model, the initial step involves a non-herkogamous ancestor suffering strong inbreeding, which incurs selection for the evolution of an incompatibility system before the evolution of the flower polymorphism. By contrast, Lloyd & Webb (1992a,b) suggest that the initial step is the evolution of a floral polymorphism in the response to selection for efficient cross-pollination, prompted by appropriate pollinators. In their model, the ancestral condition is approach herkogamy, the positioning of the stigma above the anthers, which evolves to distyly via a transitional style dimorphism. The latter is thought to evolve rapidly as a result of strong selection on anther height.

The model of Lloyd & Webb (1992a,b) represents a reformulation of Darwin's (1877) original ideas, which were not considered crucial in the model of Charlesworth & Charlesworth (1979). Many recent studies have explicitly been designed to test Lloyd & Webb's (1992a,b) hypothesis. Numerous population-level experiments have been undertaken to examine the factors affecting the efficiency of pollen transfer between morphs in distylous and style-dimorphic species (Stone & Thomson, 1994; Nishihiro *et al.*, 2000; Lau & Bosque, 2003; Cesaro & Thompson, 2004; Cesaro *et al.*, 2004) and morph ratio variation in style-dimorphic (Arroyo & Dafni, 1995; Baker *et al.*, 2000a,b; Arroyo *et al.*, 2002; Thompson *et al.*, 2003) and heterostylous (Barrett *et al.*, 2004) species. Style polymorphism and ontogeny of flower morphs have also been compared in this context (Faivre, 2000). A few studies have examined the frequency of the gain and loss of self-incompatibility and polymorphism at the phylogenetic level in heterostylous groups (e.g. Kohn *et al.*, 1996; Schoen *et al.*, 1997). The evolution of style polymorphism in *Narcissus* has been thoroughly analysed (Graham & Barrett, 2004; Pérez *et al.*, 2004) and indicates that monomorphic approach herkogamy preceded style dimorphism, which itself preceded distyly, as predicted by Lloyd & Webb (1992a). However, the relative roles of pollinators and breeding system changes in the evolution of heterostyly in *Narcissus* are not well understood: understanding their influence on the evolution of heterostyly is needed to assess Lloyd and Webb's hypothesis.

*Narcissus* sect. Apodanthi provides an ideal study system to test the pertinence of the above-mentioned evolutionary models. This group of seven species contains three main types of floral form (Arroyo, 2002; Pérez *et al.*, 2004): style dimorphism (*Narcissus calcicola*, *Narcissus scaberulus*, *Narcissus rupicola*, *Narcissus cuatrecasasii* and *Narcissus marvieri*), heterostyly (*Narcissus albimarginatus*), and monomorphism (*Narcissus watieri*). The most striking results published on this plant group (Pérez *et al.*, 2004) indicate that style dimorphism is the ancestral condition to distyly and that variation in style polymorphism is associated with perianth morphology. Although the ancestral condition (approach herkogamy) is not present, this natural group represents an excellent opportunity to test whether the reported stylar transitions are associated with either different pollinators or different breeding systems. Using the seven

species of *Narcissus* sect. Apodanthi, the objective of this study was to reconstruct the sequence of evolutionary transitions in the evolution of heterostyly and thus undertake a comprehensive test of Lloyd & Webb's (1992a,b) model. Specifically, we asked whether evolutionary transitions from stylar dimorphism to distyly are unlinked to shifts in self-incompatibility, and whether those are instead related to pollinator types. A second aim was to check if style polymorphism is lost, and whether this loss is associated with changes in self-incompatibility. All species were subject to experimental work to obtain comparable data on breeding systems and pollinator fauna relevant to evolution of heterostyly. These data were compared with previous results on style and perianth polymorphism of these species (Pérez *et al.*, 2004). Reproductive shifts in the course of evolution were investigated by means of phylogenetic analysis of plastid sequences of the *trn*T-*trn*L, *trn*L-*trn*F and *trn*Q-*trn*R spacers.

## Materials and Methods

### Study species

The seven species of *Narcissus* sect. Apodanthi grow at elevations ranging from 250 to 2500 m above sea level (a.s.l.) in the western Mediterranean. They typically occur in soil pockets in rocky habitats, on both acidic and basic soils. *Narcissus calcicola* Mendonça and *Narcissus scaberulus* Henriq. grow at lower elevations and start blooming in late January or early February. *Narcissus albimarginatus* D. Müll.-Doblies & U. Müll.-Doblies, *Narcissus cuatrecasasii* Fern. Casas, Laínz & Ruiz Rejón, *Narcissus rupicola* Dufour, M. Roem. & Schult. f., *Narcissus marvieri* Jahand. & Maire and *Narcissus watieri* Maire grow at elevations between 800 and 2500 m a.s.l. and flower from late February to early June depending on altitude and latitude (Table 1). The seven species are some of the earliest species blooming in Mediterranean communities, where they are frequently among the first to flower (Herrera, 1995). Additional information can be found in Pérez *et al.* (2004).

### Breeding system

Given that flower initiation frequently fails in common glasshouse conditions, we collected plants in the field with flower buds ready to open. Single plants were collected at least 1.5–2 m apart to avoid repetition of genotypes resulting from vegetative propagation. Plants were cultivated in pots with a mixture of peat and perlite (3 : 1) and generously watered. Previous observations and published studies showed that growing and flowering, once started, of most *Narcissus* species are largely insensitive to transplantation (Arroyo & Dafni, 1995; Barrett *et al.*, 1997; Arroyo *et al.*, 2002; Pérez-Barrales, 2005).

We performed self-pollinations for each morph (L-self and S-self ), and crosses within (L × L, S × S) and between (L × S,

**Table 1** Populations of *Narcissus* sect. Apodanthi, number of flowers used for study of incompatibility system and dates for pollinator censuses

| Species | Sex polymorphism | Population | Coordinates | Elevation (m.asl) | No. of hand pollinations* | | | Pollinator censuses |
|---|---|---|---|---|---|---|---|---|
| | | | | | | L | S | |
| *N. albimarginatus* | Distyly | 1. Morocco: Rif Mountains, Jebel Bouhachem | 35°14′49″N, 5°26′25″W | 1650 | (1) | 11 | 15 | 7–9 March 2004 |
| | | | | | (2) | 10 | 12 | |
| | | | | | (3) | 10 | 17 | |
| *N. cuatrecasasii* | Style dimorphism | 2. Spain: Jaén, Mancha Real | 37°46′36″N, 3°36′00″W | 900 | (1) | 27 | 9 | 20–21 March 2004 |
| | | | | | (2) | 30 | 13 | |
| | | | | | (3) | 17 | 12 | |
| | | 3. Spain: Jaén, Nava de las Correhuelas | 37°55′40″N, 02°52′06″W | 1600 | | – | – | 11–13 May 2003 |
| | | 4. Spain: Jaén, Cerrada de San Ginés | 38°04′23″N, 02°53′10″W | 1300 | | – | – | 8 April 2003 |
| | | 5. Spain: Jaén, Nacimiento del Guadalquivir | 37°50′25″N, 02°58′20″W | 1300 | | – | – | 9 April 2003 |
| *N. calcicola* | Style dimorphism | 6. Portugal: Leiria, Serra de Santo Antonio | 39°32′17″N, 8°32′26″W | 600 | (1) | 10 | 13 | – |
| | | | | | (2) | 12 | 13 | |
| | | | | | (3) | 17 | 17 | |
| *N. scaberulus* | Style dimorphism | 7. Portugal: Viseu, Oliveira do Conde | 40°26′14″N, 7°57′24″W | 250 | (1) | 21 | 10 | – |
| | | | | | (2) | 21 | 12 | |
| | | | | | (3) | 21 | 18 | |
| *N. rupicola* | Style dimorphism | 8. Spain: Ávila, Puerto de Mijares | 40°20′10″N, 4°48′40″W | 1450 | (1) | 17 | 16 | 17 May 2003; 23–24 April 2004 |
| | | | | | (2) | 16 | 16 | |
| | | | | | (3) | 16 | 16 | |
| | | 9. Spain: Ávila, Plataforma de Gredos | 40°17′5″N, 5°13′18″W | 1650 | | – | – | 23 April 2004 |
| | | 10. Spain: Madrid, Bola del Mundo | 40°47′5″N, 3°59′21″W | 2200 | | – | – | 18 May 2004; 6 June 2004 |
| | | 11. Spain: Ávila, Puerto del Pico | 40°19′07″N, 05°00′36″W | 1300 | | – | – | 23 April 2004 |
| | | 12. Spain: Madrid, Miraflores de la Sierra; Sierra de Cuerda Larga | 40°24′00″N, 03°46′00″W | 1900 | | – | – | 19 March 2004 |
| | | 13. Spain: Madrid, Colmenar Viejo, Cerro de San Pedro | 40°39′00″N, 03°46′00″W | 1400 | | – | – | 19 March 2004; 4, 8 and 9 April 2004; 5 and 13 April 2005 |
| *N. marvieri* | Style dimorphism | 14. Morocco: Taza, Jebel Tazzeka | 34°4′50″N, 4°10′41″W | 1720 | (1) | 8 | – | 1 March 2004 |
| | | | | | (2) | 9 | – | |
| *N. watieri* | Monomorphism | 15. Morocco: High Atlas, Oukaimeden | 31°12′42″N, 7°51′8″W | 2500 | (1) | 11 | – | 10 April 2001 |
| | | | | | (2) | 13 | – | |

