DECONSTRUCTING HETEROSTYLY: THE EVOLUTIONARY ROLE OF INCOMPATIBILITY SYSTEM, POLLINATORS, AND FLORAL ARCHITECTURE

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Darwin's early work on heterostyly and related style polymorphisms (the presence of two or three style morphs within a population) generated much interest to understand how precise interactions between ecological and genetic mechanisms influence the evolution of floral diversity. Here we tested three key hypotheses proposed to explain the evolution of heterostyly: (i) the presence of self-incompatibility; (ii) the role of pollinators in promoting dissasortative mating; and (iii) floral architecture, which restricts pollinators' movements and ensures more exact pollen deposition on their bodies. We combined data from experiments, field observations, and published studies to test whether evolution of style polymorphism in *Narcissus* is driven by the incompatibility system, pollinator guilds, or floral architecture, within a phylogenetic framework. Neither differences in pollinator environment nor the presence of genetic self-incompatibility were correlated with presence of style polymorphism. However, our results indicate that the evolution of style polymorphism was driven by the presence of a narrow and long floral tube.

KEY WORDS: **Evolutionary transitions, floral architecture, heterostyly, incompatibility system, pollinators.**

Heterostyly and related style polymorphisms have fascinated biologists since Darwin provided an evolutionary explanation of the mechanisms suggesting that they serve to promote outcrossing in hermaphroditic plants (Darwin 1877). Heterostyly is characterized by the presence of two (distyly) or three (tristyly) flower morphs with reciprocal displacement in the height of the stigmas and the anthers within a flower. Style dimorphism is a polymorphism related with heterostyly, where two stylar morphs exist without concomitant reciprocal variation in stamens. Evolutionary models suggest that stylar dimorphism is an intermediate step in the transition from style monomorphism to distyly (Charlesworth and Charlesworth 1979; Lloyd and Webb 1992; Graham and Barrett 2004); however, such an intermediate step is present in comparatively few taxonomical groups. Heterostyly is a convergent trait present in at least 28 angiosperm families (Barrett and Shore 2008), and the long-standing interest in this style polymorphism has generated a diversity of hypotheses involving different scenarios and selective forces to explain its evolution (Baker 1966; Charlesworth and Charlesworth 1979; Lloyd and Webb 1992).

Charlesworth and Charlesworth (1979), following Baker (1966), suggested that the trait has evolved as a mechanism to avoid inbreeding depression. According to their model, the ancestral stage is a non-herkogamous, self-compatible plant having undergone strong inbreeding depression, which would have selected for the spread of a self-sterility mutation at a single locus, dividing the population into two mating types without any separation of anthers and styles. Many pollen grains would land on incompatible stigmas and thus high waste of gametes would make

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the system inefficient. Such inefficiency would promote the invasion of reverse herkogamous (short style placed below the anthers) mutants resulting in an intermediate stage of style dimorphism, with a posterior appearance of reciprocal placement of male and female sexual organs (distyly). This mostly genetical hypothesis, expressed in a quantitative fashion, is the logical descendant of some proposals from the 20th century contradicting Darwin's original postulate that self-incompatibility was an evolutionary consequence, not a cause, of reciprocal herkogamy. This view gained wide acceptance in the second half of the 20th century because of the common presence of heterostyly and heteromorphic self-incompatibility. However, recent studies in different plant lineages have failed to find the predicted evolutionary transitions affecting stylar polymorphism and self-incompatibility (Kohn et al. 1996; Graham and Barrett 2004; Pérez-Barrales et al. 2006; Ferrero et al. 2012).

Lloyd and Webb (1992) extended Darwin's (1877) original hypothesis proposing an ecologically based model for the evolution of heterostyly where, in contrast with Charlesworth and Charlesworth's (1979) model, the ancestor was already an approach herkogamous plant (typical flower design where the stigma protrudes from the anthers), with the subsequent invasion of a second reverse herkogamous morph (Webb and Lloyd 1986). In this model, inbreeding was not considered a selective force. Lloyd and Webb (1992) argued that the herkogamous condition, despite its wide abundance among flowering plants and success in avoiding selfing (Lloyd and Webb 1986), is suboptimal and unstable because the lack of reciprocity between male and female sexual organs of any two mating plants would result in high pollen waste, and hence could lead to distyly. A major selective force in this ecological model is the role of pollinators promoting efficient cross-pollination between morphs (Darwin 1877; Lloyd and Webb 1992). Although pollinators may be important in favoring efficient gamete exchange among sexual morphs, plants are not passive actors and can play a crucial role in ensuring accurate pollen placement on the pollinator's body by constraining the access to the floral reward and therefore contact with anthers and stigma. Hence, flower shape could affect plant fitness through its attraction of particular pollinators and its effect on the functional "fit" between pollinator and flower (Campbell et al. 1996; Alexandersson and Johnson 2002). Many of the species in which heterostyly is present share a common floral morphology, involving actinomorphy (radial symmetry), with simple open petals at the mouth, and the presence of a restrictive floral tube with nectar concealed at the base (Ganders 1979; Lloyd and Webb 1992). Hence floral tubes, a common device among angiosperms, possibly play an important role in ensuring efficient cross-pollination between morphs by directly affecting pollen placement on the pollinator's body (Lloyd and Webb 1992). In fact, species with different morphology, such as numerous stamens, free carpels, open-dished shaped corollas, or exposed nectar probably lack the necessary restrictions on pollinator access to ensure accurate pollen transfer required for the evolution of heterostyly (Barrett and Shore 2008). In accord with this observation, results of a study with *Narcissus* suggest evolution of style polymorphism was concentrated in branches of the phylogeny in which species possessed a floral tube (Graham and Barrett 2004).

