

1 **Original Article**

2 **Running title:** An unusual stylar polymorphism in *Narcissus*

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4 ***Stylar polymorphism on the edge: unusual flower traits in Moroccan***

5 ***Narcissus broussonetii*** (*Amaryllidaceae*)

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24 **ABSTRACT**

25 Heterostyly and related polymorphisms (e.g. stigma-height dimorphism) have been used
26 as model systems to study the origin and maintenance of plant population variability.
27 Stigma-height dimorphism frequently occurs in *Narcissus* and it is associated to a
28 particular flower shape. Here we describe a new, peculiar case of stigma-height
29 dimorphism in *N. broussonetii*, a species in the margin of the geographic distribution
30 range of the genus. We determined the stylar condition of *N. broussonetii* and its
31 variation across populations, analyzed perianth morphology and its relation with stylar
32 variation, and compared this with other stylar dimorphic species of the genus. We also
33 studied the incompatibility system and pollination ecology of the species. *Narcissus*
34 *broussonetii* is a style dimorphic species, as suggested in early studies but later
35 neglected, and displays unusual flower morphology, with a very long flower tube and
36 virtual absence of a corona. The species presents a late (ovarian) acting incompatibility
37 system and crosses within and between morphs are fertile. We observed short-tongued
38 diurnal and long-tongued nocturnal pollinators. Our findings confirm that the presence
39 of the observed dimorphism across populations is most probably the result of the joint
40 action of a non-heteromorphic incompatibility system, extremely long and narrow
41 flower tubes, and a combined role of short- and long-tongued pollinators.

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43 **Key words:** Heterostyly, morph-ratio, *Narcissus*, perianth traits, pollinators, stigma-
44 height dimorphism, self-incompatibility.

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49 **INTRODUCTION**

50 Flower phenotypic variation occurs in plants at several scales, from lineages to
51 populations, as well as across the distribution range of a species and ultimately within
52 populations. At a population level this variation is frequently continuous and may
53 express the current action of selective and stochastic forces, which may lead to
54 speciation if effective reproductive isolation does occur (Endler 1986). In some cases
55 discrete variation in floral morphology has been described (Barrett, 2002). The traits
56 involved might affect the sexual function, with or without gender differentiation (i.e.
57 dioecy and related conditions; Geber, Dawson & Delph, 1999; style polymorphisms and
58 heterodichogamy: Barrett *et al.*, 2002), perianth color (Gigord, Macnair & Smithson
59 2001) or inflorescence architecture (Tor ang,  gren & Erlhen 2008).

60 Here we focus on heterostyly and its related stigma-height dimorphism, which
61 provide excellent opportunities for exploring inter-population variation in polymorphic
62 floral traits (Lloyd & Webb, 1992b; Barrett, Jesson & Baker, 2000). This sexual
63 polymorphism involves spatial separation of anthers and stigmas and is characterized by
64 the presence of two (distyly) or three (tristyly) morphs with reciprocal placement in the
65 height of the stigma and anther positioning, or discrete variation only in stigma height
66 with no differences between morphs in anther height (stigma-height dimorphism)
67 (Lloyd & Webb, 1992b).

68 Charlesworth & Charlesworth (1979) and Lloyd & Webb (1992a,b) recognized
69 stigma-height dimorphism as an intermediate step towards the evolution of heterostyly,
70 although only the latter authors made explicit predictions about the conditions for its
71 maintenance. Briefly, they proposed that higher levels of disassortative (crosses
72 between morphs) than assortative (crosses within morphs) mating in both morphs
73 mediated by specific pollinators are required to maintain the style dimorphism,

74 regardless of the incompatibility system, as empirically shown by Cesaro & Thompson
75 (2004). Despite an initial scarcity of cases to illustrate stigma-height dimorphism (see
76 Lloyd, Webb & Dulberger, 1990; Lloyd & Webb, 1992a) these authors predicted that
77 additional examples would be noticed. Since then, stigma-height dimorphism has been
78 described in more species and genera in which heterostylous species commonly occur
79 (i.e. *Anchusa*, *Nivenia*, *Lithodora*, *Linum*, *Narcissus*, *Quinchamalium* and *Nymphoides*;
80 reviewed in Darwin 1877; Riveros, Arroyo & Humaña, 1987; Thompson, 2005; Ferrero
81 *et al.*, 2009; Sánchez *et al.*, 2010; Haddachi 2013; Santos-Gally, Gonzalez-Voyer &
82 Arroyo 2013) providing new plant groups to test these predictions. Also, a few studies
83 have examined the predicted critical role of pollinators in maintaining higher levels of
84 disassortative mating than assortative mating (Stone & Thomson, 1994; Cesaro &
85 Thompson 2004; Pérez-Barrales & Arroyo 2010; Simón-Porcar, Santos-Gally &
86 Arroyo, 2014). These findings have provided support to the Lloyd and Webb (1992b)
87 model in determining the range of expression of style polymorphism and its relation
88 with the perianth morphology and pollination ecology (Santos-Gally *et al.*, 2013).