*(1) number of self-pollinations (L-self, S-self); (2) number of within-morph cross-pollinations (L × L, S × S); (3) number of between-morph pollinations (L × S, S × L).

S × L) morphs in the five style dimorphic species, except *N. marvieri*, and the heterostylous species. In the monomorphic species (*N. watieri*) only self- and cross-pollinations were performed (Table 1). Despite previous reports of monomorphism in *N. marvieri* (Pérez *et al.*, 2004), two populations of this species have recently been reported to be style dimorphic; however, strongly L-biased morph ratios precluded study of S-plants (Pérez-Barrales, 2005). Flowers for hand cross-pollination were emasculated with fine forceps before anthesis. The hand pollination treatments were carried out 36 and 60 h (days 2 and 3) after emasculation, by brushing an entire dehiscent anther from two different individuals on the stigma. All pollinated flowers were bagged with mesh textile to prevent uncontrolled pollination.

Fully developed seeds and unfertilized ovules in each hand-pollinated flower were counted. We were unable to count ovules in *N. albimarginatus*, *N. calcicola* and *N. scaberulus* because of the ripe stage of the fruits and collapsed unfertilized ovules. In these cases, we used the mean value of ovules per flower (calculated in a sample of other fresh flowers of the same populations) to obtain seed:ovule ratios. Seed:ovule ratios after self-pollination and cross-pollinations were used to calculate the self-compatibility index according to Lloyd & Schoen (1992).

### Flower visitors and their pollination efficiency

Pollination censuses were performed in the same populations used for crossing programmes and in additional populations for some species (Table 1). We failed to obtain data for *N. calcicola* and *N. scaberulus* because of adverse weather conditions during three consecutive seasons. We chose high flower density plots of 10 $m^2$ for each population to increase the probability of observing visits. All observations and captures were done for insects that effectively visited a flower, i.e. they touched the anthers or stigma. A census was taken so as not to disturb visiting insects for a 15-min period, followed by a second census from a new position near to the plot, and then, after a 5-min break, in a different plot in the population. Simultaneous censuses in different parts of the populations were taken by different people to increase the total time effort. We were able to capture some insects during censuses in populations of *N. albimarginatus*, *N. cuatrecasasii* and *N. rupicola*. These captures were used both for insect identification and to check that the insects effectively act as pollen carriers for *Narcissus*. Insects were assigned to morpho-functional groups, according to their morphology, size and behaviour in the flowers. The visit rate to flowers was estimated in each population as the total number of recorded effective visits per time period. We used a white light trap (Kearns & Inouye, 1993) to determine if there were nocturnal visitors in some populations of *N. cuatrecasasii*, *N. rupicola* and *N. marvieri*. Trap-captured insects were considered effective visitors if they brought pollen on their bodies. Traps were set from GMT 18:00 h until 09.00 h the next day (see Table 2 for details).

Pollen grains were counted under a stereomicroscope (×50) on six parts of the insect body: (1) the head, (2) the dorsal and (3) the ventral sides of the thorax and abdomen, (4) the legs, (5) the wings and (6) the proboscis. We established two classes of pollen grain number (more or less than 50 pollen grains) for each body part. This number was chosen because it is the average number of ovules in a flower of *Narcissus*, i.e. 50 pollen grains is the minimum approximate number needed to fertilize all ovules in a flower.

### Statistical analyses

**Breeding systems** Differences in mean seed set (as a percentage of ovules siring seed) after different experimental treatments were tested by means of nonparametric analyses of variance (ANOVAs) on raw data. When only two treatments were compared in monomorphic species (L-self and L × L cross), Mann–Whitney *U*-tests were performed. For dimorphic species there were six treatments (see the 'Breeding system' section), and hence Kruskal–Wallis *H* was used. For specific comparison of means of seed set we planned *a priori* contrasts of particular biological significance. Thus, for dimorphic species we contrasted all selfed (L-self, S-self) vs intramorph (L × L, S × S) and intermorph (L × S, S × L) outcrossed seed set. For these comparisons, even if the ANOVA did not show overall significant differences, we used least squares means to carry out a *t*-test on arcsin-transformed data of seed:ovule ratio per flower (see Quinn & Keough, 2002, for details). Data on seed set for each treatment are shown as mean values and confidence intervals (α = 0.05) obtained by bootstrapping (20 000 runs) as this variable is not normally distributed (e.g. many flowers did not set fruit).

**Pollinator efficiency** Values for the numbers of pollen grains on body parts of insects were analysed by means of two two-way independence tables. First we examined the presence of pollen grains, with insect body parts and insect taxonomic orders as main factors. In a second analysis, pollen quantity (< 50 and > 50 pollen grains) and insect taxonomic order were the main factors. Association between factors was analysed using a *G*-test (Sokal & Rohlf, 1995).

### Phylogenetic analyses and character reconstruction

We obtained plastid DNA sequences using the laboratory protocols described in Dumolin-Lapegue *et al.* (1997) and modified in Pérez *et al.* (2004). As a result, *trn*L(UAA)-*trn*F(GAA), *trn*T(UGU)-*trn*L(UAA) and *trn*Q-R spacer sequences were obtained for the seven species of sect. Apodanthi (see Table 4 below). Given the low number of characters retrieved from *trn*Q-R sequences, a matrix of *trn*L-F and *trn*T-L sequences was analysed. Maximum parsimony (MP) and Bayesian inference (BI) analyses were then performed not only for this ingroup, as described in Pérez *et al.* (2004), but also for an extended sample of *trn*L-F and *trn*T-L sequences

