American Journal of Botany 91(7): 1007–1021. 2004.

INVITED SPECIAL PAPER

PHYLOGENETIC RECONSTRUCTION OF THE EVOLUTION OF STYLAR POLYMORPHISMS IN *NARCISSUS* (AMARYLLIDACEAE)[1]

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We investigated the origin of stylar polymorphisms in *Narcissus*, which possesses a remarkable range of stylar conditions and diverse types of floral morphology and pollination biology. Reconstruction of evolutionary change was complicated by incomplete resolution of trees inferred from two rapidly evolving chloroplast regions, but we bracketed reconstructions expected on the fully resolved plastid-based tree by considering all possible resolutions of polytomies on the shortest trees. Stigma-height dimorphism likely arose on several occasions in *Narcissus* and persisted across multiple speciation events. As proposed in published models, this rare type of stylar polymorphism is ancestral to distyly. While there is no evidence in *Narcissus* that dimorphism preceded tristyly, a rapid transition between them may explain the lack of a phylogenetic footprint for this evolutionary sequence. The single instances of distyly and tristyly in *Narcissus albimarginatus* and *N. triandrus*, respectively, are clearly not homologous, an evolutionary convergence unique to Amaryllidaceae. Floral morphology was likely an important trigger for the evolution of stylar polymorphisms: Concentrated-changes tests indicate that a long, narrow floral tube may have been associated with the emergence of stigma-height dimorphism and that this type of tube, in combination with a deep corona, likely promoted, or at least was associated with, the parallel origins of heterostyly.

Key words: ancestral-state reconstructions; concentrated-changes test (CCT); floral evolution; heterostyly; *Narcissus*; *ndF*; pollination biology; stigma-height dimorphism; stylar polymorphism; *trnL-trnF*.

Flowering plants possess extraordinary diversity in reproductive traits, even among closely related species. This variation is the result of the evolutionary lability of reproductive characters and implies that there are diverse functional solutions for achieving mating and fertility. The floral diversification that has accompanied the coevolution of flowers and animal pollinators is particularly striking and has resulted in contrasting suites of floral characters associated with different pollinator groups (e.g., Grant and Grant, 1965; Armbruster, 1993; Johnston et al., 1998; Schemske and Bradshaw, 1999).

Although diversifying selection has played a prominent role in floral evolution, pervasive convergence in pollination mechanisms also characterizes many unrelated animal-pollinated groups. The multiple origins of the heterostylous genetic polymorphisms distyly and tristyly across at least 28 diverse animal-pollinated angiosperm families are a classic example of the convergent evolution of plant sexual systems (Darwin, 1877; Ganders, 1979; Lloyd and Webb, 1992a, b; Barrett et al., 2000b). Most heterostylous species are adapted for pollination by long-tongued pollinators and commonly possess actinomorphic, tubular flowers with a stereotypically reciprocal arrangement of sex-organ heights. The reciprocal herkogamy that characterizes heterostyly is a structural mechanism that functions to increase the proficiency of cross-pollination in species with perfect flowers (Lloyd and Webb, 1992a, b). Although heterostyly (both distyly and tristyly) has been studied intensively since the mid-nineteenth century (reviewed in Barrett, 1992), remarkably little is known about its evolutionary history. Only two studies have explicitly used phylogenetic approaches to investigate the origins of heterostyly (distyly—Schoen et al., 1997; tristyly—Kohn et al., 1996); neither was able to identify the ancestral states or the precise evolutionary pathways involved in the evolution of these complex pollination mechanisms.

Narcissus a small genus of animal-pollinated geophytes in Amaryllidaceae (monocots: Asparagales) with exceptional floral diversity (e.g., Figs. 1, 2), provides an excellent opportunity for examining the evolutionary history of heterostyly. Four major classes of stylar condition, stylar monomorphism, stigma-height dimorphism, distyly, and tristyly (Fig. 2), are represented among the 10 sections recognized in the genus (Henriques, 1887; Fernandes, 1935; Dulberger, 1964; Bateman, 1968; Lloyd et al., 1990; Arroyo and Dafni, 1995; Barrett et al., 1996, 1997; Arroyo and Barrett, 2000; Baker et al., 2000b, c; Arroyo et al., 2002; Thompson et al., 2003). No other heterostylous taxon displays this range of stylar variation. *Narcissus* may therefore provide one of our best opportunities for investigating the evolutionary pathways by which heterostylous polymorphisms originated. A diversity of flower shapes is found in *Narcissus* (Fig. 1), particularly associated with corona size and floral tube length. It would therefore be valuable to assess the possible functional role of floral morphology in

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[1] Manuscript received 2 September 2003; revision accepted 5 March 2004.

The authors thank John Blanchard for sharing his broad expertise of the genus and for providing material from his live collection. Juan Arroyo and Mark Chase also provided material. We also thank Juan Arroyo, Lawrence Harder, Angela Baker, John Thompson, and Luis Villar for helpful discussions and locality information. Hardeep Rai and Heath O'Brien provided substantial assistance with laboratory work. Alan Meerow and Peter Endress provided helpful comments on the manuscript. This work was funded by Discovery Grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) to S. W. G. and S. C. H. B.

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stimulating evolutionary transitions among the different stylar conditions.

Species of *Narcissus* can be classified as to whether they are monomorphic, dimorphic, or trimorphic for style length (reviewed in Barrett et al., 1996). Here we use the term ''stylar polymorphism'' to refer to populations or species that are dimorphic or trimorphic for style length, irrespective of the type of polymorphism (stigma-height dimorphism, distyly, tristyly) that they exhibit. Monomorphic species are usually composed of plants with long-styled flowers, with stigmas protruding beyond the two anther levels that typically occur in members of this genus (e.g., Figs. 1d, h; 2a). This condition is referred to as ''approach herkogamy'' (see Webb and Lloyd, 1986, for a review of herkogamy) and is the most common stylar condition in *Narcissus*. In some monomorphic species (e.g., *N. serotinus*, Fig. 1m; *N. viridiflorus*, Fig. 1n) the stigma is located just above or at the same level as the anthers. Populations with stylar monomorphism often display considerable quantitative variation in style length, but do not exhibit the clear bimodality or trimodality that characterizes species with stylar polymorphism (Barrett et al., 1996).

There are two types of stylar dimorphism in *Narcissus*. The most common, stigma-height dimorphism (Fig. 2b), involves populations with two floral morphs that differ in style length (long- and short-styled plants, hereafter referred to as L- and S-morphs), but which have anthers placed at roughly the same position at the top of the floral tube (Dulberger, 1964; Baker et al., 2000c; Arroyo et al., 2002). This polymorphism occurs in approximately a dozen species distributed among three sections of the genus (*Apodanthi*, Fig. 1g; *Jonquillae*, Fig. 1e; *Tazettae*, Fig. 1k). The second dimorphic condition, distyly (Figs. 1b, 2c), is restricted to a single species: *N. albimarginatus* of section *Apodanthi* (Arroyo and Barrett, 2000; Peréz et al., 2003). The only two known populations of this rare Moroccan species contain two floral morphs (L- and S-morphs) that differ reciprocally in stigma and anther height (i.e., reciprocal herkogamy; heterostyly). The final stylar condition, tristyly (Figs. 1a, 2d), is a more complex form of reciprocal herkogamy in which populations contain three floral morphs that differ in style length (L-, M- [mid-] and S-morphs). This polymorphism occurs in a single widespread species (*N. triandrus*, Fig. 1a) of section *Ganymedes* and its expression is unique among the six flowering plant families that have tristylous taxa (Amaryllidaceae, Connaraceae, Linaceae, Lythraceae, Oxalidaceae, Pontederiaceae; Barrett, 1993; Thompson et al., 1996). *Narcissus triandrus* possesses imperfect sex-organ reciprocity (Fig. 2d) and a system of late-acting self-incompatibility that permits both intermorph and intramorph mating (Barrett et al., 1997, 2004; Sage et al., 1999). In contrast, typical tristylous species have a trimorphic incompatibility system that prevents intramorph mating and three organ levels that are positioned reciprocally (reviewed in Barrett and Cruzan, 1994). The occurrence in *Narcissus* of these four contrasting patterns of stylar variation raises obvious questions concerning their evolutionary relationships and the selective mechanisms leading to evolutionary transitions among them.

In *Narcissus*, the evolutionary sequence(s) in which the different stylar polymorphisms originated from stylar monomorphism is not known. A simple model based on increasing floral complexity posits an evolutionary transition series from ancestral monomorphism, through stigma-height dimorphism, to distyly, and then finally to tristyly. Theoretical support for parts of this sequence of events comes from models for the evolution of distyly and tristyly. Lloyd and Webb's (1992a, b) ''pollen-transfer'' model for the evolution of distyly proposes that it evolves from a monomorphic ancestor with approach herkogamous flowers, via an intermediate stage of stigma-height dimorphism. The ''inbreeding avoidance'' model for the evolution of distyly (Charlesworth and Charlesworth, 1979) also posits an intermediate stage of stigma-height dimorphism, but the ancestral condition in this case is assumed to be a homostylous population with long styles and long-level anthers. Finally, Charlesworth's (1979) model of the evolution of tristyly commences with an ancestral monomorphic long-styled population, with a transient dimorphic stage that then becomes invaded by the M-morph, establishing stylar trimorphism. The dimorphic stage involves a population with reciprocal herkogamy, and therefore stigma-height dimorphism is not explicitly dealt with in this model. The particular evolutionary sequences in these three models are supported by detailed theoretical arguments and are certainly plausible; however, empirical support from comparative analyses of heterostylous groups of both the ancestral states and intermediate conditions postulated in the models are currently lacking.

