New Idea

Late-acting self-incompatibility and a narrow floral tube as selective forces for stylar dimorphism in *Narcissus* (Amaryllidaceae)

Violeta I. Simón-Porcar

Violeta I. Simón-Porcar (violetasp@us.es), Departamento de Biología Vegetal y Ecología, Universidad de Sevilla, Apartado 1095, 41080 Sevilla, Spain

Abstract

Most heterostylous species show self- and intra-morph incompatibility and models established for such taxa have traditionally been applied to the evolution of stylar dimorphism and heterostyly in *Narcissus*, a genus with late-acting self-incompatibility. The model of Lloyd and Webb (1992a,b) proposed that, in an approach-herkogamous ancestor, stylar dimorphism and heterostyly appeared consecutively as a result of two single mutations selected positively to enhance cross-pollination. Most polymorphic *Narcissus* are stylar dimorphic with two anther whorls, the lower positioned in the middle of a narrow floral tube, and style lengths that locate the stigmas above or below the low-level anthers. Here, I propose that in an ancestor with open-tubed flowers, late-acting self-incompatibility and variable style length, the narrowing of the floral tube increased self-pollination and ovule discounting in individuals with the stigma at the same height as the low-level anthers, imposing disruptive selection against this phenotype and causing the bimodal distribution of style lengths. This hypothesis stresses the need of avoiding self-interference for the selection of stylar dimorphism. It does not exclude the promotion of cross-pollination as a force for subsequent evolution of heterostyly in the genus nor the need of inter-morph pollination for the maintenance of polymorphism.

Keywords: Herkogamy, heterostyly, late-acting self-incompatibility, self-interference, stylar dimorphism, *Narcissus*.

Heterostyly and stylar dimorphism are similar sex polymorphisms of hermaphroditic flowers whose characteristics and frequency of occurrence differ sharply among certain angiosperm families. Typical heterostylous species show two (distyly) or three (tristyly) floral morphs in which the stigmas and anthers are positioned reciprocally (i.e. they show reciprocal herkogamy; Darwin 1877, Ganders 1979, Barrett 2002). The morphs are associated with other di- (tri-) floral morphisms in the form or size of the stigma and pollen grains, called ancillary traits, and a heteromorphic self-incompatibility system (HetSI) that causes same-morph plants to be incompatible with one another. This complete syndrome, apparently codified by a single two- (three-) allele supergene (Lewis and Jones 1992), occurs in many taxa of at least 28 families (e.g. Primulaceae, Linaceae, and Turneraceae; Barrett and Shore 2008). In stylar dimorphism, floral morphs differ in the position of the stigma (either above or below one set of anthers; for most species with stylar dimorphism there is only a single whorl) while anther position remains similar, with low reciprocity among morphs (i.e. they show either approach or reverse herkogamy; Barrett et al. 2000). This polymorphism is uncommon in most of the above mentioned 28 families but frequent in various genera in two families (i.e. *Narcissus*–Amaryllidaceae–, and *Anchusa*, *Lithodora*, and *Glandora*–Boraginaceae–) where most stylar polymorphic species lack ancillary traits or the heteromorphic self-incompatibility system, but in turn present late-acting self-incompatibility (LSI; Dulberger 1970, Schou and Philipp 1984, Sage et al. 1999, Ferrero et al. 2012).

Figure 1. (a) Evolution of heterostyly proposed by Lloyd and Webb (1992a,b) modified slightly to accommodate the two anther whorls of *Narcissus*; (b) alternative hypothesis proposed here for the genus *Narcissus* and other taxa with LSI: late-acting self-incompatibility. The morphological condition and the selective force are indicated in each step, labelled in different colours when the models differ. Same colour of reproductive organs indicates cross-compatibility. Dotted lines indicate variation in style length. Note that in all steps the two anther whorls are retained though their positions within the floral tube converge in the evolution of reciprocal herkogamy.

In LSI, self-pollen tubes arrive to the ovary and even penetrate ovules but flowers fail to set fruits (Seavey and Bawa 1986, Gibbs 2014). It allows all cross-pollinations regardless of stylar morph types, presumably due to the existence of many alleles that govern the incompatibility system (Barrett et al. 1997, Arroyo et al. 2002, Ferrero et al. 2012), but it is particularly penalizing for self-pollination because it leads to ovule discounting (Seavey and Bawa 1986, Sage et al. 1994, Gibbs 2014). The waste of female gametes results in reduced seed production by cross pollen in subsequent pollinations (Dulberger 1964, Waser and Price 1991, Vaughton 1993, Vaughton et al. 2010, Simón-Porcar et al. 2015). Thus, highly self-pollinating phenotypes with LSI suffer a severe depletion of their female fitness.