Narcissus L. (daffodils) is a geophyte genus with 60–80 species distributed throughout the Mediterranean Basin (Dorda and Fernández Casas 1989; Blanchard 1990). This genus presents unrivalled variability in floral morphology among style polymorphic taxa, both in perianth shape and sex organ position (Graham and Barrett 2004; Barrett and Harder 2005). Across species, flowers are characterized by the presence of either a long and narrow or a short and relatively wide floral tube; and a variable corona, which in two species (*Narcissus cavanillesii* and *Narcissus broussonetii*) is virtually absent. The corona of *Narcissus* is an extension of the floral tube, it begins in the insertion of the sepals, and in some species it is long and wide, thus this structure may influence plant–pollinator interactions. Interspecific variation in sex organ position (style and stamen polymorphism) covers a range spanning style monomorphism, style dimorphism, distyly (*Narcissus albimarginatus*), and tristyly (*Narcissus triandrus*) (Barrett and Harder 2005). Floral phenology in *Narcissus* species varies from autumn to spring, and species are found from lowlands and coastal plains, to cliffs and alpine vegetation, hence pollinator guild and abundance vary widely across species. Indeed, even closely related species have very different main flower visitors (Santos-Gally 2011). The high variation in floral morphology, sex organ position, and pollinators makes *Narcissus* an excellent model in which to study the traits having favored the evolution of stylar polymorphism. Furthermore, recent developments in phylogenetic relationships among taxa in this genus (Santos-Gally et al. 2012) allow for the use of modern phylogenetic comparative analyses necessary to tackle such an endeavor (Whittall and Hodges 2007; Tripp and Manos 2008).

Here we test the influence of the three key factors proposed to drive the evolution of stylar polymorphism combining experimental data and observational field studies with data from the primary literature to perform phylogenetic comparative analyses. Specifically we test the following predictions: first, that self-incompatibility is evolutionarily correlated with the presence of stylar polymorphism as proposed by Charlesworth and Charlesworth (1979). Second, that the relative abundance of precise pollinator guilds is evolutionarily correlated with the presence of stylar polymorphism, as explicitly suggested by Lloyd and Webb (1992). Finally, that the presence of a long-narrow floral tube is a necessary prerequisite for the evolution of stylar polymorphism, as envisaged by the later authors.

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Materials and Methods

TAXON SAMPLING AND PHYLOGENY

Complex taxonomy and high species diversity has precluded sound phylogenetic resolution in *Narcissus*. In our study we used a Bayesian (ultrametric) phylogenetic tree of *Narcissus*, reconstructed using plastid (*trn*L–F, *trn*T–L, and *ndh*F) sequences of 39 species with the software BEAST (Drummond and Rambaut 2007) and in which branch lengths reflect time (Santos-Gally et al. 2012). The phylogeny covers ~49–65% of the species described and all species with stylar polymorphism are represented. For all comparative analyses, we used this phylogeny with some modifications explained below.

BayesTraits v. 1.0 (Pagel and Meade 2006) can be applied to a sample of phylogenetic trees such that evolutionary models and hypotheses are tested taking phylogenetic uncertainty into account. Accordingly, we created a sample of 500 alternative phylogenetic trees by sampling the resulting posterior distribution of the Markov Chain in BEAST. Trees were sampled after burn-in from iteration 20,000,000 to iteration 70,000,000 every 100,000 iterations to minimize autocorrelation between successive iterations. Trees were pruned to include species for which information on stylar polymorphism, incompatibility system, and pollinators was available. Continuous traits (i.e., pollinator guilds) were analyzed using phylogenetic generalized least squares (PGLS; Martins and Hansen 1997) and the sample of 500 alternative trees in the continuous function in BayesTraits.

PERIANTH TRAIT AND STYLAR POLYMORPHISM CODIFICATION

We measured four traits: floral tube width and length, and corona width and height of all species of subgenus Hermione (11 species, data available in the Dryad depository doi: 10.5061/dryad.78930) and one species from section Apodanthi (*Narcissus atlanticus*). All measurements were taken from digital images of the top and side view of flowers using ImageJ (Rasband 2008). Data for the remaining species of the genus were collected from the literature (28 species; data available in the Dryad depository doi: 10.5061/dryad.78930). To examine association among species based on perianth traits, we applied phylogenetic principal component analysis (PPCA; Revell 2010b) in R (Team 2009) using code provided by L. J. Revell. PPCA incorporates the expected covariance among trait values resulting from shared ancestry into the principal component analysis (Revell 2010b). Principal component results showed clear evidence for two groups of species: the first presenting wide coronas and wide, short floral tubes, whereas the other presented narrow coronas and long, narrow floral tubes (see Fig. 2, as well as data available in the Dryad depository doi: 10.5061/dryad.78930). Based on these results, we categorized species into those presenting a wide tube and those presenting a narrow tube. Such dichotomization of the phenotypic trait was necessary for the phylogenetic comparative analyses to determine the evolutionary correlation and contingency in evolutionary transitions between floral shape and stylar polymorphism (Pagel and Meade 2006). Average floral tube width of wide tube species was 2.7 times that of narrow tube species, indicating that the dichotomization does reflect natural variation in this trait.

Here, we use the term stylar polymorphism to refer to species presenting stylar dimorphism, distyly (one species), or tristyly (one species) irrespective of the polymorphism they exhibit. We categorized species as presenting or not presenting stylar polymorphism based on information collected from the literature (27 species: Herrera 1995; Graham and Barrett 2004; Pérez et al. 2004) and from field samples collected for this study (12 species; for further details see Supporting Information).

