Pollinator visitation pattern and differential reproductive compatibilities maintain bract colour polymorphism in a nocturnal ginger from Western Ghats, India

Saket Shrotri[1] and Vinita Gowda[1]

[1]Tropical Ecology and Evolution (TrEE) Lab, Department of Biological Sciences, Indian Institute of Science Education and Research (IISER) Bhopal, Bhopal, Madhya Pradesh, India, 462066.

Corresponding author: Vinita Gowda.

Email: gowdav@iiserb.ac.in

Orchid IDs:

Saket Shrotri: https://orcid.org/0000-0002-4596-9451

Vinita Gowda: https://orcid.org/0000-0001-8533-0014

Summary

- Polymorphism, the presence of discrete floral morphs within a population, can be maintained by positive or negative frequency selection. Pollinators often show a positive frequency-dependent foraging which confers reproductive advantage to common morphs over rare morphs. However, pollinator-mediated selections often act on visual floral displays. Here, we investigate hawkmoth-mediated selection in a nocturnal *Curcuma caulina* (Zingiberaceae) with bract colour polymorphism, where the role of visual display is minimal.
- We used path analyses to explain maintenance of differential morph abundances in *C. caulina*. For this, we quantified inter-morph trait variations in floral structures, nectar traits, pollinator visitations, physiological compatibilities and reproductive fitness.
- We noted that the rarest morph was self-compatible, exhibited higher nectar reward and pollinator visits, and low reproductive fitness, whereas the two common morphs were complete to partially self-incompatible, with lower nectar rewards and pollinator visits, but higher reproductive fitness.
- We conclude that while hawkmoths show a positive response to floral rewards, self-compatibility confers lower reproductive fitness to the nectar-rich rare morphs. In contrast, self-incompatibility confers higher fitness to the common morphs despite

lower pollinator visits. Thus, colour polymorphism in nocturnal *C. caulina* is explained by floral traits associated with bract colour, which are under pollinator-mediated selection.

Keywords

Cross-compatibility, Floral colour polymorphism, Frequency-dependent selection, Hawkmoth pollination, Self-compatibility, Structural Equation Modelling (SEM)

Introduction

In plants, floral colour polymorphism (often referred to as FCP) is a common trait that is often under pollinator-mediated selection. The emergence of FCP in plants has been attributed to two factors - mutation events (Kellenberger *et al.*, 2019) and genetic recombination (Stebbins, 2019). However, its maintenance in a population is often explained by spatiotemporal variation in both biotic and abiotic selection regimes such as balancing selection, frequency-dependent selection, selection of pleiotropic traits, heterozygous advantage, and neutral or stochastic processes (Narbona *et al.*, 2018; Vaidya *et al.*, 2018; Sapir *et al.*, 2021). In plants with FCP, some of the key floral characters that are variable and are under positive selection by the pollinators are floral colour (Waser & Price, 1981, 1985; Jones & Reithel, 2001; Gigord *et al.*, 2001; Dormont *et al.*, 2010; Ortiz *et al.*, 2015), flower shape or size (GÓmez, 2000; Majetic *et al.*, 2007, 2009; Barazani *et al.*, 2019), nectar characteristics (Melendez-Ackerman & Campbell, 1998; Carlson & Holsinger, 2013), number of open flowers (Dormont et al., 2010; M. Eckhart et al., 2006), and flowering time (Brunet *et al.*, 2015). Examples where pollinators show selective preference towards a morph, and cause selection differentials within a polymorphic population are also common such as in the perennial, Mediterranean herb *Lobularia maritima*. Here, pollinators prefer white morphs with larger floral displays compared to the purple morphs that had smaller displays resulting in higher reproductive fitness in the white morphs (GÓmez, 2000). Similarly, Jones & Reithel (2001) showed that in snapdragons (*Antirrhinum majus*) bumble bees preferentially visited colour-morphs with higher nectar. These examples show that in a polymorphic population pollinators can recognize floral colour morphs via traits associated with them. .

One of the structural features of a polymorphic species is the presence of an unequal number of morphs within a population. In a comprehensive study of floral color polymorphisms in the Mediterranean region, Narbona *et al.* (2018) reported that nearly all of

their study populations exhibited unequal morph frequencies, while in a few species, morph frequencies have also been shown to vary with time (Dormont *et al.*, 2010; Tang & Huang, 2010; Tang *et al*., 2016). When floral colour polymorphisms is maintained by pollinator-mediated selection, morph abundances within a population can modulate not only pollinator visitation rates (Dormont *et al.*, 2010; Ortiz *et al.*, 2015; Wenzell *et al.*, 2023), but also outcrossing rates (Fry & Rausher, 1997; Subramaniam & Rausher, 2000; Holsinger, 2000), and ultimately the reproductive fitness of the morph within the population (Gigord *et al.*, 2001; Lau & Galloway, 2004; Koski *et al*., 2020).