The most frequently discussed models on the evolution of heterostyly *sensu stricto* include the models of Charlesworth and Charlesworth (1979) and Lloyd and Webb (1992a,b). Although both evolutionary models were developed for heterostyly with HetSI, the genus *Narcissus* has been frequently used to illustrate the model of Lloyd and Webb because it appears to embody the initial stages. Lloyd and Webb (1992a,b) proposed a scenario in which in an ancestral population of flowers showing approach herkogamy, stylar dimorphism appeared first and was followed by reciprocal herkogamy due to flowers with the dimorphism's ability to increase cross-pollination and reduce pollen wastage. HetSI would have appeared last to enhance the avoidance of self- and intra-morph fertilization (Figure 1a). This sequence of steps is based on the idea that the HetSI cannot be selected before style-length dimorphism. Without any morphological constraint to deliver pollen

reciprocally through pollinators, HetSI would lead to a high waste of pollen given the frequent pollinations between cross-incompatible individuals (Darwin 1877, Lloyd and Webb 1992a: 197). Accordingly, their proposed succession of events (reciprocal herkogamy followed by incompatibility) would not be mandatory in polymorphic species with a non-HetSI such as *Narcissus*, where all cross-pollinations are compatible (Lloyd and Webb 1992a: 197, Sage et al. 1999, Santos-Gally et al. 2015, Simón-Porcar et al. 2015). Lloyd and Webb (1992a: 202) proposed the favouring of cross-pollination as a selective force for stylar dimorphism. However, they acknowledged that for self-incompatible ancestors selection against self-pollination would probably have had a more important role than they assumed. In general, sexual self-interference is considered an important selective force in floral evolution (Barrett 2002) that may lead to the selection of herkogamous phenotypes (Webb and Lloyd 1986, Karron et al. 1997, Stone and Motten 2002, Larrinaga et al. 2009, Navarro et al. 2012).

Here, I propose a variation on the first steps of the evolutionary pathway of Lloyd and Webb (1992a,b) for stylar dimorphism in *Narcissus*. *Narcissus* flowers have one style and stigma and, in contrast with other style-dimorphic species, two anther whorls (with three anthers each). The lower anther whorl is positioned in the middle of a narrow floral tube in all dimorphic species (Figure 1a), and style dimorphism entails stigmas positioned either above or below this whorl. My hypothesis is that in an ancestor with open-tubed flowers (without floral tube, i.e. bowl- or funnel-shaped corollas), LSI and continuous variation in style length, the narrowing of the floral tube forced close contact between low-level anthers

and styles at the same height. This imposed negative selection on these due to self-pollination and ovule discounting, and therefore caused the bimodal distribution of style lengths (Figure 1b). Hence, rather than the promotion of cross-pollination as in Lloyd and Webb model (1992a,b), selection against self-pollination may have given rise to stylar-dimorphism in the group. The maintenance of dimorphism in populations, however, should still rely on between-morph cross-pollination through pollinators as determined in the model of Lloyd and Webb. The promotion of cross-pollination would also support the subsequent selection of reciprocal herkogamy and HetSI, although the former has been found in two species of *Narcissus* (Barrett et al. 1997, Arroyo and Barrett 2000), the latter has not yet been found in this genus. All transitions in the model of Lloyd and Webb, except the appearance of HetSI, are explained through the appearing and spreading of a single mutation of large phenotypic effect. Under my hypothesis, disruptive selection on a quantitative trait would account for the transition from the ancestral condition to stylar dimorphism. Subsequent gradual balancing selection would lead to reciprocal herkogamy (Figure 1b; and next section), although the action of single mutations of large phenotypic effect in this transition would not be inconsistent with my hypothesis.