INCOMPATIBILITY SYSTEM

We experimentally determined the incompatibility system in 10 species of *Narcissus* based on the seed set resulting from three hand pollination treatments: self-pollination and within- and between-morph cross-pollination. We collected about 30 bulbs per species (range = 15–60 bulbs; some dimorphic species were represented by half short-styled and half long-styled plants, depending on the population morph ratio). These plants were grown in the greenhouse at the University of Seville to perform all experiments (for further details see Supporting Information). The effect of different treatments on the seed set (seed to ovule number) was tested using a generalized estimating equation (GEE) with binomial distribution and logit link functions. We included treatment as a categorical explanatory variable and flower position in the inflorescence as a covariate (function GEE in PASW version 18; SPSS 2009). The ratio of the averages of self- and outcross-pollination treatments was used to measure the self-compatibility index proposed by Becerra and Lloyd (1992). For the remaining 18 species in our sample data on incompatibility system was collected from the literature (data available in the Dryad depository doi: 10.5061/dryad.78930).

POLLINATORS

We observed pollinator visits in representative populations of 10 species: three monomorphic (*Narcissus serotinus*, *Narcissus obsoletus*, and *Narcissus tortifolius*) and seven dimorphic (*Narcissus tazetta*, *Narcissus papyraceus*, *Narcissus elegans*, *Narcissus dubius*, *Narcissus bertolonii*, *Narcissus broussonetii*, and *Narcissus scaberulus*). Diurnal and nocturnal observations were performed in plots of 10 m^2 during several days in October 2007, January, March, and October 2008, and February, March 2009. We recorded a total of 191.4 and 87.3 h of diurnal and nocturnal pollinator censuses, respectively (for further details see

Supporting Information). Data for the remaining species of the genus were collected from the literature (10 species; data available in the Dryad depository doi: 10.5061/dryad.78930). We focus on visitation rate of pollinators because data on efficiency of pollen transfer are virtually inexistent.

For all populations, pollinators were categorized based on their potential efficiency in cross-pollination between morphs (disassortative pollination) in the hypothetical eventual appearance of a reverse-herkogamous (short-styled) mutant, following the arguments of Lloyd and Webb (1992). If pollinators were unable to access the floral reward, and therefore could not contact the sexual organs of the hypothetical reverse-herkogamous mutant, they were categorized as restricted. If particular morphology of the pollinators (presence of a proboscis as long as or longer than the floral tube) permitted access to the nectar and potentially to all the sexual organs, then they were categorized as unrestricted. We use the terminology "restricted" and "unrestricted" because it highlights the constraint imposed by floral morphology on pollinators. Note that because pollinator efficiency in cross-pollination is influenced by species floral traits, the same pollinator could be classified as restricted with respect to a species with a long, narrow floral tube and as unrestricted with respect to a species with a short, wide tube. A classification solely based on the pollinator morphology was not adequate for our purposes. Following classification of pollinators, we calculated for each species the relative frequency of visits for unrestricted and restricted pollinator types. We used the relative frequency of unrestricted pollinators as the independent variable in the comparative analyses (data available in the Dryad depository doi: 10.5061/dryad.78930); the variable was arcsine transformed prior to analysis (Sokal and Rohlf 1995).

COMPARATIVE ANALYSES

We used the programme BayesTraits, which combines Bayesian and maximum likelihood-based approaches, to test the correlation and, where applicable, contingency of character evolution, between incompatibility system and stylar polymorphism, as well as between floral tube width and stylar polymorphism. The method applies a continuous time Markov model of trait evolution that calculates the likelihood of discrete trait data under two models of evolution, one in which the traits are allowed to evolve independently of one another on a phylogenetic tree and one in which they evolve in a correlated fashion (dependent model). The independent and dependent models can be compared by means of the harmonic mean of log-likelihoods calculating 2(log[harmonic mean (independent model)] − log[harmonic mean (dependent model)]). A result greater than 2 is taken as positive evidence that the dependent model is favored, greater than 5 is strong and greater than 10 is very strong evidence (Pagel and Meade 2006). BayesTraits combines uncertainty about both the model of trait evolution and phylogenetic uncertainty by simultaneously sampling the posterior distribution of phylogenies and parameters of the model of trait evolution. The reversible-jump Markov chain Monte Carlo method constructs a Markov chain that explores the universe of possible models, visiting them in direct proportion to their posterior probabilities. The parameters of the model of trait evolution are simply the values of the transition rates between the eight possible character states in a model of correlated evolution. The posterior probability distribution of the values of the parameters provides a means of analyzing the probability that the true value of the transition parameter between two trait states is nonzero. This is simply calculated from the proportion of the evolutionary models visited by the Markov chain, which assigned a value of zero to the transition parameter (i.e., the transition does not occur), henceforth referred to as *z*-scores (Pagel and Meade 2006). The evolutionary path from the putative ancestral state to derived state of two discrete traits can thus be inferred from the posterior probability distributions of the eight transition parameters in the dependent model of evolution (Pagel and Meade 2006; Gonzalez-Voyer et al. 2008). In our analyses, the hypothesis that the evolution of a floral tube is the trigger enabling evolution of stylar polymorphism, predicts that the transition from a hypothetical ancestral state of open flowers (no floral tube) and absence of stylar polymorphism (both traits observed in the nearest ancestors of *Narcissus*: *Sternbergia colchiciflora* and *Sternbergia lutea*, and found to be the most likely ancestral state in *Narcissus*; Meerow et al. 2006; Santos-Gally et al. 2012) to a derived state of floral tube and stylar polymorphism necessarily involves first a transition from open flowers to flowers with a tube.