89 *Narcissus* L. is a Mediterranean genus of geophytes with a wide range of stylar
90 variation (Arroyo, 2002; Graham & Barrett, 2004), including style monomorphism with
91 different degrees of herkogamy, style dimorphism, distyly (*Narcissus albimarginatus*)
92 and tristyly (*Narcissus triandrus*). Unlike species with a diallelic incompatibility
93 system, which avoid intra-morph fertilizations, thereby promoting only inter-morph
94 fertilizations if pollen is successfully transferred between both morph types,
95 polymorphic *Narcissus* species usually present an inter and intra-morph compatibility
96 system (Dulberger, 1967; Barrett *et al.*, 1996; Arroyo *et al.*, 2002). Hand-pollination
97 experiments have shown that both types of crosses produced similar number of seeds
98 (Santos-Gally, Gonzalez-Voyer & Arroyo, 2013) but most dimorphic species of

99 *Narcissus* present L-biased populations, most likely due to the fact that L-plants are
100 most probably homozygous (ss) at the locus controlling style length. Assuming that
101 inheritance is governed by a single Mendelian locus, as demonstrated in *N. tazetta*
102 (Dulberger, 1967), a closely related species of *N. broussonetii* (Santos Gally, Vargas &
103 Arroyo, 2012), assortative mating of L-morph plants necessarily results in offspring
104 with L-styles. Higher levels of assortative mating can result in morph-ratio variation,
105 although the latter may also depend on the interplay between floral architecture, pollen
106 vectors and their ability to promote inter-morph pollen flow and the relative fitness of
107 morphs (Arroyo and Dafni 1995; Barrett, Lloyd & Arroyo, 1996; Barrett *et al.*, 1997,
108 Pérez-Barrales & Arroyo, 2010; Simón-Porcar, Santos-Gally & Arroyo, 2014),
109 especially when dimorphic species of *Narcissus* deviate from a perfect reciprocal
110 placement of anthers and stigmas (reciprocity). In this sense, characterization of the type
111 of styler variation presented in *Narcissus* has led to controversies among researchers
112 (e.g. *N. triandrus*; Lloyd, Webb & Dulberger, 1990; Barrett *et al.*, 1997, *N. fernandesii*,
113 *N. cyclamineus*; Barrett, Arroyo & Lloyd 1996, and other species in the genus, Valdés
114 *et al.*, 1987). This has also occurred in other plant groups where heterostyly is present
115 (Lloyd, Webb & Dulberger, 1990; Ferrero *et al.*, 2009; Sánchez *et al.*, 2010).

116 One of the species that is subject to this controversy is *N. broussonetii*. Graham
117 and Barrett (2004) classified it as monomorphic, based on herbarium specimens and
118 interpretative a priori explanations, although Fernandes (1940) and Maire (1959)
119 explicitly described the species as heterostylous, and Blanchard's (1990) description
120 implies the presence of a very short style. This species shows a particular phylogenetic
121 and biogeographical position (Santos-Gally, Vargas & Arroyo, 2012), as well as distinct
122 floral morphology (virtual lack of corona and very long floral tube). Here we aim to
123 clarify the significance of *N. broussonetii* in the evolution of style polymorphisms

124 within a genus that has provided deep insight on the evolution of sexual polymorphism
125 in plants. To do this we firstly determined the stylar condition of *N. broussonetii* and its
126 variation across populations, predicting that the species is style polymorphic as
127 suggested by previous taxonomic descriptions (Fernandes, 1940). Secondly, we
128 described the perianth morphology and compared it with other style polymorphic
129 species of the genus. Given that early descriptions of this species placed it in its own
130 genus because of the peculiar flower morphology (Gay, 1858) we predicted it would
131 present a stark contrast with the morphology of other species in the genus. Thirdly we
132 determined the incompatibility system. Lastly we identified pollinators and quantified
133 their visitation rate. With these data in hand, we contrasted flower morphology, sexual
134 traits, pollinators and incompatibility system of *N. broussonetii* with those of closely
135 related stylar dimorphic species, to help us to elucidate the underlying causes behind the
136 variable nature of style polymorphism.

137

138 **MATERIAL AND METHODS**

139 **Study species and population sampling**

140 *Narcissus broussonetii* Lag. is an endemic daffodil from Morocco, with most
141 populations located along the Atlantic coast to the Anti-Atlas mountains under an arid
142 sub-Mediterranean climate, and in an inland portion reaching Fez (Blanchard, 1990;
143 Santos-Gally, Vargas & Arroyo, 2012). This geophyte grows principally on limestone-
144 derived soils in rock pockets usually from sea level to 700 m above sea level. The
145 flowering season is associated to the autumnal rainy season of the Mediterranean
146 climate, peaking from October to early December. Given the strong interannual climatic
147 variation, flowering time and intensity is extremely variable among years. Although our
148 sampling spanned five years during the presumed flowering season, in only one of these

149 years were we successful in collecting a large enough sample size to accurately describe
150 the polymorphism. We explored a total of six populations along the Atlantic coast of
151 Morocco (see table within Fig. 4).

152

153 **Patterns of sex organ variation, flower morphology and morph ratio among**
154 **populations of *N. broussonetii***

155 We sampled plants, from six populations of Morocco, which were separated by at least
156 one meter to avoid repetition of the same genet and preserved flowers in 70% ethanol
157 until their measurement in the laboratory. To examine whether *N. broussonetii* exhibits
158 any type of style polymorphism we collected more than 100 flowers (range = 105–186;
159 total N = 760; the first flower of each umbel inflorescence) from each population. The
160 floral traits: floral diameter, tepal length, corona length and width, length and width of
161 flower tube, the height of the upper and lower-stamen whorls and the style length were
162 measured to 0.01 mm precision using digital callipers. Except the corona, all
163 measurements were made from the top of the ovary (Fig. 1).