A key point in this proposal is that narrow floral tubes should increase self-interference in comparison with open-tubed flowers, regardless of the fit of pollinators. Hence, the combination of continuous variation in style length and LSI could occur in an ancestor with open-tubed flowers because stigmas and stamens would be spaced out on the horizontal plane (Figure 1b). However, after the narrowing of the floral tube, massive incompatible self-pollination with ensuing ovule and pollen discounting in individuals with the stigmas at the same level as the lower anther whorl would select against this phenotype. This level of the floral tube would become a mal-adaptive position for the stigma and this would catalyse the appearance of the stylar dimorphism through disruptive selection. The position of upper anthers above the narrow floral tube would still allow their overlap with stigmas of the long-styled morph because they are separated horizontally and self-pollination would be of lesser magnitude. Thus, the long-styled morph includes phenotypes with stigmas located anywhere above the lower anther whorl, either below, at the same height or above the upper anther whorl (see next section and Figure 2). Eventually, the enclosure of the upper anthers in the narrow floral tube or through the convergence of stamens might lead to a second mal-adaptive position for the stigma and to the appearance of three levels of stigmas (morphs). This might well have preceded the evolution of tristylly in *Narcissus triandrus* (Bateman 1952, Barrett et al 1997).

Figure 2. Stigma and anther positions in a sample of 100 randomly selected individuals (one flower per individual) in a dimorphic population of *Narcissus papyraceus* (36° 6'N, 5° 44'W; Bolonia, Cádiz prov., Spain). a) Stigma and anther heights of flowers from different individuals ordered by the stigma height. b) Frequency distributions of herkogamy levels with respect to the lower whorl of anthers (distance between stigma and lower anthers -at the insertion point of the stamen-, in mm). Data from Arroyo et al. (2002).

The role of self-interference resulting from LSI as a selective force in the evolution of stylar polymorphism in *Narcissus* was considered by Yeo (1975) and Barrett et al. (1996). The latter authors modified the equations of the Lloyd and Webb's model (1992a) for the maintenance of a stylar dimorphism in populations by including a term for ovule discounting in floral morphs of *Narcissus*. They concluded that the low herkogamy in the long-styled morph with respect to the upper anther whorl of *Narcissus* (i.e. the short distance between stigma and upper anthers within an individual flower of this morph, see Figure 1a) would lead to self-pollination and ovule discounting. This could promote the spreading of a short-styled mutant with higher herkogamy (i.e. larger separation between the stigma and both anther whorls) and lower rates of self-pollination, and therefore to the appearance of stylar dimorphism. Though the cross-pollination hypothesis prevails, subsequent studies on polymorphism

species of *Narcissus* have also considered this viewpoint (Barrett et al. 1997, Cesaro et al. 2004). The hypothesis presented here differs significantly from the proposal of Barrett et al. (1996) as it reconsiders the ancestral morphological condition (approach herkogamy vs. continuous variation of style length, ranging both above and below the two anther whorls), the mode of appearance of stylar polymorphism (single mutations vs. disruptive selection), the subject of negative selection through ovule discounting (long-styled vs. intermediate-styled plants), and incorporates the decisive role of the narrow floral tube on triggering the negative effect of self-pollination.

Stylar polymorphism in *Narcissus*

Logical outcomes of the proposed evolutionary pathway include, first, that the stylar length may still present evident variation (i.e. a subdued bimodal distribution) rather than two absolutely discrete classes. Nevertheless, discreteness and reciprocal herkogamy would be derived states acquired later to improve precision in cross-pollination between morphs. These features may vary among populations or species according to their history and selective pressures, being likely greater in populations subjected to high selective pressures through pollinators and/or in derived species in the phylogeny. The second outcome would be that the less frequent style-length phenotype would be the one that has the stigma at the same height of the lower anther whorl, wherever the latter is located in each species (note that this is inherent to the definition of morphs in the genus either above or below this anther whorl). There is relatively abundant morphological information on the position of sexual organs in *Narcissus*. However, the majority of studies (e.g. Barrett et al. 1996, Pérez et al. 2004, Thompson et al. 2012) provide average values of sexual organ position which, although not inconsistent with my hypothesis, impede the ability to clearly ascertain the patterns of variation predicted.

In other cases, the availability of morphological information at the individual level allows me to explore my predictions. For instance, re-exploring the raw data of Arroyo et al. (2002) gave further insights into style-dimorphic *Narcissus papyraceus*, a relatively basal species in the phylogeny of the genus (Santos-Gally et al. 2012) that presents LSI (Simón-Porcar et al. 2015). Populations of this species show a bimodal but frequently overlapping distribution of style lengths in dimorphic populations, with the least frequent position located exactly at the level of the lower anther whorl (see an example population in Figure 2, data from other populations not shown). In addition, a morphological exploration reveals that stigmas situated at the same level as the lower anther whorl in this narrow-tubed *Narcissus* are very likely to be self-pollinated (Figure 3).