We used a uniform prior for the independent model and a γ hyperprior (0, 10; 0, 10) for the model of dependent evolution (Pagel and Meade 2006). Choice of prior for the dependent evolution model does not change the results. Note that for the comparison of the harmonic means of the independent and dependent models we ran the dependent model using a uniform prior whereas contingency was determined from the results of the model with a γ prior. The analyses were run for 5,050,000 iterations with burn-in at 50,000 iterations and sample frequency of 300 iterations.

The correlation between relative frequency of unrestricted pollinators (continuous trait) and stylar polymorphism was analyzed using PGLS models (Martins and Hansen 1997) in BayesTraits using a sample of 500 phylogenies as above. We compared a model without any correlation between the two traits to a correlated evolution model using the harmonic mean of log-likelihoods as above. Both models were run with a uniform prior for 1,050,000 iterations and burn-in of 50,000 iterations. PGLS models have the advantage of incorporating different evolutionary models (e.g., Brownian motion, Ornstein–Uhlenbeck), combining categorical and continuous traits in a single analysis and estimating an evolutionary parameter (in this case λ) simultaneously with model fit that adjusts the variance–covariance matrix to adequately fit the model of evolution, in our case a Brownian motion model (Freckleton et al. 2002; Revell 2010a). In the Results we present the mean value of the regression slope and 95% credibility intervals (CI), calculated after burn-in using the package coda in R (Team 2009).

Results

FLORAL TRAITS

We found that a relatively high percentage of the species presented long-narrow floral tubes and stylar polymorphism (41% of the 39 species: one distylous, one tristylous, and 14 stylar dimorphic species). None of the species with open-tube flowers presented stylar polymorphism (Fig. 1). Self-incompatible species are more frequent than self-compatible ones (20 vs. 8, respectively), however both traits are equal distributed across the phylogeny (Fig. 1). The same pattern occurs with species presenting different proportion of unrestricted pollinators (see Fig. 1).

INCOMPATIBILITY SYSTEM

There was no support for an evolutionary correlation between presence of self-incompatibility and stylar polymorphism. Our results indicated that a dependent model of evolution between incompatibility system and stylar polymorphism did not provide a better fit to the data than an independent model (difference between harmonic means = 0.062), where evolutionary transitions in character state of one trait are unrelated to the state of the second trait (Pagel and Meade 2006).

POLLINATORS

Our results did not support the hypothesis that pollinators are important in promoting the evolution of stylar polymorphism. There was no correlation between relative frequency of unrestricted pollinators and presence of stylar polymorphism (difference between harmonic means = 1.09, $\beta = 0.13$, 95% CI = −0.22 to 0.5). For one polymorphic species (*N. elegans*), pollinator observations were only available from a monomorphic population, to avoid any potential biases we repeated the analyses after excluding this species, nonetheless the results did not change (in this model the difference between harmonic means shows more support for the independent model = −1.6, $\beta = 0.10$, 95% CI = −0.27 to 0.49).

PERIANTH MORPHOLOGY

Results of the phylogenetic principal components analysis showed that floral morphology differed markedly among species (Fig. 2). Measures of the floral tube (length and width) and corona (width and height) presented negative loadings on the first principal component (PC1). Floral tube length and width and corona width loaded negatively on the second principal component (PC2) whereas corona height presented a positive loading. Hence, PC1 separated species into those with narrow floral tubes and narrow, low coronas at one extreme, and wide floral tubes and wide, high coronas at the other (Fig. 2). PC2 mostly separated species based on the length of the tube, which presented the highest loading of all measures (−0.98).

Perianth morphology and stylar polymorphism have evolved jointly in *Narcissus*. Indeed, our results indicated that there was very strong support for the dependent model of evolution (in contrast with the independent model; see Table S1), where traits evolve in a correlated fashion (difference in harmonic means between the dependent and independent models = 13.4). Furthermore, the most likely evolutionary path from the ancestral state of absence of stylar polymorphism and wide floral tube to the derived state of stylar polymorphism and narrow-long floral tube involves first a transition toward narrow-long floral tubes (compare the importance of transition $q1,2$ with that of the alternative, transition $q1,3$, as indicated by the z-scores: 0.36 vs. 0.70, where higher values indicate less likely transitions; Fig. 3), followed by a transition toward stylar polymorphism (z-score value of transition $q2,4 = 0.0$; Fig. 3). Transitions in this evolutionary path were assigned a value of 0 in 36% and 0% of the sampled Markov chains (Fig. 4). The alternative route involving first a transition toward stylar polymorphism ($q1,3$) followed by a transition toward long-narrow tubes ($q3,4$) is not supported (Fig. 3) because the first transition in this evolutionary path ($q1,3$) was assigned a value of 0 in 70% of the sampled Markov chains (Fig. 4). Thus, the results support the prediction that the presence of a narrow-long floral tube serves as a necessary evolutionary prerequisite for the evolution of stylar polymorphism in *Narcissus*. Interestingly, the results also indicate that loss of stylar polymorphism is restricted to flowers with narrow-long floral tubes (Fig. 3), strengthening the suggested importance of floral architecture for the evolution of stylar polymorphism. The transition from a state with stylar polymorphism and a long-narrow floral tube toward a state with a wide floral tube and stylar polymorphism was assigned a value of zero in 70% of the sampled Markov chain. Conversely, the alternative route involving a loss of stylar polymorphism while maintaining the narrow-long floral tube was assigned a value of 0 in only 0.002% of the sampled chain (Fig. 3).