164 To determine whether flowers could be classified into two discrete morphs, we
165 ranked them based on the length of the style relative to the height of the stamen whorls.
166 We classified flowers as L-morph (stigma among or above anther whorls) and S-morph
167 (stigma below lower anther whorl) and examined patterns of variation among traits
168 across populations. We applied a multivariate analysis of variance (MANOVA) to test
169 for overall differences in floral morphology. We included population, morph and their
170 interaction as explanatory variables. To test for differences between morphs and/or
171 populations we conducted an ANOVA for each floral trait. We applied a principal
172 component analysis (PCA) on the correlation matrix from four perianth traits (flower
173 diameter, tepal length and floral tube length and diameter) for all sampled individuals.

174 We extracted the mean and standard error of the scores of the first and second principal
175 components for each population. We excluded three variables from the MANOVA and
176 PCA, style length and height of the upper and lower-stamen whorls, because the first
177 presents an apparent bimodal distribution (see Fig.1 in Supporting Information) and the
178 second and third are highly correlated with tube length ($r = 0.92$ and $r = 0.83$, $P <$
179 0.000 , for upper and lower-anther whorl, respectively) as flowers present epipetalous
180 stamens with a filament free portion close the flower tube mouth (see Fig. 1). To
181 account for the influence of flower tube length and morph on style length and upper and
182 lower stamen height, we performed a linear mixed effects analysis. We included morph
183 as a fixed effect and population as a random effect. The length of the floral tube was
184 incorporated as a covariate. We compared two models: “model A”, which included the
185 interaction between morph and floral tube length, and “model B” which did not include
186 the interaction. Plots of the residuals revealed that these were normally distributed. We
187 calculated delta AIC (Δi) to compare between models (Burnham and Anderson 2002).
188 We adjusted style length and upper and lower-stamen height according to the formula
189 used by Baker, Thompson & Barrett (2000): adjusted data = organ position – b (flower
190 tube length – mean flower tube length), where b indicates the slope of the regression of
191 organ position and floral tube length. To calculate differences in adjusted sex-organ
192 position between morphs we conducted an ANOVA with morph as a main effect. For
193 all analyses we used R (R Core Development Team 2013) and the *lme4* package for
194 linear mixed effect analysis (Bates, Maechler & Bolker, 2012).

195

196 We used the same flowers collected for floral measurements to determine the
197 morph ratio for each population. We assessed the significance of the departure of morph

198 frequencies in each population of *N. broussonetii* from isoplethy (1:1 morph ratio) with
199 a *G* test (Sokal & Rohlf, 1995).

200

201 **Comparisons of morphology and reciprocity among style dimorphic species of**
202 *Narcissus*

203 In order to assess whether *N. broussonetii* fits within the phenotypic range of variation
204 of these floral traits, we applied a phylogenetic principal component analysis (PPCA;
205 Revell, 2010) on the correlation matrix from four perianth traits (length and width of
206 both flower tube and corona) of *N. broussonetii* and 15 stilar polymorphic species,
207 representing 80% of all polymorphic species of *Narcissus* (data were obtained for the
208 perianth trait analyses in Santos-Gally, Gonzalez-Voyer & Arroyo, 2013). PPCA
209 incorporates the expected co-variance among trait values resulting from shared ancestry
210 into the principal component analysis (Revell, 2010). We performed this analysis in R
211 (R Core Development Team, 2013) using code provided by L. J. Revell (Revell, 2012),
212 and using the phylogenetic reconstruction of *Narcissus* from Santos-Gally, Vargas &
213 Arroyo (2012).

214 Compared to heterostylous plants, style-dimorphic species are characterized by a
215 lack of reciprocity in the position between anthers and stigma. However, some style-
216 dimorphic species present higher levels of reciprocity compared to others (i.e. *N. dubius*
217 vs. *N. assoanus*; Baker *et al.*, 2000; *N. cuatrecasassi* vs. all other dimorphic species in
218 Section *Apodanthi*; Pérez, Vargas & Arroyo, 2004). In order to assess variation in the
219 degree of reciprocity, we compared measurements of *N. broussonetii* with data from
220 nine style-dimorphic and one heterostylous *Narcissus* species obtained from the
221 literature (Baker, Thompson & Barrett 2000; Pérez-Barrales, Vargas & Arroyo, 2004;
222 Aedo 2013; Santos-Gally *et al.*, 2013) and data obtained from two species for this study

223 (*N. bertolonii* and *N. elegans*). We measured stigma-anther separation and its difference
224 between morphs as an estimate of reciprocity (Barrett & Shore, 1987). Because all
225 stigma-height dimorphic species of *Narcissus* present two levels of anthers in each
226 morph, we calculated the stigma-anther separation taking the mean of the upper anther
227 level of S-morph as reciprocal of the mean of the stigma height of L-morph, and the
228 mean of the lower anther level of L-morph as reciprocal to the stigma of S-morph.