Figure 3. Unvisited flower of *Narcissus papyraceus* of intermediate style length in which the stigma shows a high level of self-pollination due to its close contact with low-level anthers.

Remarkably, a pilot study of the patterns of pollination and female fertility in a total of 104 individuals of *N. papyraceus* in the same population as Figure 2 (Simón-Porcar, unpublished) showed that the intermediate style-length phenotype receives twice the average stigmatic pollen load but has only two thirds of the seed set of either the long- or the short-styled individuals. These results are easily explained if the excess pollen is self, which due to the LSI present will cause ovule discounting.

The disruption between long and short styles seems to be consistently minor in populations of the Moroccan *Narcissus brousonetti*, another basal species in the phylogeny of the genus (Santos-Gally et al. 2012) which holds LSI and whose classification as style-dimorphic was subjected to controversy (Santos-Gally et al. 2015). In this species the lower anther whorl is presented in a relaxed manner very close to the upper whorl, above the floral tube, where this is wide. Under my hypothesis, this unusual morphology would imply lower self-pollination and hence a lower selective pressure against the intermediate style phenotype, which would explain the more continuous transition between floral morphs. On the other hand, discreteness and reciprocity between morphs seem higher, though variable, in populations of *N. assoanus* (Barrett et al. 1996, Baker et al. 2000). Baker et al. (2000) did not catalogue *N. assoanus* as heterostylous because the reciprocity was still imperfect. In contrast,

discreteness and reciprocity are at a maximum in *N. albimarginatus*, the only *Narcissus* species categorised as distylous in regards to morphology (Arroyo and Barrett 2000). More quantitative data at the individual level throughout populations and species of the genus are needed to investigate my hypothesis. However the available information seems compatible with a disruption in style length at the level of the lower anther whorl and gradual variation in sex organ positions approaching discreteness and reciprocal herkogamy in some *Narcissus* populations and species. Ultimately, the distribution of style morphs in the genus and beyond would result from the interplay between selection at the individual and species level (see Raduski et al. 2011 for an interesting discussion on this topic).

Future research

To test the hypothesis presented here, adopting a quantitative perspective to study the adaptive landscape depicted by herkogamy, and possibly correlated traits, is needed. First, extensive surveys of the patterns of pollination and female fertility of individuals with different herkogamies are required, including phenotypes with the stigma at the same level as the lower anther whorl, in various species of *Narcissus*. Studies of phenotypic selection including the costs on the female and male function would in turn provide the most accurate estimates of the magnitude of self-interference. The results from these studies could be modelled for diverse scenarios of inheritance of style length and over a large number of generations. This may shed light on the likelihood of self-pollination in phenotypes of intermediate style length as the evolutionary driver of stylar dimorphism in *Narcissus*.

The reliability of this proposal depends on the ancestral conditions of LSI, open-tubed flowers and variable stylar length in *Narcissus*, and studies of phylogenetic character reconstruction are needed to establish whether this is the case. The possibility of an ancestor with LSI in *Narcissus* is highly likely given its widespread occurrence in the genus (Dulberger 1964, Sage et al. 1999, Simón-Porcar et al. 2015) and in other Amaryllidaceae (*Sternbergia*, *Galanthus*, *Pancratium*, *Cyrtanthus*; Eisikowitch and Galil 1971, Dafni and Werker 1982, Chudzik et al. 2002, Vaughton et al. 2010; see also Lloyd and Webb 1992a: 165). Similarly, open-tubed flowers are the rule in related genera *Sternbergia*, *Pancratium*, *Galanthus*, *Acis*, *Leucojum*, and *Lapiedra*, and are the most likely ancestral condition in *Narcissus* (Meerow et al. 2006, Santos-Gally et al. 2012, 2013). This condition of open-tubed flowers proposed as the ancestral condition for *Narcissus* must not be confounded with wide-tubed flowers of some *Narcissus* sections (e.g. *Pseudonarcissus*) which are derived in the phylogeny, and monomorphic.