Discussion

Our results indicate that the evolution of stylar polymorphism in *Narcissus* is not correlated with the presence of self-incompatibility, which is counter to the genetic hypothesis of Charlesworth and Charlesworth (1979). Furthermore, our results found no support for an evolutionary association between relative

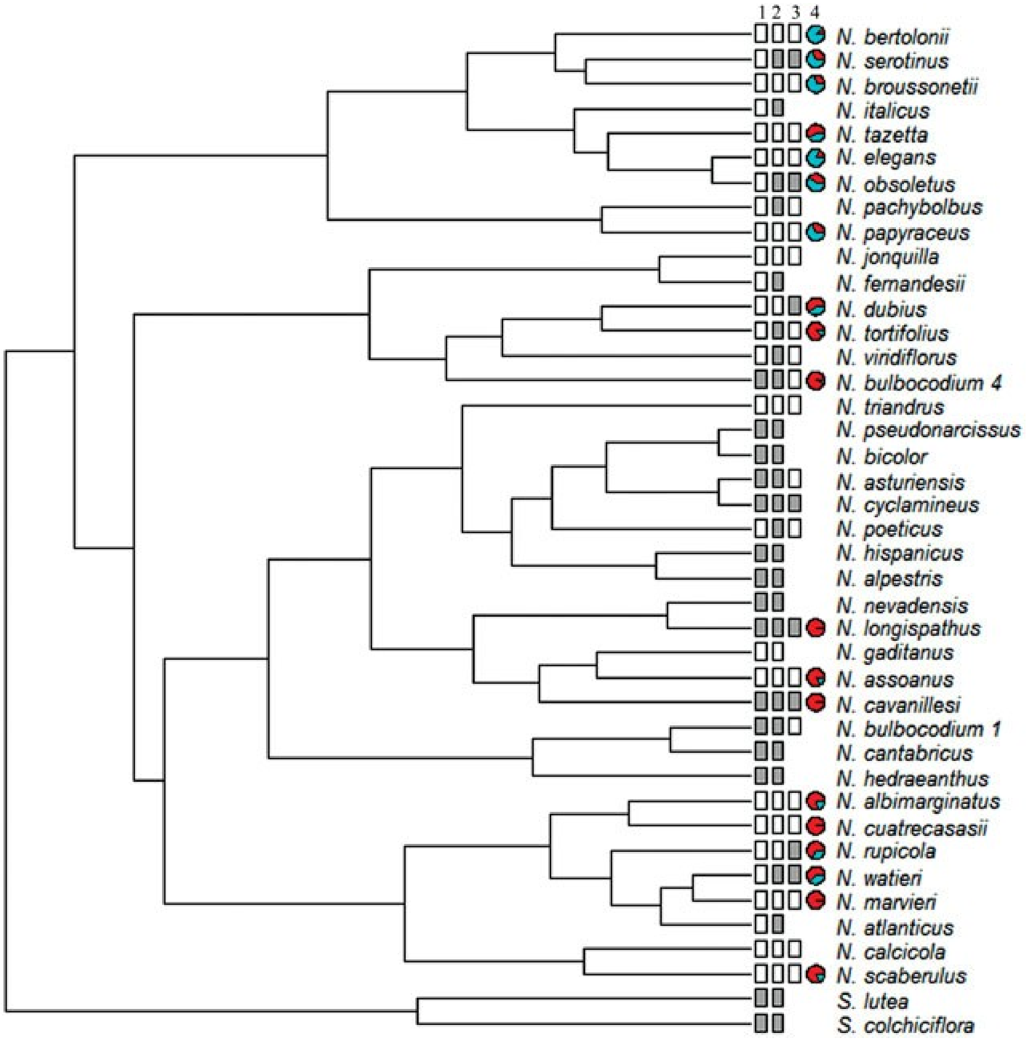

Figure 1. **Phylogenetic tree of *Narcissus* showing discrete and continuous traits used in this study. (1) Long and narrow-tube (white) and open-tube flowers (grey). (2) Stylar polymorphism (white) and stylar monomorphism (gray). (3) Self-compatible (gray) and self-incompatible. (4) Proportion of restricted (gray/blue) and unrestricted pollinators (dark-gray/red). *Stenbergia lutea* and *Stenbergia colchiciflora*, both outgroup species (not included in the analyses) are shown in the figure to highlight the absence of floral tube and polymorphism.**

abundance of distinct pollinator guilds and stylar polymorphism, contra the prediction derived from Lloyd and Webb's (1992) ecological hypothesis. However, our results provided strong support for an evolutionary correlation between floral architecture, namely the presence of a narrow-long floral tube, and stylar polymorphism. The results not only provide strong support for an evolutionary correlation between floral tube and stylar polymorphism, but also indicate that the presence of a narrow-long floral tube is a necessary prerequisite for the evolution of stylar dimorphism in *Narcissus*. Lloyd and Webb (1992) explicitly proposed such a correlation based on subjective appreciation of perianth morphology of most heterostylous groups, a view advanced early on by Darwin (1877).

INCOMPATIBILITY SYSTEM

Charlesworth and Charlesworth (1979) assumed that heterostyly evolved from a monomorphic ancestor and proposed that the primary selective force favoring the evolution of heterostyly was inbreeding avoidance, hence the necessary prerequisite of a self-incompatibility system immediately preceding the appearance of style dimorphism. Although uncommon, self-compatible heterostylous species occur in several genera. Mulcahy (1964) and Ornduff (1972) described a self-compatible system in some tristylous and distylous species of *Oxalis*. In the genus *Amsinckia*, some distylous species are known to be self-compatible (reviewed in Ganders 1979). Recently, Ferrero et al. (2012) reported absence of a strong association between style polymorphism and