229

230 **Hand-pollination treatments**

231 We calculated the seed set resulting from each of our four hand-pollination treatments:
232 self-pollination, within- and between-morph cross-pollination, and cross-pollination
233 after previous self-pollination the day before. The latter treatment is a test for ovule
234 discounting due to post-zygotic (late acting) self-incompatibility, or to early inbreeding
235 depression (Barrett *et al.*, 1997). In these conditions, ovules fertilized by self-pollen will
236 not produce seeds if the plant is self-incompatible, neither will they be available for
237 further outcross fertilization (Barrett *et al.*, 1997). Fifty-six plants (40 L and 15 S) of *N.*
238 *broussonetii* (style morph as subject effect) were collected from a natural population
239 located in the Atlantic coast of Morocco (population number 1 in Fig. 4). Bulbs were
240 grown in the greenhouse at the University of Seville. We randomly assigned each of the
241 four treatments to flowers within the inflorescence. Flowers subject to hand-pollinations
242 of the within-morph and between-morph pollination treatments were emasculated
243 before anthers dehisced. From October 17 until November 8 2007 pollinations were
244 carried out daily on inflorescences that were marked and bagged with exclusion nets
245 (0.1 mm pore size) to avoid any potential pollen contamination. Cross-pollinations
246 involved a single, randomly chosen pollen donor among the corresponding parent class.
247 We harvested fruits 6–8 weeks after pollination, counted the number of plump seeds,

248 aborted seeds and undeveloped ovules, the latter two were taken as unsuccessful events.
249 Because at the end of the experiment 31 aborted fruits were missing (from a total of 224
250 fruits) and we could not count the ovules of those fruits, they were replaced using the
251 series mean method (function SMEAN in SPSS v. 22, 2013).

252 The effect of different treatments on the seed set was analysed using generalized
253 estimating equations GENLIN for proportion data with breeding system treatments as
254 categorical predictor (function GEE in SPSS v. 22). Repeated measurements (plants)
255 and the position within inflorescence were included in the analyses as subject and
256 within subject variables, respectively. A binomial error distribution was used for the
257 dependent variable (number of ovules converted into seeds). To test model effects we
258 specified Type III sum of squares, Chi-Square Statistics group and Kernel as a log
259 quasi-likelihood function. We performed *post hoc* analyses with pairwise *t*-tests to
260 assess differences in total seeds produced by each hand-pollination treatment.

261

262 **Pollinators**

263 Given the unpredictable nature of flowering phenology, it was not possible to observe
264 pollinators during flower sampling. We therefore conducted pollinator observations in a
265 region where the species occurs naturally and during the reported flowering date
266 (population 1 in Fig. 4). Natural flowering date was confirmed by the observation of
267 three wild plants in bloom close to the study plot. We transported 22 plants of *N.*
268 *broussonetii* from the University of Seville greenhouse (where they were transported in
269 the first year of sampling) back to Morocco. Diurnal and nocturnal observations were
270 performed in seven plots with 6–11 inflorescences and 20–45 flowers from 1st to 3rd of
271 November 2009. We recorded a total of 14 and 4.45 hours of diurnal and nocturnal
272 pollinator censuses, respectively. We initiated 15 min observation periods at 10:00 am

273 and continued until 18:00 hours, when diurnal pollinator visitation noticeably declined.
274 Nocturnal pollinators were observed using a head-lamp with red light; we also recorded
275 images using a digital video camera with *near infrared* to record in the dark (Sony
276 DCR-SR70). Nocturnal observations initiated at 18:00 hours and continued until 20:00
277 or 21:00 hours, depending on the wind and cold that are crucial to the activity of
278 pollinators. We rotated observers randomly among plots, changing every 15 min. For
279 each flower visited, we recorded the visiting species and if visits were legitimate (i.e.,
280 visitor's body came in contact with anthers and/or stigma). Representative specimens
281 were captured for their identification and stored. We examined the pollen collected by
282 insects from captured specimens to assess if flower visitors could function as
283 pollinators. Pollen preparations were made for each captured insect by rubbing a cube of
284 fuchsine-stained jelly (Beattie, 1971) on the animal's body to collect any adhering
285 pollen grains. We examined pollen samples under the microscope and compared with
286 reference samples made directly from anthers of *N. broussonetii*.

287 We classified pollinators into two morphotypes based on the length of their
288 proboscis as short-tongued (ST) or long-tongued (LT). The distinction between these
289 two groups was based on whether the tongue is able to reach the nectar column or not
290 (e.g. Santos-Gally *et al.*, 2013). We calculated the visitation rate (number of visits per
291 hour) to flowers of short-tongued and long-tongued groups.

292

293 **RESULTS**

294 **Patterns of sex organ variation, flower morphology and morph ratio among** 295 **populations**

296 Despite the substantial variation in the relative position of sexual organs among
297 individuals of each population, flowers of *N. broussonetii* could be classified in two