In contrast, when FCPs are not driven by pollinator-mediated selection, the floral colour morphs are either associated with differential chemical defence (Irwin *et al.*, 2003; Strauss & Whittall, 2006) or with differential reproductive compatibilities, such as in geophytes. Here polymorphic forms within a population can be maintained by features such as clonal reproduction. For example, in *Iris lutescens* a novel floral morph may gain a selective advantage because of its higher clonality (Thompson & Eckert, 2004) even when the pollinators did not favour it (Imbert *et al.*, 2014). Further, rare morph may be maintained in a population if it is self-compatible and autogamous (self-fertilisation). This was observed in *Ipomoea purpurea* (Fry & Rausher, 1997; Subramaniam & Rausher, 2000) and in *Lysimachia arvensis* (Arista *et al.*, 2013; Ortiz *et al.*, 2015) where the rare morph was maintained due to its self-compatible and autogamous reproductive traits despite it being under pollination limitation. Thus, inter-morph differences in both physiology and reproductive compatibility can play a critical role in the maintenance of rare morphs within a population, and it highlights the importance of studying variation in physiological compatibilities among morphs.

The role of environmental factors in the maintenance of FCPs has been discussed in a few perennial and rhizomatous plants such as *Mimulus guttatus* (Twyford *et al.*, 2018), *Boechera stricta* (Vaidya *et al.*, 2018), *Anemone pavonina* (Streinzer *et al.*, 2019), *Geranium nepalense* (Tang *et al.*, 2016) and in multiple polymorphic species of British Isles (Warren & Mackenzie, 2001). These studies suggest that FCPs are more common in temperate and Mediterranean regions. This is because temperate regions have higher diversity of herbaceous species and climatic fluctuations that may select for variation in the abiotic-stress associated anthocyanin production (Warren & Mackenzie, 2001; Tang & Huang, 2010; Berardi *et al.*, 2013, 2016; Narbona *et al.*, 2018; Sapir *et al.*, 2021). However, similar studies are not available in the tropics although floral colour variation and FCP has been reported across multiple lineages in the tropics (Shrotri, 2024).

Within the Asian tropics, several genera within the family Zingiberaceae (gingers) are known to have a diversity of floral polymorphisms ranging from stylar polymorphism to colour polymorphisms. Gingers are a dominant, perennial understory herb with clonal reproduction, and both diurnal as well as nocturnal flowering. While stylar polymorphism is common in the genera *Alpinia* and *Amomum* (Raju *et al.*, 2004; Takano *et al.*, 2009; Ren *et al.*, 2022), FCP is common across several genera in the family Zingiberaceae such as *Achasma*, *Aframomum*, *Boesenbergia*, *Curcuma*, *Globba*, *Hedychium* and *Roscoea* (Poulsen & Lock, 1997; Harris *et al.*, 2000; Leong-Skornicková *et al.*, 2007; Mood *et al.*, 2013; Yadav, 2024; Yadav and Gowda, in press). Irrespective of the type, floral polymorphism has been shown to be critical in plant-pollinator interactions and critical for speciation in both the genera *Alpinia* and *Roscoea* (Takano *et al.*, 2009; Paudel *et al.*, 2019). Among the polymorphic gingers, the taxonomically diverse genus *Curcuma* is known for its floral colour variation (Leong-Skornicková *et al.*, 2007), including the nocturnal species *C. caulina* (Fig. S1).

In the tropics, nocturnal plant species are often pollinated by crepuscular and nocturnal pollinators such as long-tongued hawkmoths (Johnson & Raguso, 2016; Hahn & Brühl, 2016; Johnson *et al.*, 2017; Liu & Sun, 2019), however very little is known about their floral preferences, ability to discriminate floral traits, especially floral colours, and pollination efficiency (Weiss, 2001; Goyret *et al.*, 2008). In the nocturnal ginger *C. caulina*, we test whether FCP within the population may be maintained by pollinator-mediated selection and selection of certain reproductive traits such as autogamy, self-compatibility and cross-compatibility. To the best of our knowledge, the maintenance of FCP has not been studied in any exclusively nocturnally flowering plant species. Therefore, to understand the maintenance of FCP within a population of mass flowering population of *C. caulina* from the Western Ghats, India we addressed the following questions:

1. Do floral colour morphs show similar distribution frequency?
2. Do the floral colour morphs differ in their floral morphology, floral rewards, physiological compatibilities, and reproductive fitness?
3. Do the pollinators show similar visitation rates to the morphs?