The ancestral condition of the variable stylar length will be the more challenging to support. Style length is a trait subjected to heavy selection, and any sign of variation in style length has likely been eliminated in most current populations. This fact could bias findings towards monomorphism. Microevolutionary processes in *Narcissus* and overall occurrence across insect-pollinated species from other plant families indicate that approach herkogamy is probably the most frequently selected condition (Webb and Lloyd 1986, Baker et al. 2000, Arroyo et al. 2002, Barrett 2002). To date, there is no solid evidence regarding the ancestral stylar condition in *Narcissus*. Graham and Barrett (2004) supported the ancestral condition of approach herkogamy based on a view that the related genera *Sternbergia* and *Galanthus*, and *Narcissus broussonetii* and *N. elegans*, basal species in their phylogeny of the genus, are all approach herkogamous. Nonetheless, there is a lack of extensive population surveys on the distribution of style lengths in the related genera, and Santos-Gally et al. (2013b, 2015) found in later studies that *N. broussonetii* and *N. elegans* are stylar dimorphic. In the best of cases, more detailed morphometric studies in *Narcissus* and other related genera of Amaryllidaceae, and the use of quantitative indices for the characterization of the polymorphism in phylogenetic comparative studies, are necessary to attempt to determine the ancestral condition with regard to phenotypic variability in style length.

Finally, detailed studies are needed on the genetic architecture underlying stylar polymorphism in *Narcissus*. The genetic control by a diallelic locus proposed for *Narcissus tazetta* by Dulberger (1964) was inferred from the segregation of morph progeny obtained from controlled hand pollinations. Although heterostyly and related stylar dimorphism are conventionally thought to be discrete, and hence governed by few genes, there is increasing interest in the existence of intra-specific an intra-population variation in floral traits related to these polymorphisms (e.g. Faivre and McDade 2001, Thompson et al. 2012, Haddadchi 2013, Brys and Jacquemyn 2015), which should entail more complex genetic foundations. A quantitative trait locus analysis of stylar length could help distinguish whether variation in this trait is due mainly to a single diallelic locus in *Narcissus*, as Lloyd and Webb (1992a) suggested, or to many loci or alleles, perhaps linked, as would be expected in the case of an ancient continuous variation (see Shore and Barrett 1990, Chen and Tanksley 2004, Yoshida et al. 2011). The increasing development of genomic and transcriptomic techniques should enable this exploration as in other heterostylous groups (Li et al. 2016, Nowack et al. 2015, Arunkumar et al. 2017). However, there will be some challenges in overcoming the difficulties imposed by the great genome size of monocots such as *Narcissus* (Zonneveld 2008). Furthermore, depicting the patterns of genomic variation

nearby the loci determining style length is a first step to distinguishing between an scenario of a pure selective sweep, expected from positive selection on new mutants as proposed by Lloyd and Webb (1992a,b), or a mixed scenario of background selection, resulting from negative pressure on intermediate style length, and genetic hitchhiking associated to heterostylous morphs, as proposed here. Alternative approaches to unravel the genetic basis of stylar polymorphism include (i) the study of the ontogenetic pathways in morphs from different species (e.g. Faivre 2000); and (ii) segregation analyses of herkogamy as a continuous trait in the progeny from crosses between L- and S- morphs combined with the development of threshold inheritance models (e.g. Ehlers et al. 2005).

Stylar dimorphism beyond *Narcissus*

Apart from *Narcissus*, the combination of stylar polymorphism, narrow floral tube and non-diallelic self-incompatibility is known to occur in only three genera of the family Boraginaceae: *Anchusa* (Dulberger 1970, Philipp and Schou 1981, Schou and Philipp 1983), *Lithodora*, and *Glandora* (Ferrero et al. 2012). Though the latter genera have not been studied in detail, it is known that *Anchusa* holds LSI (Schou and Philipp 1983). Therefore, the hypothesis proposed here might also be applicable to these genera. It is notable that these taxa are also characterized by a low reciprocity of floral morphs, with a pattern of variation in style-length similar to that in *Narcissus* (Philipp and Schou 1981, Ferrero et al. 2009, Ferrero et al. 2011). In addition, in certain species of *Lithodora* and *Glandora*, populations have either stylar dimorphism or heterostyly (i.e. reciprocal herkogamy) due to a gradual variation in the positioning of the sexual organs (Ferrero et al. 2011). These shifts seem to illustrate that cross-pollination can indeed promote the gradual transition from stylar-dimorphism to heterostyly (see also Quilichini et al. 2004). Moreover, it is noteworthy that a continuous variation in stylar length from approach to reverse herkogamy occurs in *Mertensia fusiformis*, another species of the same subfamily, the Boraginoideae (Forrest et al. 2011). This species is self-incompatible but lacks a narrow floral tube, which may preclude it from having a bimodal distribution of style lengths. Boragineaceae constitutes a compelling group for studying the evolution of heterostyly in general and for the hypothesis presented here. It includes great variation in stylar polymorphisms and HetSI, sometimes of incomplete function, as a derived character in other genera (Brys et al. 2008, Ferrero et al. 2012), and stylar polymorphisms appear to have various independent origins (Cohen 2013).