298 morphs based on whether the stigma was positioned below or above the lower anther
299 level (Fig. 2), furthermore style length showed a bimodal distribution (Fig. 1 in
300 Supporting Information). The MANOVA analysis showed significant differences in
301 floral morphology among populations and morphs (Population: Pillai = 0.14, $F = 30.66$,
302 $P < 0.000$; morph: Pillai = 0.014, $F = 2.75$, $P = 0.03$). The results of the ANOVA
303 indicated that flower diameter, tube length and tube width differed significantly among
304 populations and tepal length between morphs and among populations (see Table 1). The
305 first two components (PC1 and PC2) of the PCA explained 91% of the variance in the
306 perianth traits (PC1, 56% of the variance explained; PC2 35%). Flower diameter loaded
307 negatively on PC1 and PC2 accounted for variation in flower-tube length. Both
308 components cluster populations into three groups (see Fig. 3). The remaining flower
309 traits accounted for the other low-variance components (see Table within Fig. 3). Linear
310 mixed effect analysis indicated that morph and tube length explained a significant
311 proportion of the variance in style length and upper and lower stamen height (Table 1).
312 The comparisons between the two models for each sex-organ height (as response
313 variable) indicated that there is no interaction between morph and flower tube length
314 (style length: $\Delta i = 2$; Upper and lower stamen whorl: $\Delta i = 0.8$ and $\Delta i = 1.6$,
315 respectively) (Table 1). Means of the stigma height of L- and S-morphs evaluated after
316 controlling for tube length differ significantly among morphs (L-morph = mean \pm SD:
317 34.5 mm \pm 0.19; S-morph = mean \pm SD: 18.9 mm \pm 0.34; $F_{1, 759} = 1679.8$, $P < 0.0001$,
318 see Table 2).

319 We determined the morph of a total of 760 plants from six populations of *N.*
320 *broussonetii* and all of them were dimorphic. Only one population contained an equal
321 number of S and L individuals ($G = 1.6$, $P = 0.20$). The other five populations were L-

322 biased with a percentage of the L-morph ranging from 66% to 98% (see table within
323 Fig.4).

324

325 **Comparisons of morphology and reciprocity among style dimorphic species of** 326 *Narcissus*

327 The PPCA analyses comparing floral morphology among species showed that the first
328 two components (PC1 and PC2) explained 92% of the variance in the perianth
329 measurements (PC1, 64% of the variance explained; PC2, 28%). Measures of the floral
330 tube (length and width) presented negative loadings on the first principal component
331 axis (-0.96 and -0.57, respectively) while corona height and width presented positive
332 loadings (0.41 and 0.73, respectively). Results of the PPCA showed clear evidence for
333 distinct morphology of *N. broussonetii* when compared with other species from the
334 genus, characterized by the absence of a corona and by a very long flower tube (Fig. 5).
335 With respect to the sexual organs, it is noteworthy that in L- and S-flowers the upper-
336 and lower-stamen whorls were markedly closer to each other (Fig. 1) as compared to
337 other *Narcissus* species (Fig. 6). This placement of the stamen whorls has a strong
338 influence on the reciprocity, especially for the S-morph where the difference in height
339 between the lower-stamen and the style exceeds 5.47 mm (Fig. 6). This reciprocity
340 value clearly contrasts with that of other stylar polymorphic species of the genus, as can
341 be seen by the fact that *N. broussonetii* presents the largest values (variation range of all
342 species: 0.19–5.47 for S-morph and 0.14–2.15 for L-morph) for the difference between
343 sexual organ heights of reciprocal male and female organs in the L- and S-morphs (Fig.
344 6).

345

346 **Hand-pollination treatments**

347 The proportion of seeds sired varied between hand-pollination experiments (χ^2 Wald =
348 76.9, d.f. = 7, $P < 0.001$). The species is self-incompatible in both morph types and inter
349 and intra-morph compatible (Fig. 7). Average seed set following the different
350 outcrossing treatments was similar in each morph type ($P = 1$ for both morphs) but
351 contrasted markedly with out-crossed pollination one day after self-pollination ($P =$
352 0.02 and 0.05 for L within and between cross-pollinations, respectively; $P < 0.001$ for S
353 within and between cross-pollinations). Ovule discounting by self-pollination reduced
354 the seed set by 45% and 57% in L within and between cross-pollinations, respectively;
355 and by 49% and 56% for S within and between cross-pollinations, respectively (Fig. 7).
356 The mean proportion of seeds sired by S-morph was higher than the L-morph and
357 differed markedly in out-cross pollinations ($P < 0.01$ for all cases), whereas the
358 difference was not significant between L and S self-pollination treatments ($P = 0.8$).

359

360 **Pollinators**

361 During diurnal observations we only detected activity of short-tongued pollinators,
362 mainly pollen collecting pollinators, such as hoverflies and some bees (*Amegilla* sp.,
363 *Apis mellifera*, both in the Apidae) (Table 2 and see Figure 2 in **Supporting**
364 **Information**). Diptera (*Stomorhina* sp. in the Calliphoridae, and *Eupeodes luninger* in
365 the Syrphidae) and hymenoptera (*Amegilla* sp., *Apis mellifera* and *Ceratina* sp.) were
366 the most frequent orders of diurnal short-tongued visitors, both accounting for 30.7 %
367 and 46.3 % of visits, respectively (see Table 2 in **Supporting Information** for details).
368 In contrast, the nectar appeared to be efficiently exploited by the most abundant
369 nocturnal long-tongued pollinators: *Autographa gamma* and *Agrius convolvuli*, who's
370 long proboscis (1 and 4 cm respectively) apparently enables them to reach the nectar.

371 All slide preparations observed under the microscope presented pollen of *N.*
372 *broussonetii*, thus all reported insects can be considered as potential pollinators.