A common feature of the two models for the evolution of distyly is the existence of an intermediate stage of stigma-height dimorphism (and see Richards, 1997). This polymorphism is a very rare condition in flowering plants, with occasional cases reported from both non-heterostylous (e.g., *Epacris*, *Kalmiopsis*—Ericaceae; *Chlorogalum*—Agavaceae) and heterostylous families (e.g., *Anchusa*, *Lithodora*—Boraginaceae; reviewed in Barrett et al., 2000b). In the heterostylous families in which stigma-height dimorphism occurs, there is no phylogenetic evidence linking this polymorphism to the origin of distyly. Lloyd and Webb (1992a) proposed that stigma-height dimorphism is rare or missing in heterostylous groups because the polymorphism is an unstable transitional state. According to this view, the polymorphism should be easily modified through selection for anther dimorphism, and it is therefore a short-lived stage that is quickly passed through during the evolution of distyly (and see Charlesworth and Charlesworth, 1979). This argument is largely based on functional grounds, because of the apparent maladaptiveness of uniform anther height for pollen transfer in populations with two stigma heights (but see Stone and Thomson, 1994). If this scenario were true in *Narcissus*, we would not expect stigma-height dimorphism to be common and distyly rare, the scenario that actually occurs. Stigma-height dimorphism in *Nar-*

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Fig. 1. Flowers of representative *Narcissus* species illustrating the wide diversity in floral design. This variation is associated with different pollinator groups, as discussed in the text. Section names are indicated parenthetically: (a) *N. triandrus* var. *cernuus* (*Ganymedes*), long-styled morph; (b) *N. albimarginatus* (*Apodanthi*), short-styled morph; (c) *N. alpestris* (*Pseudonarcissi*); (d) *N. hedraeanthus* (*Bulbocodii*); (e) *N. gaditanus* (*Jonquillae*); (f) *N. cavanillesii* (*Tapeinanthus*); (g) *N. rupicola* (*Apodanthi*); (h) *N. bulbocodium* (*Bulbocodii*); (i) *N. cyclamineus* (*Pseudonarcissi*); (j) *N. poeticus* (*Narcissus*); (k) *N. tazetta* (*Tazettae*); (l) *N. dubius* (*Tazettae*); (m) *N. serotinus* (*Serotini*); (n) *N. viridiflorus* (*Jonquillae*) Photographs are by S. C. H. Barrett, except (n), by Juan Arroyo (Universidad de Sevilla).


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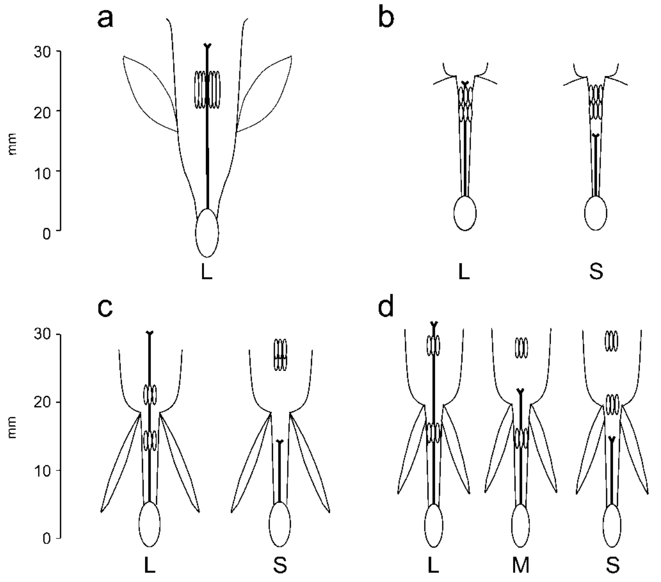

Fig. 2. Examples of the four classes of stylar variation in *Narcissus*: (a) stylar monomorphism in *Narcissus longispathus*; (b) stigma-height dimorphism in *N. assoanus*; (c) distyly in *N. albimarginatus*; (d) tristyly in *N. triandrus*. *Narcissus longispathus* exhibits approach herkogamy, with stigmas exserted beyond the two anther levels. The sex organs are located within a funnel-like corona. *Narcissus assoanus* populations contain two floral morphs that differ in style length. Note that there are small differences in the height of the lower anther levels. The flowers possess long floral tubes with small coronas. *Narcissus albimarginatus* populations contain two floral morphs that differ in style length and in anther position. Stigmas and anthers are reciprocally positioned, and flowers possess long, narrow floral tubes and deep coronas. *Narcissus triandrus* populations usually contain three floral morphs that differ in style length. There are three anther levels that correspond in height to the three stigma heights, but unlike other tristylous species sex-organ reciprocity is imperfect, because of the anomalous position of the upper-level anthers of the long-styled morph. Flowers have long, narrow floral tubes and deep coronas.

cissus appears to be a stable sexual strategy and not simply an ephemeral stage on the evolutionary pathway to distyly. Whether the origin of distyly in *Narcissus* involved an ancestor with stigma-height dimorphism, as theoretical models also predict, needs to be determined. The occurrence of both stigma-height dimorphism and distyly in section *Apodanthi* may permit the determination of whether this evolutionary sequence has indeed occurred.

In tristylous groups there is no comparative evidence that stylar dimorphism (a term that can include polymorphic conditions that range from stigma-height dimorphism to fully-fledged distyly) is an intermediate stage in the evolution of tristyly. Distylous species (Lythraceae, Oxalidaceae) and dimorphic populations (Pontederiaceae) occur in these well-studied tristylous families. However, microevolutionary studies clearly indicate that these are derived rather than ancestral conditions (reviewed in Weller, 1992). Evolutionary transitions from tristyly to distyly or other dimorphic conditions commonly result from the loss of style morphs from tristylous populations, as a result of stochastic and/or deterministic forces (Ornduff, 1972; Weller, 1976; Barrett et al., 1989; Eckert and Barrett, 1992, 1995). The loss of style morphs is often accompanied by evolutionary modifications to the remaining floral morphs, including changes to sex-organ position, incompatibility relations, and pollen size (reviewed in Weller, 1992). The absence of ancestral dimorphism in tristylous families may be explained for the same reason that stigma-height dimorphism is rare in distylous groups; the intermediate dimorphic stages were transient and are therefore not represented among extant taxa. The relationship between stylar dimorphism and trimorphism may potentially be evaluated using phylogenetic approaches in *Narcissus*.

Here we investigate the evolutionary history of stylar variation in *Narcissus* by constructing phylogenetic trees based on two relatively rapidly evolving regions of the plastid genome. We use character optimizations of floral traits to address the following specific questions arising from the theoretical and empirical work discussed in this section: (1) Is stylar monomorphism the ancestral condition in *Narcissus*? (2) How many times has stigma-height dimorphism evolved in the genus? (3) Has distyly evolved from stigma-height dimorphism, as proposed by Lloyd and Webb (1992a, b)? (4) Has tristyly evolved from an intermediate dimorphic stage, as proposed by Charlesworth (1979)? (5) Is there evidence of reversion from stylar polymorphism to monomorphism? (6) What is the role of floral morphology in promoting transitions among stylar conditions? We begin by providing a brief review of the systematics and natural history of *Narcissus* to provide a context for our historical reconstructions. We then use new molecular systematic data from the plastid genome to infer phylogenetic relationships in *Narcissus* and to reconstruct the evolution of stylar polymorphisms and associated floral characters in the genus.

GENERAL BACKGROUND ON *NARCISSUS*

Narcissus includes all species known as daffodils and narcissi and is comprised of approximately 65 species of perennial geophytes, geographically concentrated in the Mediterranean region, particularly the Iberian Peninsula, southern France, and Morocco (Blanchard, 1990). There are more than 20 000 registered names that represent over a century's worth of breeding effort directed towards plant improvement, and a history of cultivation dating back at least to the seventeenth century, and possibly substantially earlier (Wells, 1989; Blanchard, 1990; Jefferson-Brown, 1991). *Narcissus* is one of the most economically important ornamental plants (Jefferson-Brown, 1991; Hanks, 2002), and yet remarkably little is known about the floral and pollination biology of members of the genus. Phylogenetic relationships in the genus also are largely unknown (e.g., Peréz et al., 2003).

The taxonomy of *Narcissus* is unsettled, and species circumscriptions vary widely. Early workers recognized up to 160 species or as few as 16 (reviewed in Blanchard, 1990). Blanchard recognizes ~65 species. With some minor exceptions, Blanchard (1990) used the classification scheme of Fernandes (1968a), and he also summarized recent taxonomic work in the genus. We follow Blanchard's treatment (1990) here. Blanchard viewed Webb's (1980) classification of *Narcissus* in *Flora Europaea* as overly conservative in the number of taxa recognized at the species level. Webb's classification has the additional disadvantage that it does not deal with the non-European taxa. The most recent classification scheme of *Narcissus* (Mathew, 2002) incorporates elements of Fernandes' and Webb's schemes. Mathew (2002) also noted that substantial revisionary work is needed for some taxa in the genus.

Fernandes (1968a) divided *Narcissus* into two subgenera (*Hermione* with base chromosome number $x = 5$, and *Narcissus* with $x = 7$) and 10 sections (*Apodanthi*, *Aurelia*, *Bul-*

bocodii, *Ganymedes*, *Jonquillae*, *Narcissus*, *Pseudonarcissi*, *Serotini*, *Tapeinanthus*, *Tazettae*) based on his extensive chromosomal studies of the genus, conducted over more than four decades (summarized in Fernandes, 1967, 1968a, b, 1975). He also suggested pre-cladistic phylogenetic schemes for the genus (summarized in Fernandes, 1951). Until very recently (see Discussion), there have been no efforts to evaluate systematic relationships in *Narcissus* using modern phylogenetic approaches.