**Table 2** Pollinator censuses performed in species of *Narcissus* sect. Apodanthi to quantify effective visits (insects touching any sex organs)

| Species | Population | Time effort | Flower visitors | | No. of visits | Visit rate (visits h$^{-1}$) |
|---|---|---|---|---|---|---|
| *N. albimarginatus* | 1 | 24 h | Hymenoptera | *Bombus* spp. | 6 | 4.42 |
| | | | | *Anthophora* spp. | 73 | |
| | | | | Unidentified solitary bees | 5 | |
| | | | | *Apis mellifera* | 22 | |
| *N. cuatrecasasii* | 4 | 1 h 45 min | Hymenoptera | *Anthophora* spp. | 3 | 2.86 |
| | | | | Unidentified solitary bees | 2 | |
| | 5 | 3 h 40 min | Hymenoptera | *Anthophora* sp. 1 | 5 | 1.36 |
| | 3 | 8 h | | *Anthophora* sp. 2 | 1 | 0.125 |
| | 2 | 23 h 45 min | Hymenoptera | *Anthophora* sp. 3 | 180 | 8.46 |
| | | | Lepidoptera | Several butterflies | 13 | |
| | | | | Hawkmoths | 8 | |
| *N. rupicola* | 9 | 7 h 15 min | Diptera | *Eristalis* sp. | 1 | 1.1 |
| | | | | Unidentified syrphids | 2 | |
| | | | Hymenoptera | Unidentified solitary bees | 5 | |
| | 11 | 3 h 15 min | Diptera | Unidentified syrphids | 1 | 1.23 |
| | | | | Unidentified solitary bees | 3 | |
| | 8 | 20 h 15 min | Diptera | *Eristalis* spp. | 57 | 3.9 |
| | | | | Unidentified syrphids | 14 | |
| | | | Hymenoptera | Unidentified solitary bees | 5 | |
| | | | Lepidoptera | Hawkmoths | 3 | |
| | 10 | 11 h | Diptera | *Eristalis* spp. | 9 | 22.6 |
| | | | | Unidentified syrphids | 8 | |
| | | | Hymenoptera | *Anthophora* spp. | | |
| | | | | Unidentified solitary bees | 13 | |
| | | | | *Apis mellifera* | 2 | |
| | | | Lepidoptera | Several butterflies | 11 | |
| | | | | Hawkmoths | 206 | |
| | 12 | 2 h | Hymenoptera | *Apis mellifera* | 1 | 0.5 |
| | 13 | 12 h 20 min | Lepidoptera | Hawkmoths | 24 | 1.98 |
| *N. marvieri* | 14 | 12 h | Hymenoptera | Anthophoridae | 32 | 2.83 |
| | | | | *Eucera* sp. | 2 | |
| *N. watieri* | 15 | 4 h | Diptera | Other syrphids | 5 | 3.5 |
| | | | Lepidoptera | Several pierid butterflies | 3 | |
| | | | | Hawkmoths | 6 | |

For population numbers and location and dates of censuses, see Table 1.

of the tristylous *N. triandrus* complex. All parsimony analyses were conducted using Fitch parsimony (as implemented in PAUP*; Swofford, 1999) with equal weighting of all characters and of transitions/transversions. Branch and bound analyses were performed to obtain optimal trees in exhaustive searches following initial heuristic methods. Internal support was assessed using 1000 replicates of full bootstrapping. Bayesian phylogenetic inference was computed with MRBAYES 3.04b4 software (Ronquist & Huelsenbeck, 2003). To determine the simplest evolutionary model for sequences to fit the data set the following criteria were implemented: hierarchical likelihood ratio test (HLRT) and Akaike information criterion (AIC) (Posada & Crandall, 2001; Nylander, 2002). Bayesian inference was obtained after $10^6$ generations and the following parameters: four Markov chain Monte Carlo (MCMC) samplings, chain temperature = 0.2, sampling frequency = 100, and burn-in < 500. A 50% majority-rule consensus tree was calculated using a random sampling and the *sumt* command, which gives a final Bayesian estimation of

the phylogeny. Posteriori probability (PP) values were included as an alternative estimate of branch robustness (Alfaro *et al.*, 2003).

We made a particular effort to obtain plastid sequences for the same populations of the seven species of sect. Apodanthi used to investigate breeding systems and pollinator visits. To reconstruct character evolution on the most plausible phylogeny we chose the MP tree congruent with the BI analysis. Patterns of evolution were explored using the character-state optimization function of MACCLADE 4.06 (Maddison & Maddison, 1999), assuming Fitch parsimony. Character reconstruction of breeding systems and predominant pollinators was explored in MACCLADE by implementing ACCTRAN (which maximizes reversion events), DELTRAN (which maximizes parallelism events) and 'all most parsimonious states' (which implements both ACCTRAN and DELTRAN) optimizations (Maddison & Maddison, 1999). Breeding system data for outgroup species were taken from the literature and unpublished data (see Table 4 for further details). Two classes were reconstructed

**Table 3** Results of tests of differences in seed set among all hand-pollination treatments in all species of *Narcissus* sect. Apodanthi for analysis of the incompatibility system and some specific *a priori* planned comparisons between some particular treatments

| Species | Overall differences between means | | Planned comparisons | |
| --- | --- | --- | --- | --- |
| | Statistic | df | Self vs outcross (*t*) | Inter- vs intramorph outcross (*t*) |
| *N. albimarginatus* | 16.48 (*H*)** | 5 | 4.30*** | −0.69 ns |
| *N. cuatrecasasii* | 17.09 (*H*)** | 5 | 3.95*** | 1.52 ns |
| *N. calcicola* | 22.63 (*H*)*** | 5 | 5.63*** | −0.43 ns |
| *N. scaberulus* | 25.41 (*H*)*** | 5 | 4.84*** | −0.88 ns |
| *N. rupicola* | 4.38 (*H*) ns | 5 | 0.94 ns | −0.53 ns |
| *N. marvieri* | −1.78 (*U*)* | 1 | – | – |
| *N. watieri* | −1.13 (*U*) ns | 1 | – | – |

*H*, Kruskall–Wallis tests for species with six treatments; *U*, Mann–Whitney tests for species with only two treatments (see text for explanations of different hand-pollination treatments).
df, degrees of freedom; ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

for breeding systems: self-incompatibility and self-compatibility. *Narcissus* species were classified according to predominant pollinators in censuses as follows: (1) syrphids and lepidopterans and (2) solitary bees. When *N. marvieri* was properly coded as dimorphic, analysis of style polymorphism under 'all most parsimonious states' optimization retrieved a similar reconstruction as that presented in Pérez *et al.* (2004; see their fig. 6).

## Results

### Breeding systems

Five species are mostly self-incompatible (*N. albimarginatus, N. cuatrecasasii, N. calcicola, N. scaberulus* and *N. marvieri*), as there were significant differences in seed set between selfed and outcrossed treatments in planned comparisons (Table 3 and Fig. 1). All the species showed some (although low) seed set after self-pollination, which ranged from 0.8% in *N. scaberulus* to 7.7% in *N. albimarginatus* (Fig. 1). These dimorphic species showed low values for the self-compatibility index, ranging from 0.03 (*N. scaberulus*) to 0.24 (*N. marvieri*). All dimorphic self-incompatible species showed intramorph compatibility, as all intramorph cross-pollinations showed similar seed set to intermorph cross-pollinations. Two species are self-compatible, the style-dimorphic *N. rupicola* (self-pollination, 24.6%; cross-pollination, 28.5%) and the monomorphic *N. watieri* (self-pollination, 46.7%; cross-pollination, 52.1%). The self-compatibility index was high for these two species: 0.86 for *N. rupicola* and 0.90 for *N. watieri*.