373

374 **DISCUSSION**

375 In this study, we confirmed that *N. broussonetii* is a style dimorphic species, as
376 previously claimed by Fernandes (1940) and Maire (1959) although neglected by
377 Graham & Barrett (2004). However, this polymorphism is unusual among *Narcissus*
378 species, and perianth features are also particular to this narrow range, the southernmost
379 range of daffodils. High herkogamy, particularly in the S morph, low sex-organ
380 reciprocity, virtual lack of a corona, and extremely long flower tubes are the most
381 distinctive traits with respect other style dimorphic species in the genus, including
382 closely related species such as *N. tazetta*, *N. papyraceus* and *N. elegans* (Santos-Gally,
383 Vargas & Arroyo, 2012). However, the latter style dimorphic species share with *N.*
384 *broussonetii* other features, such as morph-compatible but-self-incompatible breeding
385 system, a wide array of pollinators including long- and short-tongued insects, and a
386 variable morph-ratio in populations where the L-morph tends to predominate, although
387 monomorphic populations are not reported (Santos-Gally, Gonzalez-Voyer & Arroyo,
388 2013; Santos-Gally *et al.*, 2013). All sampled populations were dimorphic for style
389 length. Furthermore, 5 of the 6 populations were L-biased and the degree of bias
390 showed a gradient of progressive increase towards the south. Interestingly, the L-bias
391 gradient is also linked with floral morphology, with populations in the south presenting
392 flowers with longer floral tube and wider flower diameter (see Fig. 3 and Table 2 in
393 **Supporting Information**). Although we investigated pollinator rate and incompatibility
394 system in a single population of *N. broussonetii*, deviation from isoplethy (L:S) could
395 be the joint result of flower morphology of L-morph, which presents reduced anther-

396 stigma separation, incompatibility system and efficiency of short pollinators in the
397 transfer of pollen within the L morph. This pattern was recently shown for *N.*
398 *papyraceus* (Pérez-Barrales & Arroyo 2010; Simón-Porcar, Santos-Gally & Arroyo,
399 2014).

400

401 ***Unusual floral features of Narcissus broussonetii***

402 Phylogenetic principal component analysis showed that tube length and the virtual
403 absence of a corona result in a strong contrast of this species with the rest of the style
404 dimorphic species of the genus. Furthermore, *N. broussonetii* displays the longest floral
405 tube of any *Narcissus*, even considering monomorphic species of *Narcissus* with large
406 floral displays (i.e. *N. pseudonarcissus*, *N. hedraeanthus*, and *N. poeticus*; Aedo, 2013).
407 The virtual absence of a corona, very infrequent in *Narcissus*, and the extreme length of
408 sexual organs, allows for these to be completely exerted from the tube, except the
409 stigma of the S-morph, which remains deep within the tube. It is worth noting that the
410 lower-stamen whorl is very close to the upper-stamen whorl, and the filament of the
411 stamens of the upper whorl presented the longest free portion among all dimorphic
412 species of *Narcissus* (R. Santos unpublished data). This design of the male sexual
413 organs has a strong effect on the level of reciprocity, which is particularly low for the S-
414 morph stigma (Fig. 4). One feature that characterizes the stylar dimorphism is the low
415 reciprocity between the heights of sexual organs (Lloyd & Webb, 1992b), although
416 there is wide variation in reciprocity among species with this type of polymorphism
417 (Faivre & Mcdade, 2001; Pérez-Barrales, Vargas & Arroyo, 2006; Ferrero *et al.*, 2011).
418 Specifically, in some style-dimorphic *Narcissus* the lower anther level from the L-
419 morph is placed closer to the stigma of the S-morph than the lower anther level of the S-
420 morph, which increases reciprocity of the species (Baker, Thompson & Barrett, 2000;

421 Pérez, Vargas & Arroyo, 2004). In this sense, *N. broussonetii* is the polymorphic
422 species in the genus with the largest herkogamy and the lowest reciprocity. It is
423 noteworthy that in the clade in which *N. broussonetii* is found, style dimorphism has
424 evolved numerous times in morphologically distinct species (R. Santos-Gally,
425 unpublished). The sister species of *N. broussonetti* is monomorphic and self-compatible
426 (*N. serotinus*), however there are no notable differences in the pollinator groups
427 between these two species (both are visited by long- and short-tongued insects) (Santos-
428 Gally, Gonzalez-Voyer & Arroyo, 2013). This latter fact highlights the importance of
429 studies analyzing the efficiency of different pollinators on male fitness, through direct
430 estimation of pollen transfer patterns (by paternity analyses or assessing pollen
431 deposition and removal by pollinators), to understand the evolution of sexual
432 polymorphism.

433 In addition to a virtual lack of corona, the striking height of the three sexual
434 whorls can be explained by ontogenetic allometric relationships with flower tube length,
435 because style and stamens, the latter being epipetalous (attached to the flower tube), are
436 strongly influenced by the development of the flower tube (Faivre & Mcdade, 2001).
437 Long tubes, styles and stamens, as those in *N. broussonetii*, increase the variance in
438 these allometric relationships, which directly affect sex organ reciprocity and probably
439 morph ratio through assortative and disassortative mating rates. Further ontogenetic
440 work and estimates of pollen flow are necessary to determine the proximate causes and
441 the ultimate consequences of this peculiar flower arrangement. It may be hypothesised
442 that a trend towards increased L frequency should be associated to increased assortative
443 mating in this morph and perhaps to some differences, even if small, in the reciprocity
444 of morphs across populations (Thompson *et al.*, 2012). Assortative mating has been
445 detected in related species of *N. broussonetii* such as *N. papyraceus* and *N. tazetta*

446 (Arroyo & Dafni, 1995; Simón-Porcar, Santos-Gally & Arroyo, 2014). Large-flowered
447 *N. broussonetii* and its variable population morph-ratio (0.56–0.91) offer excellent
448 opportunities for study.