All *Narcissus* species are winter-growing and summer-dormant bulbs that are commonly found in open sites from low elevation marshes, through rocky hillsides, to high elevation montane pastures. Most species flower in late winter and spring, although five species flower in the autumn (*N. broussonetii*; *N. cavanillesii*, Fig. 1f; *N. elegans*; *N. serotinus*, Fig. 1m; *N. viridiflorus*, Fig. 1n). Little detailed information is available on the pollination biology of species of *Narcissus* (for an exception see Herrera, 1995). All species are insect-pollinated, with the majority possessing showy flowers (Fig. 1), some of which are highly scented (Dodson et al., 1997). The major pollinators visiting flowers of *Narcissus* species are bees, butterflies, flies, and hawkmoths (Arroyo and Dafni, 1995; Herrera, 1995; Barrett et al., 1996; Baker et al., 2000a; Worley et al., 2000; Arroyo et al., 2002; Peréz et al., 2003; Thompson et al., 2003). Most species have white, pale yellow, or deep yellow flowers (Fig. 2), although the only night-flowering species in the genus, *N. viridiflorus* (Fig. 1n), has highly scented green flowers that are pollinated by crepuscular moths (Vogel and Müller-Doblies, 1975). Flowers in *Narcissus* vary in diameter from ~12 to 125 mm and are borne either solitarily (e.g., section *Bulbocodii* and most members of section *Pseudonarcissi*) or in inflorescences that can contain as many as 15–20 flowers (*N. papyraceus* and *N. tazetta*, Fig. 1k). The most prominent features of *Narcissus* flowers are the corona, a cylindrical cone extending beyond the tepals, and the floral tube. Coronal morphology is variable, ranging from a tiny ochre-to-yellow pigmented disk in *N. serotinus* (Fig. 1m) and a rudimentary structure in *N. cavanillesii* (Fig. 1f) to long trumpet-like structures (e.g., *N. alpestris*, Fig. 1c; *N. cyclamineus*, Fig. 1i) in section *Pseudonarcissi* (hence, the name ''trumpet daffodils''). Floral tubes range from long and narrow in species of sections *Apodanthi* and *Jonquillae* (Fig. 1e, n) to virtually absent in *N. cavanillesii* (Fig. 1f). Finally, flower orientation varies from pendant (*N. triandrus*, Fig. 1a; *N. alpestris*, Fig. 1c) to horizontal (*N. gaditanus*, Fig. 1e; *N. poeticus*, Fig. 1j) or erect (*N. cavanillesii*, Fig. 1f; *N. serotinus*, Fig. 1m).

Field observations of pollinators visiting flowers of *Narcissus* species over the past decade establish three primary functional groups (L. D. Harder, University of Calgary, and S. C. H. Barrett, personal observations). The first group, exemplified by members of sections *Pseudonarcissi* (Figs. 1c, i) and *Bulbocodii* (Fig. 1d, h), possesses flowers with large funnel-like coronas and short, wide, or highly funnelform floral tubes. These flowers are pollinated by a wide range of small- and large-bodied bees that generally forage for pollen from anthers enclosed within the corona (Herrera, 1995). The second involves species with long, narrow floral tubes, relatively shallow coronas, and horizontally orientated, highly fragrant flowers (e.g., sections *Jonquillae*, Fig. 1e; *Apodanthi*, Fig. 1g; and *Narcissus*, Fig. 1j). Members of these sections are primarily adapted for pollination by long-tongued Lepidoptera, mostly sphingid moths (e.g., *Macroglossum* spp.), although flowers are also visited by long-tongued bees, butterflies, and flies. Nectar serves as the main floral reward in these species. The final group combines structural elements from the first two groups, as flowers possess both well-developed and narrow floral tubes and extended coronas. This combination of floral traits only occurs in two species: distylous *N. albimarginatus* (Fig. 1b) and tristylous *N. triandrus* (Fig. 1a). *Narcissus triandrus* is pollinated primarily by long-tongued solitary bees (primarily *Anthophora* and *Bombus* spp.), which forage for nectar and pollen. No pollinator information is currently available for the rare Moroccan endemic *N. albimarginatus*.

MATERIALS AND METHODS

Taxon sampling and DNA sequencing—We examined 32 species from the 10 sections of *Narcissus* recognized by Fernandes (1968a, 1975) and four outgroup species for DNA sequence variation in two rapidly evolving plastid regions (see Appendix in Supplementary Data accompanying the online version of this article). The outgroup species sampled include representatives from both genera in tribe *Galantheae* (= *Galanthus* and *Leucojum*) and *Narcisseae* (= *Narcissus* and possibly *Sternbergia*) as defined by Dahlgren et al. (1985), who also speculated that these tribes deserve combination. A close relationship between *Narcissus* and *Sternbergia* is supported by chromosomal evidence (Flagg and Flory, 1962). *Lapiedra martinezii* was included because of its possible sister-group relationship to *Narcissus* as noted by Meerow et al. (1999) based on plastid evidence. In *Narcissus* multiple populations were sampled for a number of species, including three of its four monotypic sections (*Aurelia*, *Ganymedes*, *Tapeinanthus*, but not *Serotini*). Although noted in the Appendix, we excluded replicate populations from the phylogenetic analysis, except for an unusual population of *N. bulbocodium* (see Results: Phlyogenetics of *Narcissus*) and *N. triandrus*, for which we included representatives of three of four varieties recognized by Blanchard (1990).

One of the plastid noncontiguous regions examined is a rapidly evolving portion at the 3′-end of *ndhF*, the gene for subunit F of plastid NADH dehydrogenase. The other region, referred to here as *trnL-F*, spans two plastid tRNA transferase (*trn*) genes, *trnL*(UAA) and *trnF*(GAA), and consists primarily of two noncoding regions; the *trnL*(UAA) intron and an intergenic spacer between the two *trn* genes. We generated the majority of the *ndhF* sequences using a Sequenase version 2.0 kit (United States Biochemical, Cleveland, Ohio, USA) with 35-S labeled dATP (following manufacturer instructions) and using DNA amplification and sequencing protocols outlined in Graham et al. (1998). Automated sequencing was used to generate sequences for the *trnL-F* region, and a subset of those from *ndhF*, following protocols in Graham and Olmstead (2000). Primers used for amplification and sequencing were designed by Olmstead and Sweere (1994) and Graham et al. (1998) for *ndhF*, and by Taberlet et al. (1991) for *trnL-F*.

Analysis—We performed alignments using criteria outlined in Graham et al. (2000). Alignment gaps were coded as ''missing data'' for phylogenetic analysis, but we included two binary characters representing parsimony-informative insertion/deletions (indels) in the intergenic spacer region between *trnL* and *trnF*. Across the 37 taxa of *Narcissus* considered in the first phylogenetic analysis, representing 34 species, there are 72 parsimony-informative characters. Of these, 37 are from *ndhF*. Using *N. papyraceus* as a reference taxon, the *ndhF* region examined here is 490 base pairs [bp] long, and the unaligned portion of the *trnL-F* region that we examined is 971 bp in length. Because we had to sample nearly twice as many characters in the *trnL-F* region compared to *ndhF* to obtain a comparable amount of parsimony-informative variation, the latter protein-coding region thus evolves approximately twice as fast, per nucleotide, as the predominantly noncoding *trnL-F* region.

We conducted maximum-parsimony analysis of the DNA sequence data using PAUP* version 4.0 beta 10 (Swofford, 2002). All characters and character-state changes were equally weighted. Heuristic searches were conducted using 10 000 random addition replicates and tree bisection-reconnection (TBR) branch-swapping, with the ''MulTrees'' option activated. We included or excluded several taxa of known or suspected hybrid origin, including *N. dubius* and *N. tortifolius* (see Results: Phylogenetics of *Narcissus*). Branch support was evaluated using bootstrap analysis (Felsenstein, 1985), considering only a single random addition sequence for each of 100 replicates. Analyses were performed on combined data from both regions, because inspection of bootstrap profiles from separate analysis of each region indicated no serious conflict among them (i.e., there were no conflicting regions with more than 50% bootstrap support in both data sets; data not shown).

We also estimated branch support using MrBayes 3.0 beta 3 (Ronquist and Huelsenbeck, 2003). Modeltest version 3.06 (Posada and Crandall, 1998) was used to determine the DNA substitution model that best fits the data, among those available. The model chosen for our *Narcissus* data using hierarchical likelihood ratio tests was ''K81uf + Γ.'' This model has three distinct substitution rate categories (one transition rate, two transversion rates) and accounts for among-site rate variation using the gamma (''Γ'') distribution. The least complex substitution model implemented in MrBayes that permits at least three substitution rates is the general time reversible (GTR) model. We ran four Markov chains on the combined plastid data for one million generations with the number of substitution types set to six and with among-site rate variation accounted for using the gamma distribution (i.e., the ''GTR + Γ'' model). Chains were heated using the default temperature (0.2), and were sampled at intervals of 1000 generations. The log likelihood sum appeared to stabilize before the first 100 000 generations. We discarded trees sampled up to this point to calculate posterior probabilities of individual clades.