### Flower visitors and pollination efficiency

The populations of the different species of *Narcissus* sect. Apodanthi for which censuses were performed presented different pollinator compositions (Table 4). The primary visitors

of *N. albimarginatus* and *N. cuatrecasasii* flowers were solitary bees, mostly *Anthophora* sp., and to a lesser extent other solitary bees and *Bombus* spp. In both *Narcissus* species these bees collected pollen and nectar. Most *Anthophora* bees were males, which marked flowers and patrolled a territory delimited by these flowers. Honey bees (*Apis mellifera*) contributed to the pollinator fauna of *N. albimarginatus* (*c.* 20% of visits) and collected only pollen, although they are probably a consequence of common bee hiving of rural settlers. In *N. cuatrecasasii*, diurnal and crepuscular Lepidoptera, and occasionally small syrphid flies, contributed to less than 8% of pollinator fauna (see Fig. 2). *Narcissus marvieri* had as main flower visitors anthophorid bees, including some of the genus *Eucera* (Fig. 2). *Narcissus rupicola* showed a pollinator array more diverse than that of the *N. marvieri*. Insects with a long proboscis (butterflies and moths) accounted for > 60% of visits. Syrphid flies and small bees made a smaller contribution of 30%, and collected only pollen in these flowers. By contrast, solitary bees of medium to large size were almost irrelevant (Fig. 2). *Narcissus watieri* presented an array that was functionally similar to that of *N. rupicola*, given that moths and pierid butterflies were the main pollinators, although there was also a high frequency of syrphid flies. Short-tongued insects only collected pollen in all these species as a result of their long flower tubes. Hence, these two self-compatible species showed morpho-functional pollinator arrays (either short- or long-proboscis insects) different from those of self-incompatible species, which are dominated mostly by bees of medium tongue length (Fig. 2). In all species, visitation rates were very low. The lowest rate occurred in *N. cuatrecasasii* (0.125 visits h$^{-1}$) and the highest in *N. rupicola* (22.0 visits h$^{-1}$). In general, the rates calculated from the censuses were approx. 1–4 visits h$^{-1}$ (Table 4).

Insects captured during censuses belonged to three orders: Hymenoptera, Diptera and Lepidoptera (diurnal). Identification of these insects is included in the Appendix (Table A1).
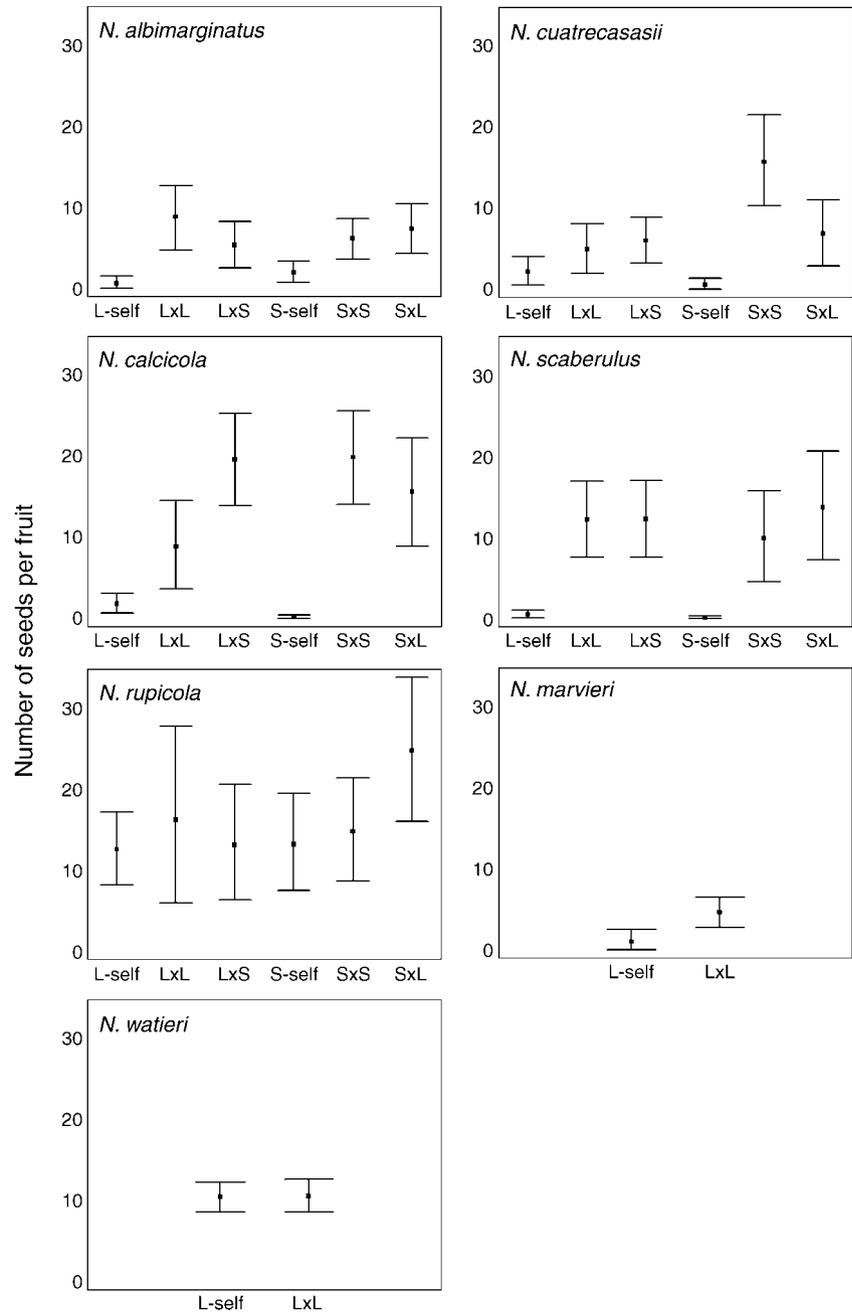
**Fig. 1** Mean seed set in long-styled (L) and short-styled (S) morphs of all species of *Narcissus* sect. Apodanthi after hand pollination. Pollination treatments were: L-self, S-self, L × L, S × S within-morph cross-pollinations, and L × S, S × L between-morph cross-pollinations. The mean ± the confidence interval is shown (obtained by bootstrapping after 20 000 runs at $\alpha = 0.05$).

Most Hymenoptera were *Anthophora* males, Diptera were mostly syrphid flies of genus *Eristalis*, and most Lepidoptera were diurnal moths (*Macroglossum stellatarum*). Light traps in populations of *N. cuatrecasasii*, *N. rupicola* and *N. marvieri* captured eight, one and 14 insects, respectively. They were moths (17) and Trichoptera (six). The latter were found in Moroccan (population 14; Jebel Tazzeka) *N. marvieri* and Spanish (population 2; Mancha Real) *N. cuatrecasasii* populations. Only 13 (seven moths and six Trichoptera) of the 23 insects captured carried pollen of *Narcissus* on their bodies, but their abundance was unrelated to flower tube length

(*N. rupicola* had the longest tubes and the lowest frequency of these insects).

The number of pollen grains found on insect bodies was counted to provide a measure of their pollination potential, while the body part where pollen was carried was noted as this may indicate a fit between insect parts and flower sex organs. A significant association among pollen, taxonomic insect order and body part was found when only presence or absence of pollen grains was considered [$G = 13.5214$, degrees of freedom (df) $= 10$, $P < 0.005$]. Dipterans tended to carry pollen mostly on the ventral thorax and abdomen,