Finally, we use a path analysis approach to identify selection regimes that are critical in shaping and maintaining floral colour polymorphism within the *C. caulina* population.

Materials and Methods

Study species and study site

Curcuma caulina (Zingiberaceae) is a rhizomatous, mass flowering, perennial herb that flowers between July to October (Fig. S1). The plant is 30–50 cm tall with a single shoot bearing a terminal inflorescence which consists of 10-18 fertile 'lateral bracts' that bear flowers (Fig. S1). Flowers are borne in a cincinni (1–3 flowers per cincinni), are nocturnal (anthesis at 18:00), and last 12–14 hours only. The stigma is funnel-shaped and projects beyond the anther, and fruits mature within the bracts. *C. caulina* is endemic to the northern Western Ghats of India and this study was carried out on Kaas Plateau (कास पठार; KAS; 17°43'12.36" N, 73°49'28.0956" E, elevation 1225m, Fig. S1), Satara, Maharashtra, India between 2019 and 2022. Kaas Plateau is an elevated ferricrete outcrop (red laterite crust) and a UNESCO Natural World Heritage site recognized for its floral diversity and high endemicity (Watve, 2013).

Floral colour morphs and variation in traits

We identified three floral colour morphs based on the extent of the reddish-pink coloration on the first (basal) lateral bract and their abundance across ten 10 x 4 m^2 transects in the population. The three morphs are Green-White (GW), Green-Red (GR) and Red-White (RW; Fig. S1). To morphologically characterise the three morphs, we measured a total of 22 vegetative and floral characters using digital vernier calliper and rulers (n=30 per morph; character list in Table S1). To assess morphological variations among the morphs, we ran a non-metric multidimensional scaling (nMDS) analysis with Bray–Curtis dissimilarity using the VEGAN package (Dixon, 2003) in R (4.3.0; R core Team, 2021; Table S2). We used the first three dimensions, to construct three-dimensional (3D) plots, which were used to visualise both intra-morph and inter-morph variations. The statistical difference among the overlapping clusters (morphs) was measured using the ANOSIM test where the Bray–Curtis dissimilarity matrix with 1000 permutations was used (VEGAN package; Dixon, 2003).

We measured nectar volume and concentration in 106 bagged flowers (32 to 38 unopened buds per morph) between 18:00 and 18:30 hrs by destructive sampling. The nectar volume (µl), concentration (% sucrose), and nectar energy (cal) were compared among the three morphs. Nectar volume was measured using calibrated glass microcapillary tubes (Drummond Scientific Company), and the sucrose concentration in nectar was determined using a handheld refractometer (ERMA BRIX 0~55%). The brix sucrose % value was then

converted into sugar (mg) per 1 ml of nectar using the table given in the CRC Handbook of Chemistry and Physics (Lide, 2004) and as outlined by Bolten *et al.*, (1979). This value was used to calculate the amount of sugar in a given volume of nectar, and it was converted into calories (1 mg of sucrose = 4 calories as given in Heinrich, 1975). Nectar traits were tested for normality using the Shapiro-Wilk test prior to performing a non-parametric comparison using the Kruskal-Wallis test between the groups. Dunn's test was then used as a post-hoc test with a significance set at $p<0.05$; analysis was carried out in R (4.3.0; R Core Team, 2021).