Character codings for ancestral-state reconstructions—Based on our field observations of the majority of taxa and herbarium surveys, we coded the stylar condition of taxa according to whether they are monomorphic, dimorphic for stigma height (but without reciprocal herkogamy), distylous, or tristylous. A few *Narcissus* species exhibit populations with two stylar conditions (e.g., *N. dubius*, *N. papyraceus*, and *N. tazetta* have stigma-height dimorphic and monomorphic populations [Baker et al., 2000b; Arroyo et al., 2002; Arroyo and Dafni, 1995, respectively]; *N. triandrus* has trimorphic and dimorphic populations [Barrett et al., 1997, 2004]). However, as discussed earlier for other tristylous groups, microevolutionary evidence suggests that in such cases monomorphism (Arroyo et al., 2002) and dimorphism (Barrett et al., 2004) are likely to be recently derived intraspecific phenomena associated with the loss of style morphs from populations, and so we did not score these taxa as having among-population variation in the class of stylar polymorphism. Based on Fernandes' (1940) review of 19th century descriptions of *N. broussonetii* it is possible that this species also possesses a stigma-height dimorphism. However, our own examination of herbarium specimens of this species and the tendency of earlier workers to misclassify *Narcissus* species as heterostylous (see Barrett et al., 1996) lead us to believe that this species does not have stigma-height dimorphism, and so we score it as monomorphic for style length. All outgroup genera considered here are uniformly monomorphic for style length.

The floral tube in *Narcissus* is the narrow, nearly cylindrical to funnelform perianth structure formed by fusion of the six tepal bases. Floral tubes are coded here either as ''long and narrow'' (at least 10 mm and less than 5 mm wide) or ''all other floral tube types.'' We group in the latter functional category those taxa with very short tubes or that lack them completely (such as *N. cavanillesii*), with taxa possessing long and wide tubes (most members of section *Pseudonarcissi*) to highly funnelform tubes (section *Bulbocodii*). In our judgement none of these forms permit very precise depth-probing by pollinators, in contrast to taxa with long, narrow tubes. The corona (paraperigone) in *Narcissus* is a tubular outgrowth above the floral tube that develops from the perianth (Arber, 1937; Singh, 1972). Coronas are coded here as either ''deep'' (at least 10 mm) or ''absent or shallow'' (''shallow'' coronas are ~5 mm or less in depth). Our size cut-offs relate to what we understand to be functionally important with regard to plant–pollinator interactions and refer to natural measurement breaks across the taxa. We used the floral measurements provided by Blanchard (1990), supplemented with personal observations.

Reconstructions of character evolution—We performed character mappings using MacClade 4.03 (Maddison and Maddison, 2001). All character reconstructions were performed with character-state changes equally weighted (Fitch, 1971; Hartigan, 1973) and with taxa of known or suspected hybrid origin excluded (i.e., with *N. dubius* and *N. tortifolius* and a suspected case of introgression involving *N. bulbocodium* removed; see Results).

None of the most parsimonious (MP) trees we obtained were fully resolved; three of the four MP trees (see Results: Phylogenetics of *Narcissus*) were partial resolutions of the fourth (the least-resolved one). We interpret polytomies on our trees as ''soft'' (corresponding to a lack of evidence for resolving speciation events; see Maddison, 1989), rather than being ''hard'' (= multiple and simultaneous speciation events). We removed some polytomies that are not relevant to the evolution of the morphological characters considered here prior to reconstructions of their evolution, by including only one species in instances where two or more species had identical plastid sequences (or in the case of *N. scaberulus* parsimony-uninformative variation); such species were removed only if they possessed stylar and floral character states identical to the representative species left behind. Two MP trees inferred from this reduced taxon set were identical to the four trees inferred from the more complete set, when deleted taxa in the reduced data set are pruned from the latter trees. However, the least resolved of these two trees still contains three polytomies (two trichotomies and a tetrachotomy). Different resolutions of these polytomies can potentially result in different character reconstructions. We therefore bracketed the actual reconstructions that would be inferred on the fully resolved plastid-based tree by considering all possible resolutions of the different polytomies on the least resolved tree (requiring 135 different resolutions [$= 3 \times 3 \times 15$] from two trichotomies and one tetrachotomy). Enumeration of character-state changes across this set of trees was performed using the ''State Changes and Stasis'' tool in MacClade. Trees that represent all possible polytomy resolutions were obtained by completely but randomly resolving polytomies on the least resolved MP tree, using the ''Create Trees'' tool in MacClade. The ''Condense Trees'' option in PAUP* was then used to eliminate nonidentical topologies from a large pool of these resolutions, yielding the 135 possible resolved trees.

Concentrated-changes tests—The concentrated-changes test (CCT; Maddison, 1990) can detect whether particular types of transition to a derived state in a dependent character are more frequent than would be expected by chance, with regards to the proportion of branches that possess a given state for the independent character and the amount and type of change in the dependent character. The CCT as implemented in MacClade requires binary characters, fully bifurcated trees, and unequivocal parsimony-based character reconstructions. The latter two conditions are not met in our case, but we bracketed the probability estimates that would be obtained on the fully bifurcated plastid tree by repeating all CCTs on the 135 possible resolutions of the polytomies on the least resolved MP tree and by assessing two contrasting schemes of character optimization where reconstructions were partly equivocal (see later).

An a priori decision must also be made on the state in the independent character that may be associated with a particular type of change in the dependent character. We used the CCT to examine whether certain types of stylar condition (dependent character) evolve more commonly in the context of particular types of floral tube and corona (independent character). In one set of CCTs, we addressed whether polymorphic stylar conditions (stigma-height dimorphism, distyly, and tristyly, considered collectively) are more likely to evolve when the flower has a long, narrow floral tube. If so, this would be consistent with the hypothesis (see Discussion) that a floral morphology that allows high-precision depth probe pollination is a necessary requirement for the evolution of any stylar polymorphism. A second set of tests was used to investigate whether heterostyly is more likely to evolve when flowers have both long, narrow floral tubes and deep coronas. This floral morphology should permit greater segregation of pollen deposition on pollinator's bodies and therefore permit the evolution of reciprocal stigma-anther positioning (heterostyly) in *Narcissus* (Barrett et al., 1996; Arroyo and Barrett, 2000).

The probability of concentrated change was estimated in each case using

MacClade, via simulation (Maddison and Maddison, 2001); actual changes were counted across 5000 simulations in each case. We considered both accelerated and delayed transformation (ACCTRAN and DELTRAN) resolutions (Swofford and Maddison, 1987) when equivocal character reconstructions were encountered for a particular polytomy resolution, but used the same character optimization scheme across the two characters when both were partly equivocal. Although outgroups were included to permit character reconstruction, we conducted the CCT on *Narcissus* only, excluding all outgroups from the test group to reduce the proportion of white branches and hence minimize type I error (see Lorch and Eadie, 1999).

Prior to performing each CCT, the dependent character (''stylar condition'' for the first tests, ''heterostyly'' for the second) was scored visually to count the number of changes to the state of interest (''stylar polymorphism'' from ''stylar monomorphism'' for the first tests, ''heterostyly'' [distyly or tristyly] from any other stylar condition for the second) on branches that also possessed the distinguished state in the independent character. The independent character for the first set of CCTs is ''floral-tube,'' with a ''long and narrow'' tube its distinguished state (the alternative state is: ''all other floral tube types''). In the second CCTs, the independent character reflects the combination of floral tube and corona status, with the distinguished state being a ''deep corona and long, narrow tube'' (the alternative state is a placeholder for any other combination of floral tube and corona type).

One problem with performing the test concerns how to score cases where there is a ''simultaneous'' change to the derived state in both the dependent and the independent characters. Such event classes may or may not support the hypothesis of dependence, because there is no way with parsimony to determine the relative order of change on the branch (Donoghue, 1989; with parsimony these ''simultaneous'' changes can be considered as occurring in either order anywhere along the branch). It can be argued, therefore, that the count should be restricted to non-simultaneous cases. However, Donoghue (1989) and others (Armbruster, 1993; Frumhoff and Reeve, 1994) have also pointed out that we might expect traits with the strongest evolutionary associations to result in rapid, successive changes: the stronger the evolutionary association, the more likely we would be to see ''simultaneous'' transitions in independent and dependent characters on the tree. We therefore performed the first set of CCTs (concerning whether polymorphic stylar conditions are more likely to evolve when there is a long, narrow floral tube) with ''simultaneous'' changes either excluded or included from the character change score. For the second set of tests, which address whether heterostyly is more likely to evolve when flowers have long, narrow floral tubes *and* deep coronas, only ''simultaneous'' changes were inferred and therefore used in CCTs.

RESULTS

Phylogenetics of Narcissus—Four MP trees were inferred from the full data set (Fig. 3), and two from the reduced taxon set used in reconstructions of character evolution (see Figs. 4, 5). The four MP trees from the full data set differ from each other only in two polytomous regions, one involving the three populations of *N. triandrus* (Fig. 3). The two MP trees inferred using the reduced taxon set (not shown) are completely consistent with the four MP trees from the larger data set, subject to tree pruning in the latter case. Bootstrap support values and Bayesian posterior probabilities are presented in Fig. 3; the two support measures generally appear to be strongly correlated with each other, although the percentage values reported for posterior probabilities are often somewhat higher that the corresponding bootstrap values. To simplify the presentation here, we generally note only bootstrap support values in the text. With the rooting among outgroups employed here (at tribe *Galantheae*), *Narcissus* and *Sternbergia* are depicted as sister taxa (with good support; bootstrap support value, BV, 95%; Fig. 3) among the genera considered. The monophyly of *Narcissus* is moderately well supported (BV = 86%; and a Bayesian posterior probability estimate of 100%).