**Table 4** Plant material of species of *Narcissus* sect. Apodanthi and outgroups for the analysis of *trn*L-*trn*F, *trn*T-*trn*L, and *trn*Q-R sequences, with a summary of breeding systems, style-length variation, and predominant pollinator types for every population

| Species (source) | Population | Plastid sequences: *trn*L-F *trn*T-L *trn*Q-R | Incompatibility system | Style polymorphism | Predominant pollinator classes |
|---|---|---|---|---|---|
| Outgroup species | | | | | |
| *N. papyraceus* (1) | Aznalcázar (Spain) | DQ490224 AY481579 – | Self-incompatible | Style dimorphic | Syrphids and lepidopterans |
| *N. bulbocodium* (2) | Cartaya (Spain) | AY490181 AY481578 – | Self-incompatible | Monomorphic | Bees |
| *N. triandrus* (3) | Zamora (Spain) | AY490199 DQ485969 – | Self-incompatible | Heterostylous (tristylous) | Bees |
| *N. pallidulus* (3) | Aldeaquemada (Spain) | AY490198 DQ485970 – | Self-incompatible | Heterostylous (tristylous) | Bees |
| *N. lusitanicus* (3) | Pena Cova de Oliveira (Portugal) | AY490200 DQ485971 – | Self-incompatible | Heterostylous (tristylous) | ? |
| Species in sect. Apodanthi | | | | | |
| *N. albimarginatus* (4) | Djebel Bouhachem (Morocco) | AY490177 AY481580 DQ490227 | Self-incompatible | Heterostylous (distylous) | Bees |
| *N. calcicola* (4) | Santo Antonio (Portugal) | AY490185 DQ485972 DQ490228 | Self-incompatible | Style dimorphic | ? |
| *N. cuatrecasasii* (4) | Sierra Mágina (Spain) | DQ490225 DQ485967 | Self-incompatible | Style dimorphic | Bees |
| | Sierra de Grazalema (Spain) | DQ490232 | | | |
| *N. marvieri* (4) | Djebel Tazzeka (Morocco) | AY490188 DQ485968 | Self-incompatible | Style dimorphic | Bees |
| | Djebel Zerekten (Morocco) | DQ490229 | | | |
| *N. rupicola* (4) | Gredos (Spain) | DQ490226 DQ485966 | Self-compatible | Style dimorphic | Syrphids and lepidopterans |
| | Bola del Mundo (Spain) | DQ490231 | | | |
| *N. scaberulus* (4) | Ervedal (Portugal) | AY490183 AY481585 DQ490233 | Self-incompatible | Style dimorphic | ? |
| *N. watieri* (4) | Oukaimeden (Morocco) | AY490189 AY481583 DQ490230 | Self-compatible | Monomorphic | Syrphids and lepidopterans |

Sources for incompatibility system and pollinators of the species: (1) Arroyo *et al.* (2002) and Pérez-Barrales (2005); (2) Bateman (1954), Barrett *et al.* (1997) and L. Navarro (pers. comm.); (3) Barrett *et al.* (1997) and C. Gomez & J. Arroyo (unpublished data), and (4) Pérez-Barrales (2005). GenBank accession numbers are given for plastid sequences.

lepidopterans on their head, ventral thorax, abdomen and proboscis, and Hymenopterans equally on all parts of their bodies. When the amount of pollen was considered (in two classes; more and less than 50 pollen grains), a significant association between insect order and amount of pollen was also found ($G = 101.6350$, df = 2, $P < 0.001$). Dipterans varied in the amount of pollen they transported, lepidopterans carried mostly pollen loads of less than 50 grains, and Hymenopterans loaded in general more than 50 grains (Fig. 3).

### Phylogeny and character reconstruction

*trn*T-L sequences of 17 new accessions reached similar variation values for *Narcissus* sect. Apodanthi as those described in Pérez *et al.* (2004). The three accessions of the *N. triandrus* complex displayed relatively high degrees of pairwise sequence divergence (0.31–0.60% Kimura 2-parameter distance) and pairwise nucleotide variation (four substitutions and an interesting 32-bp indel) for this marker. Only 100 bp at the 3′ end from the *c.* 3.5 kb of the *trn*Q-R spacer was obtained for sect. Apodanthi
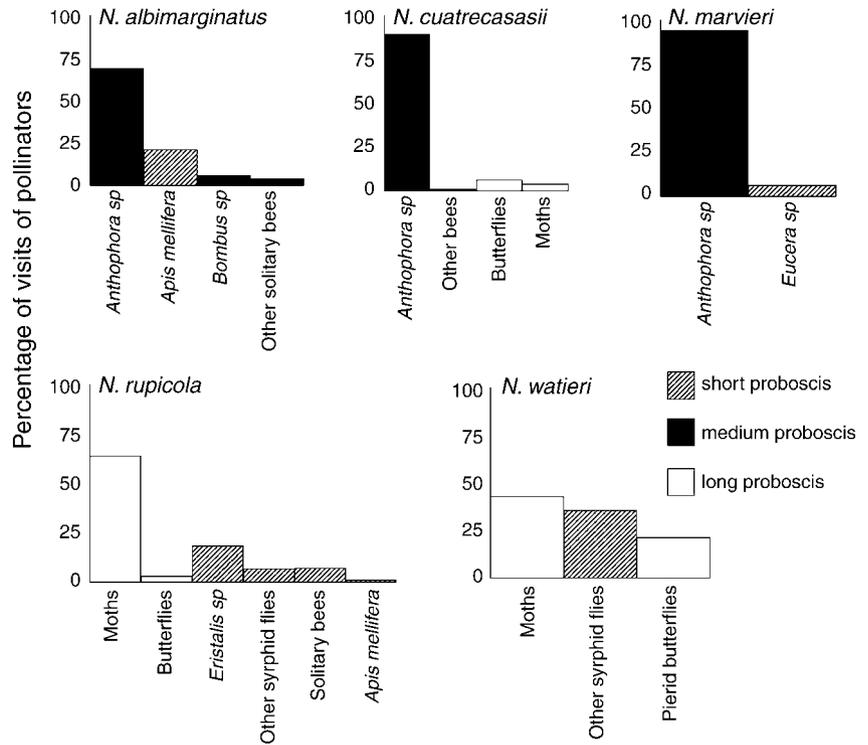
**Fig. 2** Percentage of pollinator visits to flowers of species of *Narcissus* sect. Apodanthi (see also Table 4).
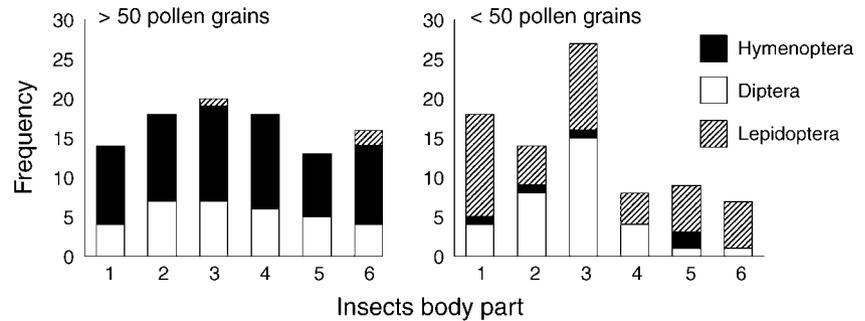


**Fig. 3** Amount of pollen grains on body parts of flower visitors of *Narcissus* sect. Apodanthi: 1, head; 2, upper thorax and abdomen; 3, lower thorax and abdomen; 4, legs; 5, wings; 6, proboscis.

populations, as a result of a poly A sequence of more than 10 bp that prevented the retrieval of an unequivocal chromatogram. We found two shared substitutions in the 5′-end fragment in *N. scaberulus* and *N. calcicola* (results not shown). No further variation was found in the *trn*L-F sequences herein presented and not included in Pérez *et al.* (2004) (Table 4).

Cladistic analysis of combined *trn*L-F and *trn*T-L sequences resulted in three most parsimonious trees (consistency index, CI = 0.88, excluding noninformative characters; retention index, RI = 0.93), which differed only in the relative position of *N. bulbocodium* (Fig. 4). The most likely of the shortest trees was chosen based on congruence with the BI tree under the simplest model of sequence evolution (GTR + G) obtained with MODEL-TEST (Posada & Crandall, 1998). This tree is also in agreement with the phylogenetic hypothesis of Graham & Barrett (2004) based on *ndh*F and *trn*L-F sequences. The three species of the *N. triandrus* group and the seven of the sect. Apodanthi form two

well-defined monophyletic groups. The evolution of all most parsimonious states for the breeding system on the most plausible phylogeny is illustrated in Fig. 5(a), and reconstruction of pollinator type as classified into two classes is illustrated in Fig. 5(b).