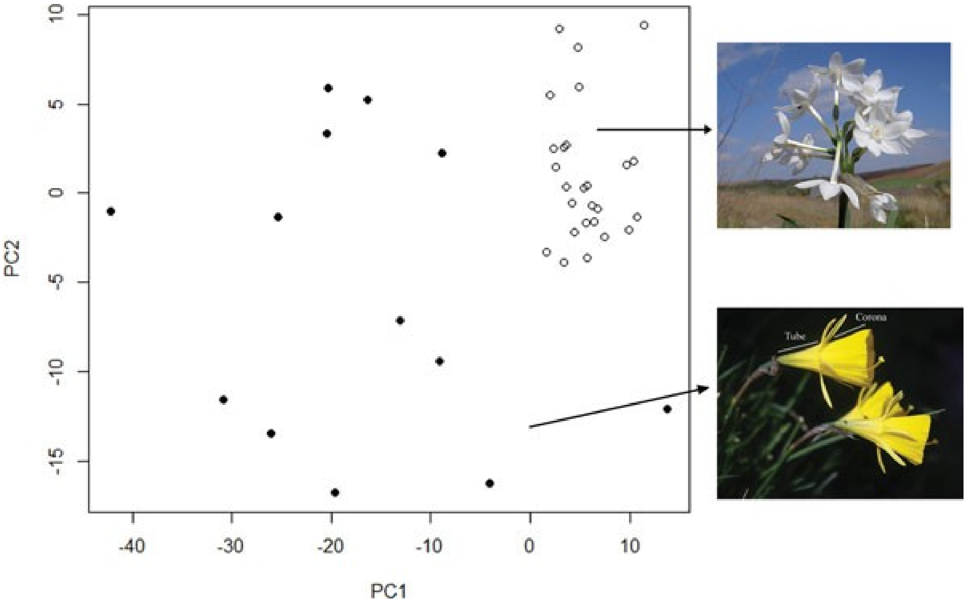

Figure 2. **Phylogenetic principal components analysis of perianth variables of *Narcissus*. Examples of the two main perianth phenotypes are shown (*N. bulbocodium*, lower-right, with open-tube flowers and *Narcissus papyraceus*, upper-right, with long and narrow-tube flowers).**

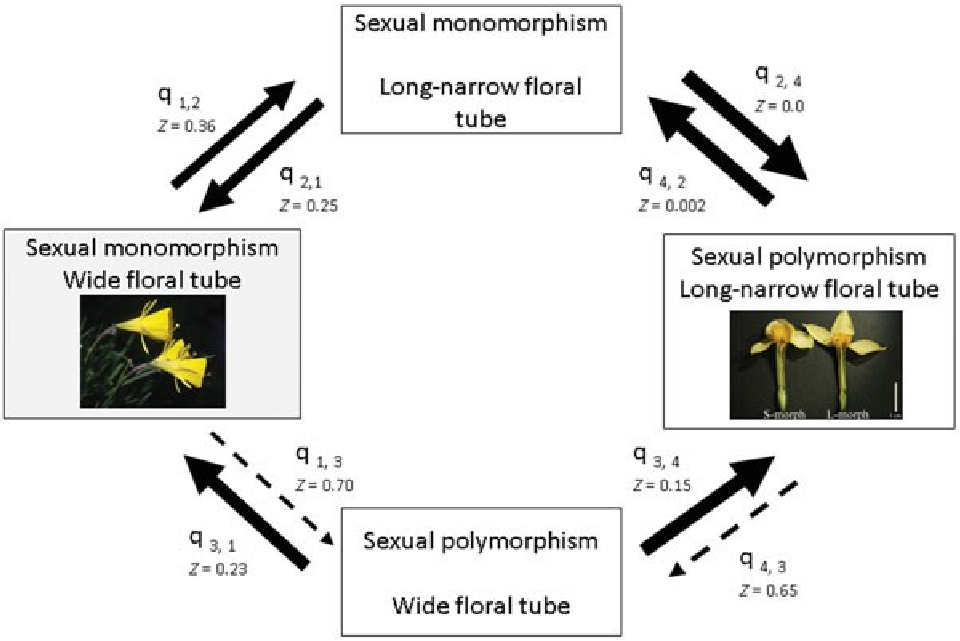

Figure 3. **Flow diagram showing the most probable evolutionary pathway from the ancestral state of stylar monomorphism and wide floral tube (gray-shaded box) to the derived state of stylar polymorphism and long-narrow floral tube. Arrow thickness is proportional to the likelihood of the transitions based on the *z*-score (the higher the *z*-score, the more unlikely the transition; see Materials and Methods for details). Photos represent *Narcissus bulbocodium*, left, and *Narcissus bertolonii*, right.**

self-incompatibility in *Glandora*, where three of the four studied distylous species were self-compatible. Self-compatibility has also been found in species of *Psychotria* (Barrett and Richards 1990; Bjorkman 1995). Finally, *Salvia brandegeei*, a distylous species, is a noteworthy self-compatible example because there are apparently no reliable reports of self-incompatibility in the Lamiaceae (Barrett et al. 2000).

An important challenge associated with tests of Charlesworth and Charlesworth's (1979) hypothesis is the fact that it is difficult to determine whether self-compatibility in heterostylous