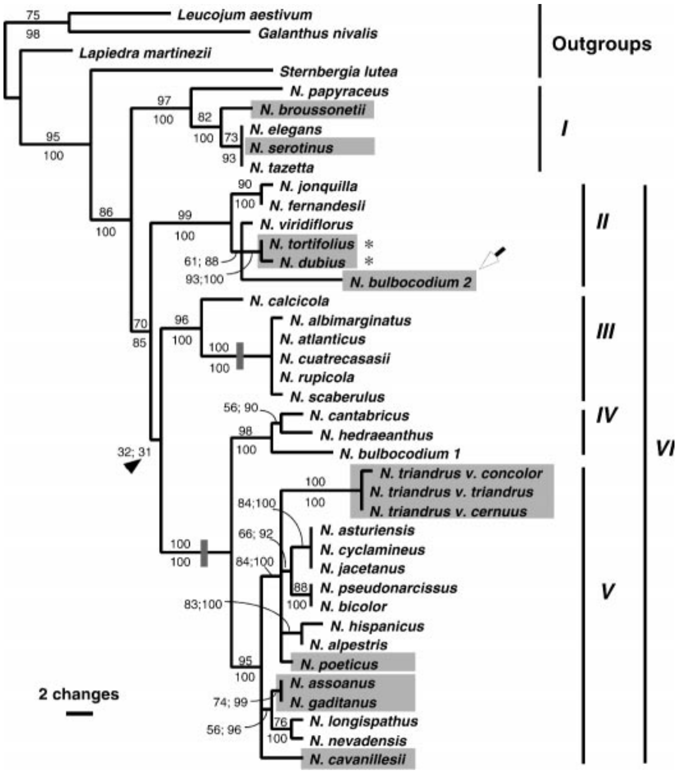

Fig. 3. Phylogeny of *Narcissus* based on parsimony analysis of combined *ndhF* and *trnL-trnF* DNA sequence data, including two indels. The tree (one of four most-parsimonious) is presented as a phylogram, with branch lengths computed using ACCTRAN optimization (length = 197 steps; consistency index = 0.878; retention index = 0.945). Trees were rooted at *Galanthus* and *Leucojum* equivalent to tribe *Galantheae* (as circumscribed by Dahlgren et al., 1985). The branch marked with an arrowhead collapses in two most-parsimonious trees, and two of three varieties of *N. triandrus* (*cernuus* and *concolor*) are resolved as a clade on two most-parsimonious trees (subtended by a branch of length one step under DELTRAN optimization). Bootstrap support values and Bayesian posterior probabilities presented as percentages are indicated beside branches (the former either above branches or to the left of paired values; the latter below branches or to the right of paired values). Several numbered clades (I–VI) correspond approximately or exactly to named infrageneric taxa; highlighted lineages disrupt the monophyly of individual sections (see text). The asterisk indicates putative allopolyploid species noted by other authors; an unfilled arrow indicates a probable introgressant population of *N. bulbocodium* (individual 2 here). Gray bars indicate two indels in the intergenic spacer region between *trnL*(UAA) and *trnF*(GAA) that act as unreversed synapomorphies for their respective clades.

We briefly summarize our phylogenetic findings within the genus with respect to the infrageneric taxa recognized by Fernandes (1968a, 1975). Two clades defined by the well-supported basal split in *Narcissus* correspond to subgenera *Hermione* (clade I, 97% BV; Fig. 3) and *Narcissus* (clade VI, 70% BV; Fig. 3), respectively. Section *Tazettae* in subgenus *Hermione* is not monophyletic, because it includes *N. broussonetii* and *N. serotinus* (corresponding respectively to two monotypic sections, *Aurelia* and *Serotini* highlighted in clade I; Fig. 3). *Narcissus elegans* is identical to *N. serotinus* (= section *Serotini*; Fig. 1m) and *N. tazetta* (Fig. 1k) for the plastid regions examined here (Fig. 3).

Only one section in subgenus *Narcissus* (clade VI) is clearly monophyletic (*Apodanthi* corresponding to clade III in Fig. 3; 96% BV). The plastid data define two major lineages within *Apodanthi*. *Narcissus calcicola* is the sister group of the re-

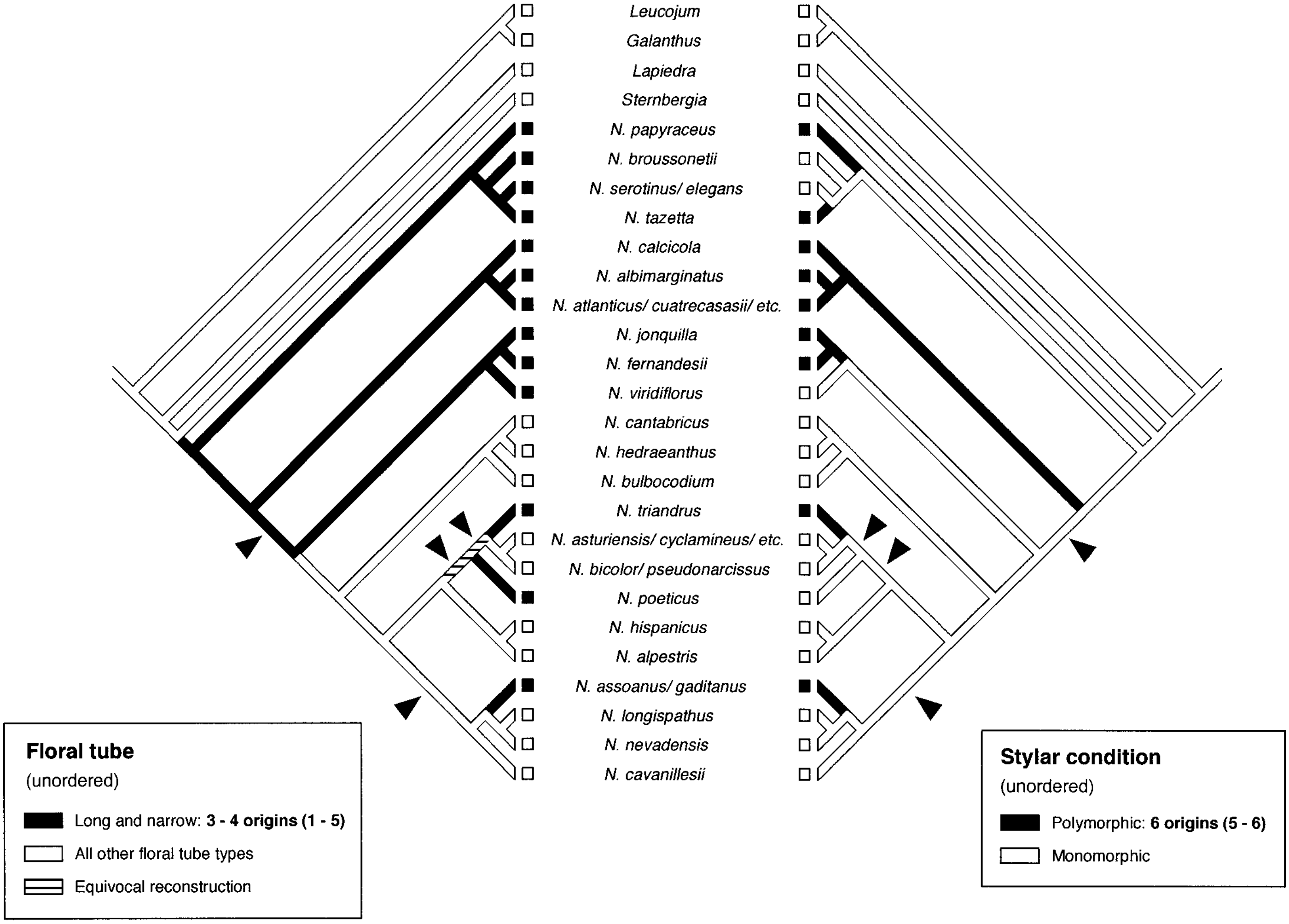

Fig. 4. Relationship between floral tube status and stylar condition in *Narcissus*. Some taxa with identical floral conditions are reduced here to single termini; taxa of suspected hybrid origin are excluded (see text). The tree is one of 135 resolutions of the least resolved most parsimonious tree; resolved branches are indicated with arrowheads. Changes in floral tube status are reconstructed on the left-hand reconstruction (with three or four origins of long, narrow tubes indicated under ACCTRAN and DELTRAN optimization, respectively). The right-hand reconstruction illustrates shifts in stylar condition. Six origins of "stylar polymorphism" (stigma-height dimorphism, distyly, and heterostyly; see text) are inferred. Numbers in brackets indicate ranges across all 135 possible polytomy resolutions for the derived character states.

mainder of the section. This basal split is well supported by bootstrap analysis (100% BV) and by a unique, unreversed 4-bp indel (Fig. 3) located in the intergenic spacer region between *trnL*(UAA) and *trnF*(GAA). The indel is inferred to represent an insertion. Three of five remaining species of *Apodanthi* considered here are indistinguishable from each other for the plastid regions examined (Fig. 3); the two species with parsimony-uninformative variation in this polytomy are *N. albimarginatus* (Fig. 1b) and *N. scaberulus*.

A deep trichotomy observed in half of the MP trees for subgenus *Narcissus* is resolved in the others (Fig. 3). This resolution is very poorly supported (32% BV). Clade II (99% BV) corresponds partly to section *Jonquillae*, except that it excludes subsection *Juncifoliae* A. Fernandes (represented here by *N. assoanus* and *N. gaditanus*, part of clade V; Fig. 1e) and includes two intersectional allopolyploids (involving sections *Jonquillae* and *Tazettae*; Fernandes, 1937, 1967; Romero et al., 1983); *N. dubius* (Fig. 1l) and *N. tortifolius*. These two taxa are stable, fertile allopolyploids (Blanchard, 1990) that are nearly identical to each other for the plastid regions considered here (Fig. 3). Clade II also includes a representative of a population of *N. bulbocodium* that presumably was derived by introgression with an unknown member of section *Jonquillae* and that gained its plastid genome in the process. We hypothesize this introgression because of the disjunct position of this *N. bulbocodium* sample in clade II relative to the other sampled representatives of section *Bulbocodii* (clade IV, discussed later). The introgressant appears to be indistinguishable from other *N. bulbocodium*, although its morphology and genetics warrant closer examination. Five other individuals sampled in the same population possess the same introgressed plastid genome (S. W. Graham, unpublished data). The other members of section *Jonquillae* in clade II (*N. fernandesii*, *N. jonquilla*, and *N. viridiflorus*, Fig. 1n) correspond to subsection *Jonquillae*. Subsection *Jonquillae* is monophyletic if the putative hybrids (the allopolyploids and presumed introgressant) are excluded from consideration (e.g., Figs. 4, 5). The same basic tree topologies are inferred if these taxa are excluded from tree searches (not shown).