Natural pollinator visitations

To determine if pollinators exhibit differential visitation towards a particular morph, pollinator visits were monitored manually and by night vision CCTV cameras (Ae Zone CCTV IR day/night vision USB camera) between 18:00 hrs and 06:00 hrs. These observations were carried out in 18 randomly selected observation plots on the plateau where each plot consisted of three to six individuals. All morphs were observed for a total of 648 hrs (i.e., 216 hrs per morph). Cameras were positioned approximately 1 to 1.5 m away from the focal plants such that all inflorescences were visible in their entirety in the field of view. The recordings from the night vision CCTV cameras were manually analysed and the following information was extracted, total number of flowers present, total number of visits by each pollinator, and the duration of each visit. We next calculated the pollinator visitation rate as the total number of visits per flower per hour for each morph. The visitation rates were summed for every hour between 18:00 to 06:00 hrs in order to record the temporal shift in pollinator visitation rate. Natural pollinator visitation rates (number of visits per flower per hour) were compared among the morphs on a temporal scale using the circular statistical analysis in ORIANA (v. 4.02 Kovach Computing Services; Kovach, 2011), and the analysis generated an average vector (μ), average vector length (r), circular variance, circular standard deviation and circular standard error of the mean with a 95% confidence interval (Zar, 1999). Since the data was found to be non-parametric, temporal differences in visitation rates between the morphs were tested using the Mardia-Watson Wheeler test (or uniform score test; Zar, 1999).

To test for inter-morph differences in pollinator visitation rates we used only the peak visitation rates from 19:00 hrs to 22:00 hrs, which accounted for ~85% of all pollinator visits. We used the Kruskal-Wallis test followed by Dunn's test as a post-hoc test at $p<0.05$. Prior to

the Kruskal-Wallis test, the data was tested for normality using the Shapiro-Wilk test. All statistical analyses were carried out in R (4.3.0; R Core Team, 2021).

Physiological compatibility and natural fruit set

To comprehensively evaluate the physiological self-compatibility and cross-compatibility rates in the three floral colour morphs, we carried out a total of 15 hand-pollination treatments. Each morph was treated separately and the pollination treatments included three autogamy, three self-pollination, and nine cross-pollinations that included three intra-morph cross-pollinations and six inter-morph cross-pollinations. Thus, a total of 471 hand pollinations were carried out (with $n \geq 30$ per treatment) where the three intra-morph cross-pollination treatments were: GW x GW, GR x GR, and RW x RW (male x female morph) and will be interchangeably referred to as 'cross-pollination' treatments. Inter-morph cross-pollination treatments were categorised into three pairs to investigate the effect of male and female parents in each treatment. These crosses are (male x female) identified as pair a: GW x GR and GR x GW, pair b: GW x RW and RW x GW, and pair c: GR x RW and RW x GR.

Autogamy treatment included unmanipulated, bagged flowers that were checked for fruit set after four weeks. For cross-pollination treatments, flowers were emasculated and mixed pollen grains from 10-15 individuals were manually transferred on marked flowers. Flowers were observed for fruit set in ~4 weeks and percent success rate in reproduction was measured for each treatment using the number of fruits per treatment as well as the number of seeds per fruit (seed count). We statistically tested the reproductive success rates between self- and cross-pollination treatments for each morph using chi-square tests. Similar comparative analyses were also carried out for the seed counts from self- and cross-pollination treatments using the Kruskal-Wallis test followed by Dunn's post-hoc test with significance set at $p < 0.05$.

We quantified the reproductive success of natural pollination events within the KAS population at the end of the flowering season in mid-October by measuring fruit set in ≥ 500 individuals per morph and seed counts in ≥ 50 per morph. For each inflorescence, we estimated the total number of flowers per inflorescence by multiplying the number of lateral bracts with the number of flowers estimated in a cincinni. Fruit-set per inflorescence was then calculated as the ratio of the number of matured fruits to the total number of flowers estimated for a particular inflorescence. We used Kruskal-Wallis test to compare both natural

fruit set and natural seed count among morphs. Dunn's post-hoc test was used with a significance set at $p < 0.05$.

Path analysis to model the maintenance of floral colour polymorphism

To understand the role of different traits in the maintenance of polymorphism within *C. caulina* we used directly measured traits (observed variables) and indirectly constructed traits (latent variables) in the Structural Equation Modelling (SEM) framework. . The SEM analysis was carried out on the combined data from all three morphs as well as for each morph separately, and the traits used were distribution frequency of the morphs, nectar traits, pollinator visitation rate, reproductive compatibility and natural fitness. The role of each trait was estimated at each step within the model, which represents the strength and direction of the relationships between the variables being examined for both observed variables and latent variables. The significance of each estimate in the path was determined using z-values and p-values, with significance set at $p < 0.05$. The analysis was performed in R (4.3.0; R Core Team, 2021) and R studio (ver. 2023.06.0+421) using *lavaan* package (Rosseel, 2012).