## Discussion

The analyses performed in the present study, coupled with previous and complementary results (Pérez *et al.*, 2004), provide significant support for the Darwinian hypothesis of the determinant role played by pollinators in flower evolution. In particular, a quantitative model, as reformulated by Lloyd & Webb (1992b), is supported for *Narcissus* considering the three main components of the model in a phylogenetic context: style polymorphism, breeding systems and pollinator efficiency. Previous studies which focused on style polymorphism and perianth morphology to infer pollinator type suggested
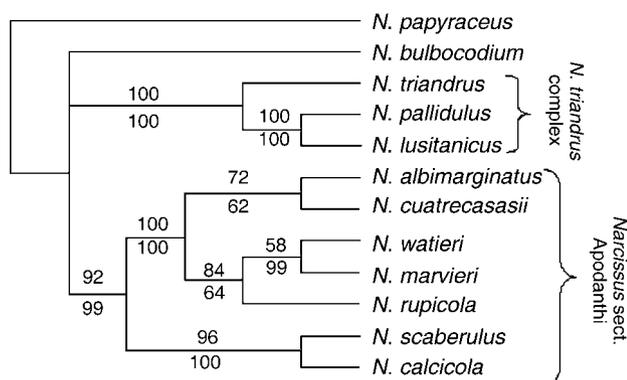
**Fig. 4** Strict consensus tree of 131 steps (consistency index, CI = 0.88; retention index, RI = 0.93) from the combined analysis of *trn*L-*trn*F and *trn*T-*trn*L sequences. *Narcissus papyraceus* served as the outgroup taxon based on a previous reconstruction (Graham & Barrett, 2004). Numbers above branches are bootstrap values. Numbers below branches show posterior probabilities from the Bayesian analysis under the GTR + *G* model of DNA substitution selected by MODELTEST 3.06 (Posada & Crandall, 1998).

that approach herkogamous monomorphism precedes style dimorphism in the course of evolution in *Narcissus*, followed by distyly and/or tristyly (Graham & Barrett, 2004; Pérez *et al.*, 2004). Our results are congruent with this sequence of style evolution, providing further support for the idea that style dimorphism is ancestral to heterostyly, and that the evolutionary transition is more closely related to changes in pollinators than breeding system evolution.

### Self-incompatibility in *Narcissus*

Barriers to selfing as revealed by our experiments in *Narcissus* sect. Apodanthi agree, to a great extent, with the incompatibility system reported for other *Narcissus* species (Bateman, 1954; Dulberger, 1964; Barrett *et al.*, 1997; Sage *et al.*, 1999; Baker *et al.*, 2000b; Arroyo *et al.*, 2002). *Narcissus albimarginatus, N. cuatrecasasii, N. calcicola, N. scaberulus* and *N. marvieri* are primarily self-sterile, in congruence with no link between the incompatibility system and style polymorphism (Table 4); crosses within and between morphs set equivalent number of seeds. All self-incompatible species had values of self-compatibility index much lower than 0.75, whereas self-compatible species had higher values, as expected according to this cut-off rule proposed by Lloyd & Schoen (1992) to discriminate between self-compatible and self-incompatible species. However, different seed-set success was retrieved in some intra- and intermorph crossings without any particular direction (Fig. 1). Morph-inconstant skewness is probably unrelated to morph-specific differences in incompatibility (but see Ornduff, 1988) and probably reflects some uncontrolled effects during sampling or experimental pollination, for example biparental inbreeding as a result of genetic substructuring of source populations, especially those that are small (cf. Hodgings & Barrett, 2006), or differential dichogamy between morphs, as reported for
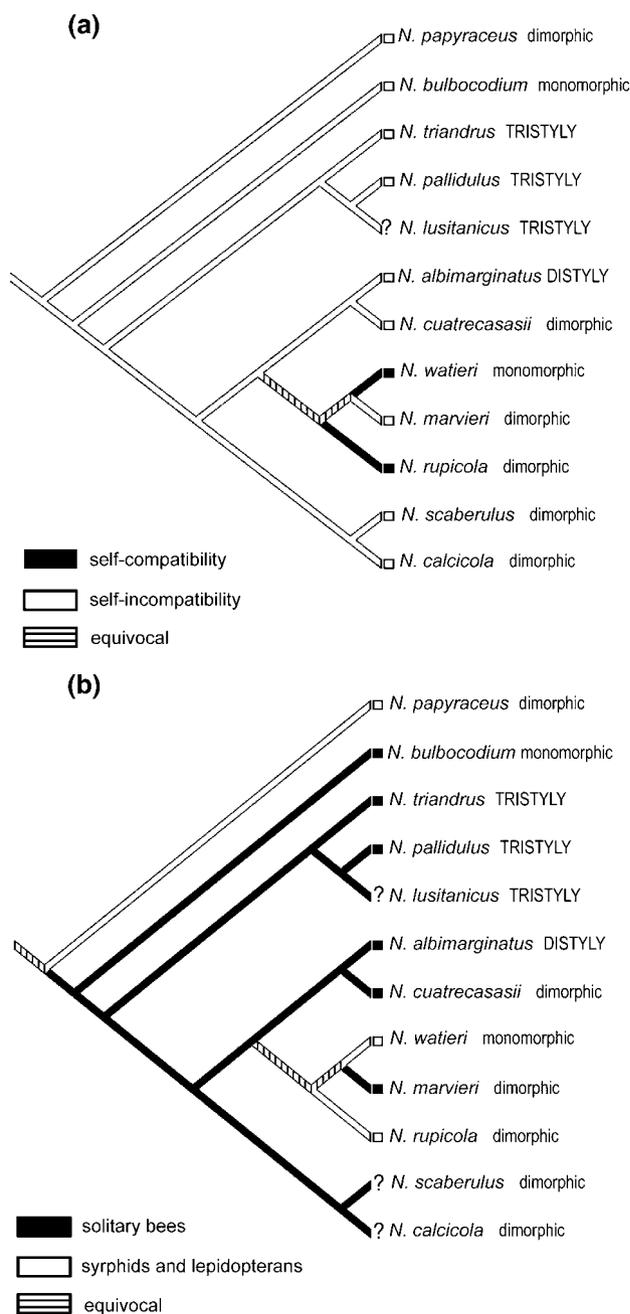


**Fig. 5** Hypothesis of character evolution for incompatibility systems (a) and predominant pollinator types in two classes (b). This maximum parsimony (MP) tree of the combined analysis of *trn*L-*trn*F and *trn*T-*trn*L sequences chosen for character reconstruction, onto which the two characters have been mapped, is congruent with the Bayesian inference (BI) tree (see text) and Graham & Barrett (2004). Character reconstruction was obtained after implementing the 'all most parsimonious states' optimization of MACCLADE (Maddison & Maddison, 1999), which is the least restrictive implementation. The stylar polymorphism condition is marked for all species. Note that reconstruction of pollinator types is coded as missing data (?) for *Narcissus calcicola, Narcissus lusitanicus* and *Narcissus scaberulus*.

*N. assoanus* (Cesaro *et al.*, 2004). Self-sterility in these apparently self-incompatible species could also be achieved by the effects of inbreeding depression. Although the results reported in this study agree with those for a few self-incompatible *Narcissus* species investigated in detail (particularly *N. tazetta* by Dulberger, 1964, and *N. triandrus* by Sage *et al.*, 1999), it would be desirable to carry out an appropriate crossing programme and a developmental study to confirm these findings.

*Narcissus rupicola* and *N. watieri* are predominantly self-compatible, as reported in a few other *Narcissus* species (Baker *et al.*, 2000b; Medrano *et al.*, 2005, but see also reviews in Bateman, 1954, and Barrett *et al.*, 1996). Both species are Mediterranean mountain species distributed at the highest elevations (up to 2500 m a.s.l.) and occur in either the most northern (*N. rupicola*) or southern (*N. watieri*) parts of the geographic range of sect. Apodanthi, where weather conditions are harsh during their early blooming season and pollinator reliability may be low. Under such conditions, the loss of incompatibility may have been selected via reproductive assurance (Baker, 1955). By contrast, the closely related *N. marvieri* is mostly self-incompatible, at least for the L-morph. Further searches for populations large enough to provide a significant number of S-morph plants are needed to test whether both morphs are self-incompatible in this species, as in all other self-incompatible dimorphic *Narcissus* species tested to date (Barrett & Harder, 2005).