The third major lineage in subgenus *Narcissus* is comprised

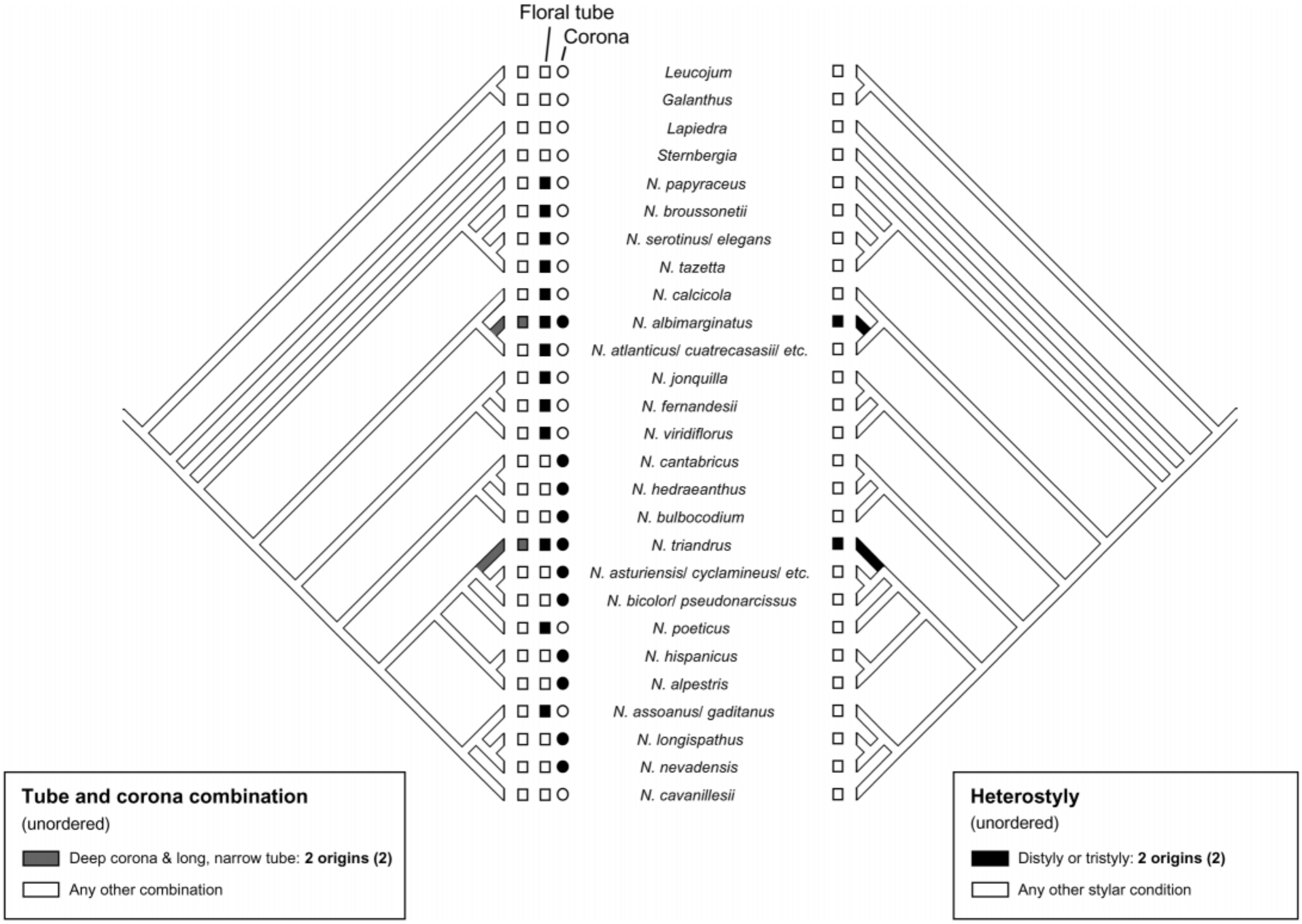

Fig. 5. Relationship between floral morphology and the origin of heterostyly in *Narcissus*. The tree is one of 135 resolutions of the least resolved most parsimonious tree (see Fig. 4). Flowers are characterized on the left-hand reconstruction as possessing either a deep corona and long, narrow floral tube or any other combination of tube and corona type (floral tube scores correspond to those used in Fig. 4; filled circles represent deep coronas and unfilled circles absent or shallow coronas). A reconstruction of the origin of heterostyly is depicted on the right; all taxa with stigma-height dimorphism or monomorphism are bracketed under a state encompassing these non-heterostylous conditions. All reconstructions across the 135 tree resolutions (see text) depict non-homologous origins of heterostyly in *Narcissus* (distyly in *N. albimarginatus*; tristyly in *N. triandrus*) and origins of heterostyly that occur ''simultaneously'' with the origin of the long-narrow floral tubes and deep corona combination. Numbers in brackets indicate ranges across all 135 possible resolutions for the derived character states.

of clades IV and V. This lineage is well supported in bootstrap analysis (100% BV) and by an additional unique and unreversed 19-bp indel located in the intergenic spacer region between *trnL*(UAA) and *trnF*(GAA), inferred to be a deletion. Clade IV corresponds approximately to section *Bulbocodii* (98% BV; Fig. 3). This section can be regarded to be monophyletic if the putative introgressant *N. bulbocodium* in clade II is excluded from consideration.

The final major lineage considered here (clade V, supported by 95% BV; Fig. 3) incorporates members of section *Pseudonarcissi* and several additional lineages (section *Ganymedes* [*N. triandrus*, Fig. 1a], section *Narcissus* [*N. poeticus*, Fig. 1j], section *Tapeinanthus* [*N. cavanillesii*, Fig. 1f], and *N. assoanus* and *N. gaditanus*, the two species that correspond to subsection *Juncifoliae*). Some of the substructure in clade V is well supported by bootstrap analysis. Two members of section *Pseudonarcissi* that are morphologically distinctive (*N. longispathus* and *N. nevadensis*) in possessing multi-flowered inflorescences are weakly supported (56% BV) as part of a clade with *N. assoanus* and *N. gaditanus* (Fig. 1e). This clade is part of a trichotomy in clade V, along with the morphologically distinctive *N. cavanillesii* (Fig. 1f) and a moderately well supported lineage (84% BV) that comprises the remainder of the clade. The latter lineage includes the sole tristylous species of *Narcissus*, *N. triandrus* (Fig. 1a), along with *N. poeticus* (Fig. 1j, section *Narcissus*) and the remainder of section *Pseudonarcissi*. While interrelationships are generally poorly resolved and supported among these taxa, several well-supported clades are apparent. These include a small clade comprising the three varieties of *N. triandrus* considered here (100% BV) and several clades of *Pseudonarcissi* (with 66–88% BV). The species that is perhaps the most distinctive trumpet daffodil florally, *N. cyclamineus* (Fig. 1i), is indistinguishable from two other members of *Pseudonarcissi* sampled here (*N. asturiensis* and *N. jacetanus*) at the level of the two plastid regions that we considered.

Reconstructions of character evolution—Our reconstructions across 135 resolutions of the least-resolved MP trees indicate that stylar monomorphism is ancestral in *Narcissus* and that stylar polymorphisms arose on multiple occasions within the genus (5–6 to six origins across the 135 resolutions). An uncertainty in reconstructions of stylar condition concerns whether or not stigma-height dimorphism is homologous within several groups. Depending on how the deepest trichotomy in *Narcissus* is resolved (the branch marked with an arrowhead in Fig. 3), stigma-height dimorphism may or may not be homologous between *N. jonquilla-N. fernandesii* and the instances of its occurrence in section *Apodanthi* (i.e., in those resolutions with *Apodanthi* and *N. jonquilla-N. fernandesii-N. viridiflorus* depicted as sister taxa and using DELTRAN vs. ACCTRAN optimization, respectively). However, when it is inferred to be homologous, stylar monomorphism in *N. viridiflorus* then represents the only possible instance of reversion from stylar polymorphism to monomorphism in *Narcissus*. Figure 4 (right-hand reconstruction) illustrates a tree resolution in which stigma-height dimorphism is unequivocally not homologous between these two clades.

The two instances of heterostyly in the genus, distyly in *N. albimarginatus* and tristyly in *N. triandrus*, originated independently of each other (Fig. 5). Because of the relatively nested position of *N. albimarginatus* in section *Apodanthi* the distylous condition is inferred to be derived from stigma-height dimorphism (e.g., Figs. 4, 5, right-hand reconstructions). Tristyly in *N. triandrus* is inferred to have evolved directly from stylar monomorphism across all 135 resolutions (e.g., Figs. 4, 5, right-hand reconstructions).

It is not clear whether instances of long, narrow floral tubes are homologous in *Narcissus*. Between one and five origins and 0–4 losses of a long tube can be inferred across the 135 polytomy resolutions, depending on how equivocal optimizations are addressed. Consequently, the primitive floral tube condition in *Narcissus* is equivocal (one possibility is shown in Fig. 4). However, a long, narrow floral tube is always found to be homologous within subgenus *Hermione* (clade I) and between section *Apodanthi* (clade III) and the portion of clade II included in character reconstructions (e.g., Fig. 4).