449

450 **Hand-pollination treatments**

451 *Narcissus broussonetii* presented self-incompatibility in both morphs and an inter and
452 intra-morph compatibility system, such as that found in most dimorphic species within
453 the genus *Narcissus* (Barrett, Lloyd & Arroyo, 1996). We also found that the
454 incompatibility system could be a result of late-acting ovarian self-incompatibility,
455 another feature common to many species from this genus (Arroyo *et al.*, 2002; Sage *et*
456 *al.*, 1999, Barrett *et al.* 1997, Cesaro *et al.*, 2004), although independent early-acting
457 inbreeding effects cannot be ruled out. Indeed, there was a decrease of nearly 50% in
458 the seed set in the cross-pollination treatment involving prior self-pollination when
459 compared to purely outcrossed hand-pollination treatments (see Fig. 7). The presence of
460 intra-morph compatibility in this species supports the hypothesis that complete loss of
461 one of the morphs could occur in nature, assuming that L-plants are homozygous (ss) at
462 the locus controlling style length, as has been documented for *N. tazetta* (Dulberger,
463 1964). In addition, the results suggest that variation in morph ratio may depend on the
464 relative levels of assortative and disassortative mating (Brys, Jacquemyn & Beeckman,
465 2008), and variable self-pollination degree between morphs, which might limit their
466 fitness.

467 Seed production from hand-pollinations tended to be lower in L-morph maternal
468 plants than in S-morph plants (Fig. 7). Within *Narcissus*, such differences in fecundity
469 between morphs have only been reported in style dimorphic *N. assoanus* (Cesaro *et al.*,
470 2004). These authors attributed this difference to the delayed receptivity of the L-morph

471 stigma due to strong protandry, in comparison with non-dichogamous S-morph flowers,
472 and these effects disappeared between one and two days after flower opening. Despite
473 having performed hand-pollinations twice with a one-day delay, it is possible that a
474 strong differential dichogamy also occurs in *N. broussonetii*, and that we did not control
475 the effect in these flowers. In related heterostylous plants, when there are differences
476 between morph in fecundity after natural pollination, the L-morph usually shows higher
477 female fecundity than the S-morph, due to its highly exposed stigmas receiving more
478 pollen (Ganders, 1979). The L-morph could compensate this through an enhanced male
479 function (pollen delivery), which is improbable in *N. broussonetii* due to similarly
480 exposed anthers in both morphs, and post dispersal differential fitness between morphs.
481 We are currently investigating these possibilities further. Unlike other style dimorphic
482 daffodils, the long exertion of L-stigma and very low reciprocity (compared to other
483 dimorphic species) can potentially result in low pollen arrival to L-stigmas, although we
484 ignore the fecundity of L- and S-morphs in natural conditions, which is particularly
485 difficult to determine given the erratic nature of blossoming in this species.

486

487 **Pollination ecology**

488 We found a high abundance of short-tongued pollinators, which accounted for nearly
489 80% of total pollinator observations. Although our observations were conducted in a
490 single location, a potential explanation of the L-bias in most of the populations studied
491 is that short-tongued pollinators probably transferred more pollen between plants of L
492 morph, which potentially increases the frequency of this morph following intra-morph
493 fertilizations. Short-tongued pollinators being mostly pollen-feeding insects which
494 cannot reach the base of the flower tube and do not search within the tube, cannot
495 pollinate S-stigmas. On the other hand, the most abundant nocturnal pollinators

496 *Autographa gamma* and *Agrius convolvuli*, probably transfer pollen to the concealed
497 stigmas of the S-morph, as shown for long-tongued pollinators by an empirical study
498 with *N. papyraceus* (Simón-Porcar, Santos-Gally and Arroyo *et al.*, 2014). Interestingly,
499 *N. papyraceus* (a species within the same clade of *N. broussonetii*, Santos-Gally, Vargas
500 and Arroyo, 2012) has a southern distribution range overlapping with that of *N.*
501 *broussonetii*. *Narcissus papyraceus* populations within this range display longer floral
502 tubes and lower reciprocity than populations from the northern range in the Iberian
503 Peninsula (see *N. papyraceus* # 2 in Fig. 6). Surprisingly, the S-morph is never lost in
504 Moroccan populations of *N. papyraceus*. In contrast, in the northern range, we found an
505 association between high abundance of short-tongued pollinators and the loss of the S-
506 morph (Pérez-Barrales, Arroyo & Armbruster, 2007; Pérez-Barrales & Arroyo, 2010;
507 Santos-Gally *et al.*, 2013). Further work on pollinator variation across populations and
508 phenotypic selection studies are needed to confirm this possible pollinator mediated
509 variation in morph ratio, as has been reported in other daffodil species (Arroyo & Dafni
510 1995).

511

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524

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655

656 Figure 1. Long-styled (right) and short-styled (left) flower morphs of *Narcissus*
657 *broussonetii*. Numbers correspond to the following measurements for both floral
658 morphs: (1, 9) tube length, (2, 10) lower anther height, (3, 11) upper anther height, (4,
659 8) stigma height, (5, 12) flower diameter, (6, 13) tepal length and (7, 14) tube diameter
660 of L and S-morph, respectively. For simplicity, only two out of three stamens per whorl
661 are drawn.