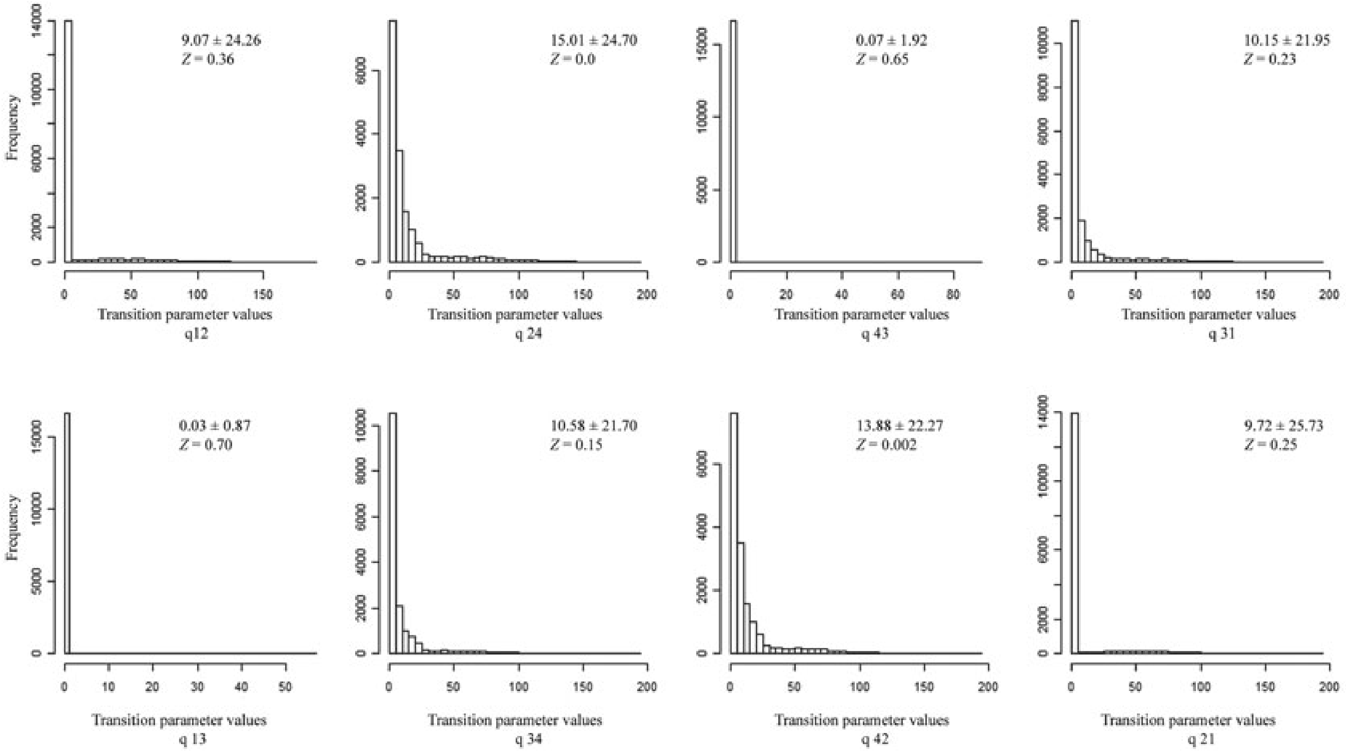

Figure 4. **Posterior probability distributions of the values of the transition rate coefficients of the model of correlated evolution between flower morphology and stylar polymorphism. *z* values present the proportion of the sampled runs from the Markov chain in which the parameter was assigned a value of 0. Shown are the average and standard deviation of the parameter values from the sampled runs and the *z*-score (see Materials and Methods for details).**

species is an ancestral or a derived trait (Barrett and Shore 2008). Nevertheless, when properly analyzed in a phylogenetic framework, self-compatibility in heterostylous groups is frequently associated to secondary homostyly (e.g., Guggisberg et al. 2006; Mast and Conti 2006). The ancestral state of the compatibility system in *Narcissus*, given available data presented here, remains undetermined because the reconstruction recovered equal probability for compatibility and self-incompatibility (ancestral state probability = 0.5 and 0.5, respectively). The null correlation between self-incompatibility and stylar polymorphism observed here is unlikely to result from reversion to self-compatibility after stylar polymorphism had evolved, because polymorphism associated with self-compatibility is very rare in our dataset; it was found only in two species (*N. dubius*: Baker et al. 2000; *N. rupicola*: Pérez-Barrales et al. 2006). Hence, our results add to the available evidence against the hypothesis that self-incompatibility is a necessary prerequisite for the evolution of heterostyly, as argued by Lloyd and Webb (1992).

POLLINATORS

The hypothesis of Lloyd and Webb (1992) assumes that an approach herkogamous ancestor was sufficient to avoid self-interference, and thus discards the need to propose self-incompatibility as an initial stage in the evolution of heterostyly. However, such a system requires efficient cross-pollination between anthers and stigma at different levels (Barrett 2002). Implicit in this evolutionary model is the crucial role of pollinators ensuring pollen transport and proficient cross-fertilization (Lloyd and Webb 1992). Our results do not support the ecological hypothesis, because we did not find a significant correlation between the relative abundance of unrestricted pollinators and stylar polymorphism in *Narcissus*. Even after having repeated the analyses excluding species that do not present any restrictions to pollinator access (i.e., species without a narrow-long floral tube), the correlation between relative abundance of unrestricted pollinators and stylar polymorphism remains nonsignificant (difference between harmonic means = 0.19, $\beta = -0.07$, 95% CI = -0.48 to 0.34). A previous study of a smaller group of *Narcissus* (sect. Apodanthi) found an apparent association between particular types of style polymorphism (style dimorphism and distyly, of different reciprocity) and pollinators (long and short tongued; Pérez-Barrales et al. 2006). However, the group was too small to allow for reliable estimation of phylogenetic correlations. Here our interest lay in exploring the role of pollinators in the evolution of style polymorphism, whatever its type. To our knowledge, this is the first study where this question is addressed across species. We were unable to quantify the precise role of each pollinator type in promoting legitimate pollen transfer, because *Narcissus* lacks the pollen dimorphism typical of other style polymorphic plants (Ganders 1979). Ongoing experiments on pollen transfer by individual pollinators from and to morph-specific plants will ascertain this possibility.