The model consisted of three paths where path 1 included three variables: abundance, nectar energy (total floral reward), and pollinator visitation rate, and the latent variable total floral rewards or β1 connected the two variables abundance and nectar energy. We next carried out a regression between the latent variable β1 and the third variable in path 1 that is pollinator visitation rate. Path 2 tested the link between pollinator visitation rate and the physiological compatibility (self or intra-morph cross), and the resultant natural fitness was calculated as fruit-sets in each morph. The contributions of self-pollination (path 2.1) and cross-pollination (path 2.2) towards reproductive fitness was examined using these two paths. The last Path 3 tested the role of reproductive fitness in defining the morph abundances within the population. Here, we estimated the latent variable β2, that is reproductive fitness by taking the product of natural fruit set and natural seed count. Finally, using the regression function, we tested the role of β2 in determining morph abundances. We constructed the SEM model to be cyclical and β2 completes the cycle of variables that may be involved in the maintenance of morphs within the KAS population. Morph specific SEM analyses were also conducted and detailed in the supplementary file method S1.

Results

Floral (bract) colour polymorphism and morphological variability

The flowers were consistently white in colour in *C. caulina*, irrespective of the morph type. Since we detected polymorphism in bract colours and not floral colours, henceforth we use the term bract colour polymorphism interchangeably with floral colour polymorphism. Within the KAS population, we report here morphological traits of the three morphs Green-Red (GR morph), Green-White morph (GW morph), and the Red-White (RW morph; insets in Fig.1a), wherein the morphs have been identified based on the colour of the lateral bracts of the inflorescence. The abundance of the three morphs differed significantly within the population with Green-Red (GR) morph being the most common morph with 98.6 ± 7.2 individuals (~44 %), the second most-common morphs was the Green-White morph (GW morph) with 75.7 ± 7.2 individuals (~29 %) , and the rarest of the three morphs was the Red-White morph (RW morph) with 24.2 ± 2.0 individuals (~13 %) in the population. A few bract colour variants (~ 14%) were extremely rare and infrequent across years and each variant was present in less than five percent of the population. Across all the transects,the number of individuals was not significantly different between the GR and GW morphs (Kruskal-Wallis $\chi 2$ = - 0.692, df = 1, p = 0.2446), the RW morph had significantly fewer individuals when compared to both GR and GW (RW vs GR, Kruskal-Wallis $\chi 2$ = - 2.427, df = 1, p = 0.0076 and RW vs GW, Kruskal-Wallis $\chi 2$ = 1.736, df = 1, p = 0.0413 respectively).

The non-metric multidimensional scaling (nMDS) analysis of the resulting 22 floral and vegetative morphometric characters (Table S1; Fig. S2) failed to form distinct clusters separating the morphs by their morphological traits. Further, the Analysis of Similarities (ANOSIM) test (p = 0.4835) and the associated ANOSIM R-value (ANOSIM R = - 0.0038) also indicated no statistical significance in the differentiation of these clusters for each morph suggesting.

Floral colour polymorphism, nectar trait variability, and differential visitation by hawkmoths

Nectar is the primary pollinator reward in *C. caulina*, and there was no significant difference between the total nectar rewards (assessed in calories as mean ± s.e.) of the two common morphs GR (16.74 ± 1.47) and GW (18.04 ± 2.2). However, the total nectar reward was highest for the rare RW morph (31.31 ± 2.25) due to both its higher nectar volume as well as higher nectar concentration (Fig. 1a; Table S2; Kruskal-Wallis $\chi 2$ = 33.53, df = 2, p < 0.0001). Flowers of *C. caulina* were visited by two species of hawkmoths with long proboscis from the Sphingidae family: *Agrius convolvuli* (~12.4 cm) and *Hippotion rafflesii* (~4.6 cm, Fig. S3). During foraging, the moths insert their proboscis into the floral tube, hover over the flower, and the sticky pollen is carried on the proboscis of *A. convolvuli* (Fig.

S3) or on the head in the case of *H. rafflesii*. We did not notice the curling proboscis during foraging as observed by Smith et al. (2022). The average time spent by each pollinator per flower was ~ 1.5 seconds for *A. convolvuli* and ~ 2.5 seconds for *H. rafflesii*. Since the pollen of *C. caulina* is sticky, pollen removal could be identified by the rupture in anther theca of flowers that had been visited by hawkmoths.