Deep coronas evolved at least twice in *Narcissus* and perhaps up to five times, with 0–3 losses (reconstructions not shown here, but see state codings in Fig. 5). Some resolutions of polytomies on the MP trees result in a deep corona being inferred to be homologous across all instances of its occurrence among clades IV and V (not shown). However, the deep corona in *N. albimarginatus* is not homologous with that seen in the rest of the genus on any of the polytomy resolutions. As a consequence, the combination of a long floral tube and deep corona clearly evolved twice; once in *N. albimarginatus* and once in *N. triandrus* (Fig. 5; left-hand reconstruction).

Hypotheses of concentrated change—Between three and six origins of stylar polymorphism in *Narcissus* arose in lineages that possessed long and narrow floral tubes. Whether or not there was a significantly elevated number of origins of stylar polymorphism in these lineages depends mostly on whether instances with ‘‘simultaneous’’ change in these two character states are included in the CCTs. When these changes are excluded, in most cases no significantly concentrated origin of stylar polymorphism is inferred in lineages with long, narrow floral tubes ($P > 0.08$). However, in a subset of polytomy resolutions (14 of 135; not shown) where long, narrow tubes are potentially inferred to be homologous across all taxa with this state, no simultaneous changes are inferred, and in these cases there is a significant concentration of change ($P < 0.05$). In the remaining 121 polytomy resolutions, the origin of stylar polymorphism is also inferred to be influenced by the presence of a long and narrow floral tube ($P < 0.05$) when simultaneous changes are included in the CCTs. In the example shown in Fig. 4, four origins of stylar polymorphism are inferred following the origin of a long, narrow floral tube under DELTRAN optimization, with no concentrated change of the former character state if two simultaneous changes are excluded ($P = 0.2756$), but significantly concentrated change ($P = 0.0054$) if the simultaneous origins are included. Under ACCTRAN optimization of floral tube status, five origins of stylar polymorphism are inferred to arise following the origin of a long, narrow floral tube ($P = 0.1066$ if simultaneous changes are excluded; $P = 0.0080$ if one simultaneous origin is included).

Only simultaneous changes to heterostyly (distyly or tristyly) and flowers with a combination of a long, narrow floral tube and a deep corona are observed. The two instances of heterostyly (distyly in *N. albimarginatus*, tristyly in *N. triandrus*) evolved independently of each other, and both evolved on terminal lineages that also exhibited switches to flowers with long, narrow tubes and deep coronas (Fig. 5). There is a highly significant concentration of ‘‘simultaneous’’ switches to distyly or tristyly, and a long, narrow floral tube with deep coronas ($P < 0.005$ across all arbitrary resolutions examined). This provides further support for a direct causal relationship between perianth and corona morphology and the evolution of heterostyly in *Narcissus*. However, this finding is nearly independent of our knowledge of *Narcissus* phylogeny: so long as these two taxa are not each other's closest relatives, similar CCT probability values can be found on fully resolved random trees with this number of taxa and two simultaneous origins of these traits (data not shown).

DISCUSSION

The evolution of stigma-height dimorphism—Our historical reconstructions of the evolution of stylar polymorphisms in *Narcissus* provide evidence of multiple independent origins of stigma-height dimorphism in the genus (Fig. 4). Four or five origins of stigma-height dimorphism are implied by the plastid-based trees (5–6 origins of stylar polymorphism, minus one non-nested origin of heterostyly), with either one or no reversions to stylar monomorphism. The primitive condition in the genus is inferred to be stylar monomorphism, and so stylar polymorphism is inferred not to be homologous between the two subgenera. Within subgenus *Hermione* stigma-height dimorphism evolved twice (Fig. 4), once in *N. papyraceus* and once in *N. tazetta*; stylar polymorphisms also evolved twice or more in subgenus *Narcissus* (the precise number of origins depends on how polytomies are resolved).

One of the most intriguing questions this raises is why stigma-height dimorphism arose so readily in *Narcissus*, and persisted across multiple speciation events in some cases (Fig. 4), when it is so rare across the angiosperms as a whole. Stigma-height dimorphism represents a relatively simple floral polymorphism when compared to the heterostylous condition. In the majority of heterostylous species the floral morphs are usually distinguished by incompatibility reactions, stigma-anther position, and pollen and stigma polymorphisms (reviewed in

Barrett, 1992). In contrast, for *Narcissus* species with stigma-height dimorphism the main distinguishing feature that separates the floral morphs is a simple difference in style length, although minor variations in anther height have also been documented in *N. assoanus* (Baker et al., 2000c). Studies of the genetic control of style length in *N. tazetta* indicate that a single diallelic locus with dominance controls the inheritance of style length (Dulberger, 1967). In common with most distylous species, the dominant *S* allele at the style-length locus governs the expression of short styles (*Ss*), with the long-styled morph homozygous recessive (*ss*). If we assume a monomorphic ancestor with long styles, as seems likely for *Narcissus*, and a similar genetic control in other species, then the origin of stigma-height dimorphism requires only that a single dominant mutation establish and spread. The genetic and developmental changes required for this event to occur are likely to be much easier than for the evolution of distyly, in which the functional grouping of associated morphological and physiological traits may often be difficult to achieve, because of constraints arising from the linkage of genes controlling multiple traits associated with the polymorphism (Charlesworth and Charlesworth, 1979). Nevertheless, despite the morphological simplicity of stigma-height dimorphism, it is a very rare condition in angiosperms, suggesting that the appropriate conditions for selection to maintain the polymorphism may be limited in most taxa. This is presumably because in these instances stylar polymorphism provides only minimal fitness benefits associated with more proficient cross-pollination, in comparison with stylar monomorphism. Why then is stigma-height dimorphism common in *Narcissus*, and what features of their flowers might favor the maintenance of this polymorphism?

Stigma-height dimorphism in *Narcissus* is strongly associated with extended, narrow floral tubes (at least 10 mm and less than 5 mm wide) and pollination by long-tongued Lepidoptera, particularly hawkmoths (e.g., *N. assoanus*, Baker et al. [2000a]; *N. dubius*, Worley et al. [2000]; *N. papyraceus*, Arroyo et al. [2002]; *N. tazetta*, Arroyo and Dafni [1995]). These associations characterize all species with stigma-height dimorphism, although several species with stylar monomorphism also possess long and narrow floral tubes and are pollinated by hawkmoths (e.g., *N. viridiflorus*, Vogel and Müller-Doblies [1975]; *N. poeticus*, L. D. Harder and S. C. H. Barrett, personal observations). Lloyd and Webb (1992a, b) proposed that heterostyly is more likely to evolve from floral monomorphism in species with floral tubes and depth-probed pollination. This is because of the greater opportunity for segregated pollen deposition on the tongues and bodies of pollinators and the role of segregation in facilitating cross-pollination when stigmas and anthers are reciprocally positioned. This argument also seems likely to apply to species of *Narcissus* with stigma-height dimorphism, although precisely how segregated pollen deposition and intermorph pollination is favored without clear sex-organ reciprocity among short-level sex organs (see Fig. 2b) is unclear.

However, evidence that stigma-height dimorphism does indeed promote effective cross-pollen transfer between the floral morphs comes from two sources. First, populations of several species visited by long-tongued pollinators are characterized by 1 : 1 floral morph ratios (Baker et al., 2000c; Arroyo et al., 2002). Equality of morph ratios demonstrates clearly that symmetrical disassortative mating arising from cross-pollination between the floral morphs can be achieved in populations with incomplete sex-organ reciprocity. Second, a recent experimental study of *N. assoanus* comparing reproductive success in monomorphic vs. dimorphic arrays indicated improved cross-pollen transfer in 1 : 1 arrays (Thompson et al., 2003). Therefore, the close association between stigma-height dimorphism, elongated floral tubes, and nectar-feeding, long-tongued pollinators seems likely to have a functional basis related to effective cross-pollination. If this is true, it may also explain the absence of stigma-height dimorphism from sections of *Narcissus* without long, narrow floral tubes (and with large coronas) (e.g., *Pseudonarcissi* [Herrera, 1995]; *Bulbocodii* [L. D. Harder, and S. C. H. Barrett, personal observations]). Species in these sections are visited primarily by short-tongued pollen-collecting bees, which enter the corona and forage in a nonstereotypical manner. The behavior of these bees and their interaction with floral morphology is thus likely not conducive to the segregated pollen transfer required to maintain stylar polymorphism.

The evolution of heterostyly—Few studies have been able to address whether heterostyly has originated on multiple occasions among closely related taxa. In taxa with many heterostylous species, this seems quite plausible, but as yet no attempt has been made to explicitly test this hypothesis using character reconstructions in such groups, despite the availability of molecular phylogenies in Rubiaceae (e.g., Bremer and Manen, 2000) and Primulaceae (Mast et al., 2001), for example, two families in which heterostyly is especially well represented. Although heterostyly is rare in *Narcissus*, our phylogenetic analysis indicates that distyly and tristyly were independently derived in the genus. Both polymorphisms are restricted to a single species (distylous *N. albimarginatus* and tristylous *N. triandrus*). Sporadic origins of heterostyly are also known from other families (e.g., Lamiaceae, Barrett et al. [2000a]; Polemoniaceae, Cochrane and Day [1994]), but no other family contains both distyly and tristyly, with each restricted to a single species.