## Pollinators

The results of insect observation satisfied the predictions of the theoretical model of Lloyd & Webb (1992a,b). In sect. Apodanthi, species with different flower morphology and stylar polymorphism (see Pérez *et al.*, 2004) have different pollinator fauna (Table 4). Males of *Anthophora* sp. were the main flower visitors in the distylous *N. albimarginatus* (68% of all visits) and in the style-dimorphic *N. cuatrecasasii* (60% of visits), in which there is a relatively high degree of reciprocity of stigma and anther heights (Fig. 2), as hypothesized in previous work (Arroyo & Barrett, 2000; Graham & Barrett, 2004; Pérez *et al.*, 2004).

Medium-proboscis bees play an important role in one more species. Data on pollinator fauna visiting *N. marvieri* (over 95% of anthophorid visits) are partial and refer to a peculiar, isolated population in Morocco (Jebel Tazekka), which has a mean flower-tube length (14.1 ± 0.2 mm) much shorter than that of the remaining populations in the Atlas Mountains (Djebel Zerekten, 24.0 ± 0.3 mm; Tizi-n-Ifar, 24.9 ± 0.2 mm). The flower morphology of typical populations of *N. marvieri* from the Atlas Mountains is very similar to that of long-tubed *N. rupicola* (see next paragraph); in fact, some authors considered the former as a mere subspecies of the latter (Maire, 1959). We do not expect a pollinator fauna dominated by bees in long-tubed populations of *N. marvieri*, a prediction to be tested in further research on the possible effect of change of pollinators on flower morphology at the within-species level (e.g. Arroyo & Dafni, 1995).

Moths and butterflies were the main flower visitors in *N. rupicola* and *N. watieri*. These long-tongued insects are nectar-feeders and contributed in both cases to > 60% of the visits. In fact, the maximum flower tube lengths of these species (*N. rupicola*: 23.0 mm; *N. watieri*: 27.2 mm) (see Pérez *et al.*, 2004) fit well with the mean proboscis length of one of their most frequent visitors (*Macroglossum stellatarum*: 27.6 mm; V. González, pers. comm.). Short-proboscis insects (mostly syrphids) contributed to the remaining insect visits, especially in *N. watieri*. These insects only collect pollen from the upper-anther whorl as the floral tube is too long and narrow for them to access nectar at the base of the tube.

Our data on nocturnal insects are partial and not directly comparable with diurnal censuses. The number of insects captured was very low and only a small fraction of insects carried pollen, which means that they are probably much lesser important visitors than diurnal insects (but see Vogel & Muller-Doblies, 1975, and Pérez-Barrales, 2005 for other *Narcissus* species). They were moths (Noctuidae, Geometridae and Depressaridae) and Trichoptera. It is worth noting the presence of Trichoptera carrying *Narcissus* pollen in one Moroccan (*N. marvieri*) and one Spanish (*N. cuatrecasasii*) species. This has rarely been documented (Petterson & Hasselrot, 1994; D. Inouye, pers. comm.) and their relative proficiency as pollinators remains an open question.

In general, bees are more efficient than lepidopterans in terms of amount of pollen grains transferred (Herrera, 1987; Hiei & Suzuki, 2001). The bees we captured usually transported more than 50 pollen grains, while lepidopterans and syrphids carried fewer than 50 grains. However, it is also necessary to know where an insect carries pollen and its accuracy when touching sexual whorls, to assess its role as an actual pollinator (Armbruster *et al.*, 1994, 2006). Most captured bees were males transporting pollen on all their body parts, whereas the other pollinators transported pollen on a narrower range of their body parts. Lepidopterans only introduce the proboscis to seek nectar and syrphids only feed on pollen from the upper anthers. Heterostylous species need pollinators to carry pollen of different morphs on different body parts in order to promote disassortative pollination (see Lloyd & Webb, 1992a, and references therein). Among all pollinators reported in this study, bees have the highest potential to transfer pollen between morphs. However, the lack of pollen dimorphism in *Narcissus* precludes direct estimation of the pattern of pollen transfer. It would be beneficial to carry out experimental studies, for example using different fluorescent dyes to track pollen movement, emasculations in controlled arrays (Cesaro & Thompson, 2004) or molecular markers. We hypothesize that pollen carried by bees on different body parts comes from stamens at different heights in the distylous *N. albimarginatus* and the close-to-reciprocal *N. cuatrecasasii* (Pérez *et al.*, 2004). Whereas intermorph pollen transfer cannot by achieved by syrphids, the role of Lepidoptera remains more complicated (Stone, 1996).

### Testing the Darwinian hypothesis of heterostyly

The present study brings further evidence to bear on Lloyd & Webb's (1992a,b) predictions of the importance of pollinators, as Darwin (1877) first suggested, for the evolution of heterostyly. Differences in the pollinator composition of species with different floral polymorphisms and the lack of association between style polymorphisms and incompatibility systems are the key elements of our results here. Style polymorphism is present in both self-compatible and self-incompatible species (Table 4). Accordingly, the incompatibility systems appear to be unlinked to genes responsible for style polymorphism (reviewed in Barrett & Harder, 2005). Taking the most reliable phylogeny into consideration (Fig. 4), it is plausible that self-incompatibility is the ancestral state, a situation that has been maintained in the three lineages of *Narcissus* sect. Apodanthi, irrespective of evolution of style polymorphism. Lack of correspondence between the evolutionary histories of the incompatibility system and style polymorphism (Fig. 5a) supports the view that the incompatibility is not critical for evolution of stylar polymorphisms, both for heterostyly (*N. albimarginatus*) and for style dimorphism (*N. cuatrecasasii, N. scaberulus* and *N. calcicola*). Joint occurrence of stylar dimorphism and extensive self-compatibility (*N. rupicola*) provides additional and strong support that incompatibility is not necessary to maintain style polymorphism. Loss of self-incompatibility is clearly derived from ancestral self-incompatible lineages in *N. rupicola*.

One unexpected result was obtained from the evolutionary reconstruction of the breeding system. We detected one possible reversion event to achieve incompatibility in the course of speciation of *N. marvieri*, the sister species to the self-compatible *N. watieri* (suggested from maximizing reversal events with MACCLADE – ACCTRAN). Alternatively, we retrieved two reversals to compatibility as maximizing parallelism (DELTRAN) (results not shown). This situation is puzzling, because it is fairly widely accepted that the evolution of selfing is quasi-irreversible (see Takebayashi & Morrell, 2001 for a review, and Igic *et al.*, 2004, 2006 for a well-documented case, but see also Routley *et al.*, 2004). Joint loss of heterostyly and self-incompatibility has been frequently reported (e.g. Kohn *et al.*, 1996; Schoen *et al.*, 1997; Truyens *et al.*, 2005) as in all these cases style polymorphism and self-incompatibility are linked. If the loss of self-incompatibility is caused by modifiers unlinked to the S-locus, a reversion to self-incompatibility is possible, but is not believed to have occurred in either at population or phylogenetic levels (e.g. Solanaceae; Stone, 2002; Igic *et al.*, 2006). An alternative explanation is that of population heterogeneity of breeding systems and style polymorphisms in the clade *N. marvieri-N. watieri-N. rupicola*, which could include both self-compatible and self-incompatible populations (see Busch, 2005). In this scenario, the ancestral condition to this clade would be self-incompatibility and style dimorphism, and loss of both conditions would have

occurred only in some populations within each species. A more extensive population sample would be needed to investigate whether these three species do have self-incompatible and compatible populations, perhaps related to different degrees of herkogamy and dichogamy (Cesaro *et al.*, 2004; Routley *et al.*, 2004), and both style-dimorphic and monomorphic populations as reported in other *Narcissus* species (Arroyo & Dafni, 1995; Arroyo *et al.*, 2002; Baker *et al.*, 2000a).