662 Figure 2. Styler dimorphism in sampled populations of *Narcissus broussonetii*. Flowers
663 are ranked by stigma height (dashed line) with upper- and lower-level anthers
664 represented by circles and triangles, respectively. Numbers within plots correspond to
665 population number in the Fig. 4.

666 Figure 3. Principal components analysis of four morphological traits from populations
667 of *Narcissus broussonetii*. The first two axes account for 56 % and 35 % of the total
668 variation, respectively. Each population is represented by mean and standard error of
669 PCA1 and PCA2 factor loadings. Loadings for all the variables of the PCA are given in
670 the top right corner. Population numbers correspond to the numbers of Fig. 4 in the
671 main text of the article.

672 Figure 4. Distribution and morph-ratio variation of six populations of *Narcissus*
673 *broussonetii* in southwestern Morocco. Each pie chart represents the morph ratio in a
674 population (black = L-morph; white = S-morph). The table within the figure gives
675 information of geographical coordinates, Voucher numbers, elevation of sampling
676 locations and G-test for significant departure from isoplethy in morph-ratio (1 : 1). Grey
677 contour represents the distribution of *N. broussonetii* (Fernandes 1940).

678 Note: morph ratio is expressed in terms of the ratio of L-morph/L- + S-morph. ^{n.s.}, Non-
679 significant; * $P < 0.05$.

680 Figure 5. Phylogenetic principal components analysis (PPCA) of four perianth traits of
681 dimorphic species of *Narcissus*. The box in the upper-left corner within the figure
682 represents loadings for all variables of the PPCA. The position of *N. broussonetii* is
683 marked with a black symbol, all other species in white. Numbers correspond to: 1) *N.*
684 *albimarginatus*, 2) *N. assoanus*, 3) *N. broussonetii*, 4) *N. calcicola*, 5) *N. cuatrecassasii*,
685 6) *N. dubius*, 7) *N. elegans*, 8) *N. fernandesii*, 9) *N. gaditanus*, 10) *N. jonquilla*, 11) *N.*

686 *papyraceus*, 12) *N. rupicola*, 13) *N. scaberulus*, 14) *N. bertolonii*, 15) *N. tazetta*, 16) *N.*
687 *triandrus*.

688 Figure 6. Differences between reciprocal sexual organs among dimorphic species of
689 *Narcissus*. Circles represent the differences between L-stigma height and S-upper anther
690 height and triangles the differences between S-stigma height and L-lower anther height.
691 *N. papyraceus* is represented by two types of population (long and short flower tube
692 length) according to Santos-Gally *et al* (2013).

693 Figure 7. Mean proportional seed set of L- and S-morphs in one isoplethic population of
694 *Narcissus broussonetii* from Safi (Morocco) after hand pollination experiments (see
695 Material and Methods for details). Values indicated are the means \pm SE of seeds sired
696 following intramorph (LxL, SxS) and intermorph (LxS, SxL) cross-pollination, self-
697 pollination (Lx, Sx) and prior self-pollination plus cross-pollination (Prior SP Lx and
698 Sx).

699

700 Table 1. Results of the mixed-model analysis with style length, upper- and lower-anther
701 height as the dependent variable, morph as a fixed effect, length of the flower tube as a
702 covariate, and population as random effect. Model A included morph, floral tube length,
703 the interaction between morph and tube length and population whereas in model B we
704 excluded the interaction between morph and flower tube length.

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Sexual trait	Source of Variation	Slope \pm S.E.	AIC	Δi
Style length	Intercept	16 \pm 1.1		
	Morph (S)	-13.7 \pm 0.3		
	Floral tube length	0.7 \pm 0.0		
	Model A		3910	
	Model B		3908	2
Upper stamen height	Intercept	6.4 \pm 0.5		
	Morph (S)	0.5 \pm 0.1		
	Floral tube length	0.9 \pm 0.0		
	Model A		2776.4	
	Model B		2774.8	0.8
Lower stamen height	Intercept	5.0 \pm 0.9		
	Morph (S)	0.8 \pm 0.2		
	Floral tube length	0.8 \pm 0.0		
	Model A		3256.1	
	Model B		3254.5	1.6

711

712 Table 2. Length of sexual organs in six populations of *Narcissus broussonetii*. Mean
713 values (mm) are given for style length, upper-stamen height and lower-stamen height.
714 Sex-organ position was adjusted to account for different-sized flowers and significant
715 differences were assessed using ANOVA (see M&M for details; *** $P > 0.001$).

716

	Long N=586		Short N=174
Style length	34.5	***	18.8
Std. Error	0.3		0.3
Range	21.2–45.8		11.6–32.1
Upper-stamen height	31.6	***	30.3
Std. Error	0.1		0.2
Range	20.9–39.5		23.9–39.1
Lower-stamen height	26.5	***	25.4
Std. Error	0.1		0.2
Range	17.5–35.8		20–32.5

717 **Supporting Information**

718 **Figure 1.** Histograms of the style length from six populations of *N. broussonetii* from
719 Morocco.

720 **Figure 2.** Pollinators observed in a population of *Narcissus broussonetii*. The two top
721 pictures show Diptera (*Eupeodes luninger* and *Stomorhina* sp.), the middle row shows
722 Hymenoptera (*Amegilla* sp. and *Apis mellifera*), the bottom row Lepidoptera (*Agrius*
723 *convolvuli* and *Autographa gamma*).

724