Heterostyly and stylar dimorphism have definitively evolved independently several times in angiosperms and we should expect that a number of diverse evolutionary

routes have been followed, with different selective pressures and ancestral conditions involved. Here I propose a novel variation in the well-established evolutionary model of Lloyd and Webb (1992a,b); many others possibly remain to be proposed and then tested.

Acknowledgements

I would like to thank J. Arroyo for his mentoring and for lending morphological data of *Narcissus papyraceus*; P.E. Gibbs, R. Santos-Gally, R. Pérez-Barrales, and S. Meeus for comments on different versions of the manuscript; J.L. Silva for assistance on the pilot field survey; D. Nesbitt for language revision, and S. Maurice and A. Faivre for their revisions.

Referees

Amy E. Faivre – aefaire@cedarcrest.edu
Cedar Crest College

Sandrine Maurice –
sandrine.maurice-oms@umontpellier.fr
Université de Montpellier

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Response to referee

I highly appreciate the contribution of Faivre (2018) in reviewing and commenting on this article. I agree with her comparison of my model with previously published hypotheses, specifically with the article by Barrett et al. (1996) and the evolutionary model of Charlesworth and Charlesworth (1979). Here, I want to highlight again the differences between my hypothesis and Barrett et al. (1996) in regards to the different *effects* attributed to the narrow floral tube (i.e. increasing self- vs. enhancing cross-pollination) and the ancestral condition (i.e. continuous variation of style lengths vs. long-styled plants). However, the question of which selective force would *cause* a wide corolla to transform into a narrow floral tube is interesting. In fact, one possibility might be an increase in the cross-pollination efficiency through a better fit with pollinators. This is not contrary to the

hypothesis that I propose because this narrowing would not originate the appearance of stylar dimorphism directly, which would instead appear through a secondary negative selection on the intermediate style lengths due to a high level of self-pollination. This concept is the main novelty of my idea, but I would like to remark that I yet again consider the promotion of cross-pollination as a main selective force in subsequent steps of the maintenance of the dimorphism and in the subsequent evolutionary pathway through heterostyly.

As Faivre (2018) acknowledges, the differences of my hypothesis compared with the model of Charlesworth and Charlesworth (1979) are prominent despite their similarity in the requirement of self-incompatibility as initial condition. In this regard, the differences in the selective forces effected by late-acting self-incompatibility and heteromorphic incompatibility are noteworthy. Late-acting self-incompatibility is highly penalizing for self-pollination but it does not impede cross-fertilization among any pair of mating individuals, and hence it does not involve pollen wastage in cross-pollinations.

The first test of my hypothesis is to model the cost of male and female function with different stylar phenotypes over multiple generations. As Faivre notes, this would require (i) to gather data of pollen transfer and seed production under natural conditions and (ii) to use these data in a demographic model to explore the prospective frequencies of stylar phenotypes. For the second test, i.e. the phylogenetic comparative studies, I agree with Faivre's suggestions of study groups. Notably I consider that, against the categorical codification of species, the use of quantitative indices for the characterization of the polymorphism would better help to determine the ancestral condition and the evolution of stylar conditions in this genus and others. Thus, the inclusion of any stylar condition, including tristylous as in *N. triandrus*, would not entail additional difficulties. In point of fact, my hypothesis is parsimonious as to explain the appearance of all described stylar conditions in *Narcissus*. A final test aiming at determining the genetic basis of the stylar dimorphism in the genus would highly benefit from the set of different approaches, including histological studies (Cohen et al. 2009), that Faivre mentions. My thought is that these approaches are likely to be used in the future in helping to unravel the evolution of these fascinating features of flowering plants.

The model of Lloyd and Webb (1992) and the articles by Barrett et al. (1996, 1997), Cesaro et al. (2004) and Santos-Gally et al. (2013), together with the early and largely forgotten work of Bateman (1952) and Yeo (1975), have all been inspirational for the hypothesis presented here.

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