If the evolution of incompatibility systems is not critical for maintaining style polymorphism, what is the evolutionary force underlying it? According to Lloyd & Webb (1992b), when the heteromorphic incompatibility system is not present, only higher levels of disassortative than assortative pollen transfer can maintain style dimorphism. Disassortative transfer is higher as the morph ratios approach equality (isoplethy). This hypothesis has positively been tested experimentally in style-dimorphic *N. assoanus* (Cesaro & Thompson, 2004). There are two main factors determining disassortative pollen transfer: reciprocity of anthers and stigma, and pollinator behaviour. Pérez *et al.* (2004) analysed the former in sect. Apodanthi, and we have focused in this paper on the latter. The most reciprocal species, distylous *N. albimarginatus* and style-dimorphic *N. cuatrecasasii* (Pérez *et al.*, 2004), have the same pollinators, bees, as the other heterostylous group in the genus (*N. triandrus* complex; C. Gomez, unpublished data), and these are very different from other species in sect. Apodanthi. A particularly puzzling feature of our results is that both self-compatible species showed contrasting nearly isoplethic style dimorphism (*N. rupicola*; 38–53% of S-plants) and monomorphism (*N. watieri*), but had similar pollinators (long- and short-proboscis insects). Pollinator information for *N. rupicola* is reliable in terms of population number, time effort and nocturnal captures and diurnal observations, but that for *N. watieri* is limited. It has been argued that long-tongued insects (i.e. Lepidoptera) may be responsible for maintenance of style-height dimorphism (Cesaro & Thompson, 2004). In contrast, short-tongued, pollen-feeding insects (mostly syrphids) may favour intramorph pollination of the L-morph, and virtually no pollination of the S-morph, thus producing morph ratio bias and possible fixation of the former in morph-compatible species (Arroyo & Dafni, 1995; Pérez-Barrales, 2005). Perhaps the relative numbers of short- and long-tongued insects in *N. watieri* allow this process to operate by syrphid activity, aided by self-compatibility (a syrphid may allow easy self-pollination of L-flowers) and a flower that is attractive to syrphids: white flat tepals with a yellow central dot (upper stamens) (Dinkel & Lunau, 2001). Again, more research in different populations of these two critical species to ascertain breeding systems, pollinators and pollen transfer patterns throughout their ranges would provide critical information on the maintenance and dissolution of style dimorphism.

Lloyd & Webb (1992a, p. 174) specifically stated that 'all attempts to date to uncover the evolutionary origins of

heterostyly have been hindered by ignorance of the exact phylogenetic relationships among the species in taxa with heterostylous representatives'. Our results using both phylogenetic information and data on breeding systems and pollinators support the idea that the evolution of flower polymorphism in *Narcissus* is mediated by the pollinator fauna. Our finding that there is a disassociation between breeding systems and style polymorphism in *Narcissus* suggests that more work on the role of pollinator shifts for the evolution of flower polymorphisms is warranted. Additionally, the common joint presence of reciprocal herkogamy and heteromorphic self-incompatibility in most heterostylous groups remains an unresolved problem in the Lloyd & Webb (1992a,b) model, which requires further investigation in a phylogenetic context; for example, do ancestral taxa consistently suffer from some inbreeding which promotes self-incompatibility?

This study supports the idea of divergent natural selection in *Narcissus* mediated by the pollinator fauna. The consistency of the association between style polymorphism and pollinators in *Narcissus* and other taxa will test the generality of Darwin's predictions about the role played by pollinator variation in flower evolution.

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## Appendix

**Table A1** Diurnal and nocturnal insects captured in *Narcissus* sect. Apodanthi populations

| Order | Insect species | Population | *Narcissus* species |
|---|---|---|---|
| Diptera | *Brachypalpus* sp. | Jebel Bouhachem, Morocco | *N. albimarginatus* |
| | *Chrysotoxum vernale** | Puerto de Mijares, Spain | *N. rupicola* |
| | *Criorhina* sp. | Jebel Bouhachem, Morocco | *N. albimarginatus* |
| | *Eristalis similis** | Puerto de Mijares, Spain | *N. rupicola* |
| | *Eristalis tenax** (7) | Puerto de Mijares, Spain | *N. rupicola* |
| | *Eupeodes corollae** (2) | Puerto de Mijares, Spain | *N. rupicola* |
| | *Eupeodes luniger** | Jebel Bouhachem, Morocco | *N. albimarginatus* |
| | *Platycheirus albimanus** | Puerto de Mijares, Spain | *N. rupicola* |
| Hymenoptera | *Andrena* sp., female* (2) | Jebel Bouhachem, Morocco | *N. albimarginatus* |
| | *Andrena* sp., female* | Puerto de Mijares, Spain | *N. rupicola* |
| | *Anthophora dispar*, male* (3) | Jebel Bouhachem, Morocco | *N. albimarginatus* |
| | *Anthophora dispar*, male* (6) | Sierra Mágina, Spain | *N. cuatrecasasii* |
| | *Anthophora acervorum* (= *plumipes*)* (3) | Sierra Mágina, Spain | *N. cuatrecasasii* |
| | *Bombus terrestris*, male* | Sierra Mágina, Spain | *N. cuatrecasasii* |
| Lepidoptera | *Conistra staudingeri* (Noctuidae)* | Jebel Tazzeka, Morocco | *N. marvieri* |
| | *Conistra staudingeri* (Noctuidae) | Plataforma de Gredos, Spain | *N. rupicola* |
| | *Depressaria* sp. (Depressaridae)* | Sierra Mágina, Spain | *N. cuatrecasasii* |
| | Geometridae* | Sierra Mágina, Spain | *N. cuatrecasasii* |
| | Geometridae (4) | Sierra Mágina, Spain | *N. cuatrecasasii* |
| | *Gonepteryx cleopatra* (Pieridae)* | Sierra Mágina, Spain | *N. cuatrecasasii* |
| | *Macroglossum stellatarum* (Sphingidae)* (4) | Bola del Mundo, Spain | *N. rupicola* |
| | *Macroglossum stellatarum* (Sphingidae) | Bola del Mundo, Spain | *N. rupicola* |
| | *Macroglossum stellatarum* (Sphingidae)* | Sierra Mágina, Spain | *N. cuatrecasasii* |
| | Noctuidae Hadeninae* (4) | Jebel Tazzeka, Morocco | *N. marvieri* |
| | Noctuidae Hadeninae (2) | Jebel Tazzeka, Morocco | *N. marvieri* |
| | *Orthosia cruda* (Noctuidae) | Jebel Tazzeka, Morocco | *N. marvieri* |
| | *Orthosia cerasi* (Noctuidae) | Jebel Tazzeka, Morocco | *N. marvieri* |
| | *Pieris brassicae* (Pieridae) | Sierra Mágina, Spain | *N. cuatrecasasii* |
| | *Spudaea ruticilla* (Noctuidae) | Jebel Tazzeka, Morocco | *N. marvieri* |
| Trichoptera | gen. sp.*(2) | Jebel Tazzeka, Morocco | *N. marvieri* |
| | Limnephilidae* | Jebel Tazzeka, Morocco | *N. marvieri* |
| | Limnephilidae* (3) | Sierra Mágina, Spain | *N. cuatrecasasii* |

*Species that carried pollen on their bodies; a single insect was captured, unless the number of insects captured is otherwise indicated (in parentheses). See text for dates and location of censuses.