Is there no role for pollinators in stylar polymorphism? Detailed microevolutionary field studies of several populations along a latitudinal gradient of *N. papyraceus*, a stylar dimorphic species with a long-narrow floral tube, suggest that even low abundance of unrestricted pollinators can suffice to maintain stylar polymorphism (Pérez-Barrales and Arroyo 2010). In contrast, in populations where the short-morph has been lost and only long-styled plants are found, restricted pollinators (usually those with a short-proboscis) were five times more abundant than unrestricted pollinators (Santos-Gally et al. 2013). In sum, rather than favoring the evolution of stylar polymorphism the key role of current pollinators might be in maintaining it after it has evolved, at least at a population level, where observations and experiments can be highly illustrative (Thompson et al. 2012). These different results at population and species level illustrate the difficulties of identifying patterns across micro- and macroevolutionary levels. In particular, pollinators operating in these groups of plants may have changed over both ecological and evolutionary time.

PERIANTH MORPHOLOGY

Previous studies having surveyed the character states of heterostylous species suggested that there are constraints on the types of flowers in which herkogamy has evolved. Most heterostylous species presented flowers with a floral tube with nectar concealed at the base, flowers also tended to present some development of a corolla tube, although corolla shape was more variable (Ganders 1979; Lloyd and Webb 1992). Such floral morphology was suggested to be of importance in positioning pollinators for efficient pollen deposition to maximize male and female fitness (Ganders 1979; Lloyd and Schoen 1992; Barrett 2002) and in fact some experimental evidence exists (Stone and Thompson 1994). Indeed, heterostyly is rarely associated with flowers with open-dish–shaped corollas and exposed nectar, as these do not impose the required restrictions on pollinators to ensure the precision in pollen transfer necessary for its evolution (Barrett and Shore 2008). Our study group included both relatively open flowers and narrow-tubed flowers allowing a test of this hypothesis. Our results support the hypothesized evolutionary correlation between perianth morphology (presence of a long-narrow floral tube) and stylar polymorphism (Lloyd and Webb 1992) as the BayesTraits analysis indicated very strong support for a dependent model of correlated evolution between these two traits. Results from a previous analysis involving 23 *Narcissus* species hinted at an association between floral morphology and stylar polymorphism, because evolutionary transitions toward a derived sexually polymorphic state were apparently concentrated in clades presenting long-narrow floral tubes, based on maximum parsimony reconstructions (Graham and Barrett 2004). Here, we show that in *Narcissus* the evolution from an ancestral stylar monomorphic state to a derived stylar polymorphic one necessarily involves first a transition toward a narrow-long floral tube (see Fig. 3). The alternative evolutionary route, involving first a transition from monomorphism toward stylar polymorphism followed by an evolutionary transition from wide-tubed flowers toward narrow-long tubed flowers is unlikely, because a key evolutionary transition is set to a value of 0 in a very high proportion of the sampled Markov chains ($z = 0.70$; see Fig. 3). In other words, evolution of stylar polymorphism in *Narcissus* is contingent on the state of the floral tube. Interestingly, according to our model, back transitions toward stylar monomorphism are also restricted to species with a long-narrow floral tube, further supporting the suggested importance of floral architecture for the evolution of stylar polymorphism.

Trait-dependent diversification could lead to apparent associations between traits. This is unlikely in our study because although 26 of the 39 species included in the phylogeny present a long-narrow floral tube, polymorphism is not equally distributed among them, 16 are polymorphic whereas 10 are monomorphic (see Fig. 1), supporting the evolutionary correlation between long-narrow floral tubes and polymorphism in *Narcissus*.

The positive transition rates leading away from a state of stylar polymorphism and wide floral tube (see Fig. 3) possibly arise because the model calculates the likelihood at internal nodes over all possible states. In this particular case we do not know of any *Narcissus* species presenting stylar polymorphism and a wide floral tube, although rare cases of stylar polymorphism in open-dished shape flowers have been reported (Rama Swamy and Bahadur 1984; Bjorkman 1995; Shore et al. 2006), and the positive transition rates ensure that the model moves out of such a state quickly (Pagel and Meade 2006). The absence of species presenting stylar polymorphism and wide floral tube, in our study, probably results from selection acting against this combination of traits in *Narcissus*.

CONCLUDING REMARKS

Our results did not support the hypothesized evolutionary correlation between self-incompatibility and stylar polymorphism, neither did they support the role of pollinators in favoring evolution of stylar polymorphism in *Narcissus*. Interestingly, our results presented strong support for the hypothesis that perianth architecture, particularly the presence of a narrow-long floral tube, has an important influence on the evolution of stylar polymorphism (Lloyd and Webb 1992). This is the first comparative correlation and contingency analysis undertaken to explicitly test main hypotheses on the evolution of heterostyly.

Although our results do not support the hypothesis that pollinators play a role in the evolution of stylar polymorphism, microevolutionary studies in one *Narcissus* species do suggest that some types of pollinators are important to avoid the loss of stylar polymorphism once it has evolved (Santos-Gally et al. 2013). It is possible that former pollinators have shaped flower architecture (e.g., long-tongued insects pollinating long-tubed flowers) and where later partially replaced by short-tongued pollen collecting insects (Arroyo and Dafni 1995), obscuring the correlation with style polymorphism. In this respect it is worth noting the possible role of the large variation in flower phenologies (Arroyo and Dafni 1995) and fragrances (Dobson et al. 1997) in some *Narcissus*, which may have a strong influence on the wide variety of pollinators involved. In addition, hybridization might also blur species differences in flower traits of importance to pollinators. Work currently under way is aimed at determining these effects.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Seed set (seed to ovule number) of 10 *Narcissus* species.

Table S1. Posterior probability distributions of the values of the transition rate coefficients of the model of independent evolution between flower morphology and stylar polymorphism.