Historical reconstructions of character evolution have provided us with some insight into the evolutionary pathways by which these polymorphisms arose in *Narcissus*. They also enable an assessment of theoretical models for the evolution of heterostyly. The evolutionary pathway by which distyly originated in *Narcissus* is consistent with Lloyd and Webb's (1992a, b) theoretical model. In section *Apodanthi*, distyly evolved from ancestors with stigma-height dimorphism and these in turn evolved from monomorphic species that most likely possessed approach herkogamy. Because most *Narcissus* species are self-sterile (Barrett et al., 1996; Sage et al., 1999), the evolution of heterostyly likely occurred in ancestors that were largely outcrossing. If this is correct, the selective forces responsible for the evolution of distyly were unlikely to be related to rates of selfing and inbreeding depression. Instead, as proposed by Lloyd and Webb (1992a, b), reciprocal herkogamy in *N. albimarginatus* was probably selected to increase the proficiency of cross-pollen transfer. Below we consider why this may have occurred in *N. albimarginatus* and not other species in the genus with stigma-height dimorphism.

The evolutionary pathway that gave rise to tristyly in section *Ganymedes* is less clear. Unlike the situation discussed for section *Apodanthi*, heterostylous *N. triandrus* is nested among monomorphic members of section *Pseudonarcissi*. Thus, there is no phylogenetic evidence that tristyly evolved via an intermediate dimorphic stage (the pathway predicted by the model of Charlesworth [1979]). Because it seems rather unlikely that tristyly would evolve de novo from a monomorphic condition, it is worth speculating on the reason for the absence of an intermediate dimorphic stage. The most probable explanation, mentioned earlier in relation to other tristylous families (see *Introduction*), is that any dimorphic stage (distyly or stigma-height dimorphism) that preceded the origin of tristyly was ephemeral and is consequently not represented among extant taxa. This seems particularly likely in *Narcissus* because of several morphological and physiological features unique to this instance of tristyly. The only difference between the L- and M-morphs of *N. triandrus* is style length, because these two morphs share similar anther heights (Fig. 2d) and have the same intramorph compatibility system. The transition from dimorphism to trimorphism may therefore have simply involved a shortening of style length in the L-morph to create the third morph. In common with the establishment of stigma-height dimorphism (discussed in the preceding section), this change may have been achieved in a relatively straightforward manner, leading to the replacement of dimorphism by trimorphism. This may well provide an example of a case where phylogenetic reconstruction is not adequate for detecting changes that occurred on microevolutionary timescales and that involved relatively simple genetic and developmental modifications.

What aspects of the floral biology of *N. albimarginatus* and *N. triandrus* distinguish them from other *Narcissus* species and might have favored the independent evolution of heterostyly in sections *Apodanthi* and *Ganymedes*? Despite occurring in different clades, *N. albimarginatus* and *N. triandrus* have remarkably similar flowers that differ in shape from other *Narcissus* species (Figs. 1, 2). Both have pendant yellow flowers of similar size and shape, with deep coronas and long, narrow floral tubes, and flowers are produced in similar numbers each season (1–3 per plant). Flowering times are comparable (March–April) and, interestingly, the flowers of both species have a distinctive fragrance (Arroyo and Barrett, 2000) that is unique within *Narcissus*. These similarities exhibit all the hallmarks of functional convergences associated with pollination biology.

Arroyo and Barrett (2000) proposed that the striking convergence between the flowers of *N. albimarginatus* and *N. triandrus* may be associated with a shift from lepidopteran to long-tongued bee pollination. Pendant flowers with deep coronas prevent long-tongued Lepidoptera from contacting sex organs during nectar foraging, and only bees visit *Narcissus* species with large coronas and/or pendant flowers. This change in pollination biology may also have been causally associated with the evolution of heterostyly in *Narcissus*. According to this hypothesis, the unique combination of deep coronas and long, narrow floral tubes in *N. albimarginatus* and *N. triandrus* facilitated the segregated pollen deposition required for efficient cross-pollen transfer between the floral morphs in heterostylous species. This is because extended coronas provide a longer vertical area for separation of the two stamen levels within a flower. Deeper flowers should enable greater segregation of pollen deposition than is possible in species with stigma-height dimorphism and shallow coronas. In the latter case (Fig. 2b), both anther levels are positioned close together at the top of the narrow floral tube, and packing constraints may limit opportunities for selective divergence in anther position (Barrett et al., 1996). Thus, according to this view, the evolution of deep coronas combined with long, narrow floral tubes (Fig. 2c, d) may have been the structural innovations that promoted the evolution of heterostyly in *Narcissus*.

Caveats with regard to reconstructions of character evolution—One assumption that is commonly used when reconstructing character evolution is that plastid phylogenetic history is a reasonable estimate of organismal phylogeny. If taxa of hybrid origin are not recognized and removed from analysis, this can seriously mislead phylogenetic inference (e.g., Doyle, 1992). As we only have genetic evidence from one linkage group (the plastid genome), our only sources of external evidence for making inferences about the existence of hybrids in *Narcissus* come from chromosomal data or from ‘‘unusual’’ phylogenetic placements. We removed two putative hybrids from analysis that were recognized previously on the basis of chromosomal evidence (*N. dubius* and *N. tortifolius*: Fernandes, 1937, 1967; Romero et al., 1983) and one that is phylogenetically disjunct from the main clade of section *Bulbocodii* and that we therefore predict arose from an introgression event (*N. bulbocodium*, individual 2, Fig. 3). There are several records of natural hybrids involving members of section *Bulbocodii* (with sections *Pseudonarcissi* and *Ganymedes*; Fernandes, 1951) and *N. bulbocodium* is one of the few taxa with extensive variation in ploidy level (e.g., Fernandes, 1967). It is possible that there are other unrecognized hybrid taxa in our analysis. Confirming the putative *N. bulbocodium* introgressant and sorting out whether other taxa are misplaced due to other undetected hybridization events will require evidence from additional multiple unlinked loci from the nuclear genome. Until these data are produced, the current results should be viewed with this strong caveat.

In previous analyses of the evolution of heterostyly, we used biased weighting schemes to reconstruct the evolution of this complex polymorphism (Graham and Barrett, 1995; Kohn et al., 1996; Barrett and Graham, 1997). Here we limited consideration of different schemes to the use of ACCTRAN and DELTRAN optimization in situations where we observed equivocal reconstructions. However, using complexity arguments to justify the use of biased weighting schemes for the origin of stigma-height polymorphisms is less defensible in *Narcissus*. Genetic evidence suggests that stigma-height dimorphism can arise relatively easily; for example, in *N. tazetta* it involves only a simple style length change governed by a single allelic difference (Dulberger, 1967). It is more difficult to explain why stigma-height dimorphism persists in the genus, because on theoretical grounds (Lloyd and Webb, 1992a, b) we would expect it to transform into distyly relatively rapidly. The explanation for the stubborn persistence of the dimorphism in the genus is likely to come from further analysis of pollinator biology.

Heterostyly is one of the most complex sexual systems in flowering plants (Barrett, 1992), and we have argued previously that this justifies the use of a biased weighting scheme when reconstructing its evolutionary origin (e.g., Kohn et al., 1996). Homology of the two instances of heterostyly in the genus (distyly in *N. albimarginatus*, tristyly in *N. triandrus*) can be forced during character reconstruction (if they are scored simply as ‘‘heterostyly’’), but the bias against the origin of heterostyly would have to be very strong (around five- to seven-fold heavier than its loss, across the 135 trees considered in the CCTs; data not shown), and multiple instances of stigma-height dimorphism in section *Apodanthi* and elsewhere would then have to be interpreted as stable evolutionary reversions to stigma-height dimorphism. The latter scenario is not supported in any current evolutionary scheme. The weight of evidence therefore supports convergent origins of heterostyly in *N. albimarginatus* and *N. triandrus*.

Since we submitted this paper, we became aware of another study that attempted to reconstruct the evolution of stylar polymorphisms in *Narcissus* using phylogenetic approaches (Peréz et al., 2003). However, the phylogenetic inferences in this study were based on very limited taxonomic and genomic samples. Only a few sections of *Narcissus* were represented by multiple species in their study, and their main phylogenetic analysis was based on a single, short plastid region (the intergenic spacer region between *trnL*[UAA] and *trnF*[GAA]; they did not sample the *trnL*[UAA] intron), which provided only limited information on relationships. The consensus tree inferred from this region (their fig. 5) was consequently largely unresolved. Peréz et al. (2003) used this tree to infer convergent origins of heterostyly in *N. albimarginatus* and *N. triandrus*. Unfortunately, their tree lacks the robustness needed to validate such a conclusion. Indeed, polytomies and poorly supported branches ($<$50% bootstrap support) on their consensus tree can be resolved in such a way that it is ambiguous whether heterostyly is convergent across *Narcissus* (S. W. Graham, unpublished data).

General conclusions—Although not completely resolved, our plastid-based phylogeny of the genus *Narcissus* provides substantial new insights into the evolutionary history of stylar polymorphisms in this genus. Stylar monomorphism is the ancestral condition in *Narcissus* and stigma-height dimorphism appears to have evolved multiple times, perhaps with one loss, and yet it clearly has also persisted across many speciation events in the genus. The single instance of distyly in the genus, in the rare Moroccan endemic *N. albimarginatus*, likely evolved directly from stigma-height dimorphism, a transition scheme that is consistent with the evolutionary hypothesis of Lloyd and Webb (1992a, b). If tristyly evolved from an intermediate dimorphic stage, as proposed by Charlesworth (1979), this left no clear phylogenetic trace in our data, but it is quite possible that the microevolutionary timescale involved in such a transition was simply too rapid to have left a macroevolutionary footprint. Floral morphology and pollinator relationships seem likely to have played an important role in the evolution of stylar polymorphisms in the genus. Long, narrow floral tubes (correlated with relatively precise depth-probed pollination by Lepidoptera) probably promoted the evolution of stigma-height dimorphism. Finally, the unusual conjunction of long, narrow floral tubes and deep coronas, part of a suite of floral features associated with pollination by long-tongued solitary bees, likely facilitated the convergent origins of heterostyly in *Narcissus*.

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