The circular statistical analysis of visitation rates across the anthesis time showed no temporal differences in pollinator visitations among the three morphs (Rayleigh Test; $p < 0.0001$; Fig. S4). The mean pollinator visitation time (denoted by the circular mean vector; μ) was noted to be between 21:00 to 22:00 for all morphs (Fig. S4). All the three morphs showed highest hawkmoth activity during the first few hours of anthesis, between 19:00 hrs to 00:30 hrs, which gradually decreased until there was no activity by 06:00 hrs, the following morning (Fig. S4). Pollinator visitation rates of the three morphs during the peak visitation hours were statistically different from each other with RW morph showing the highest visitation rate (mean number of visits per flower per hour ± s.e.; 5.14 ± 0.96), followed by the GW morph (2.8 ± 0.59), and GR morph (2.19 ± 0.3; Fig. 2b; Kruskal-Wallis chi-square = 6.52; df = 2; $p = 0.03839$; Table S3)).

From video recordings of pollinator visitations, we also observed that hawkmoths revisit the same flowers within an RW morph approximately 10-12 times, once they have discovered the nectar-rich inflorescence. In contrast, the two common morphs GR and GW with low nectar rewards received lower visitation rates and pollinators rarely revisited the flowers. Thus within-inflorescence visits were higher in the rare RW morph compared to the negligible revisits observed in the common GR and GW morphs.

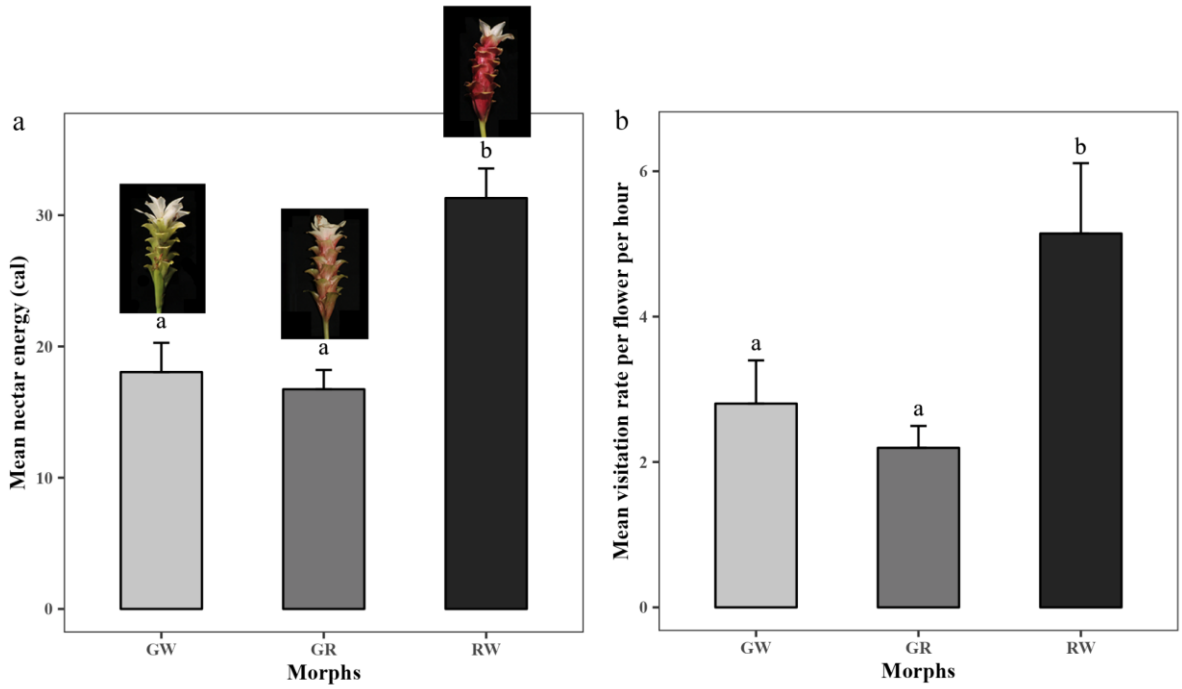

Fig. 1. Nectar energy and natural pollinator visitation rates for the three morphs of *C. caulina* (inset). From left, GW, GR, and RW morphs. a. Mean (± s.e.) nectar energy (cal) and b. Mean (± s.e.) pollinator visitation rates during the peak pollinator activity period. Small letters indicate significant differences between morphs ($p < 0.05$). Statistical results of between morphs comparison are given in Table S2 for nectar energy and in Table S7 for visitation rates.

Physiological compatibility and natural fitness vary among the morphs

From a total of 478 hand-pollination treatments which consisted of autogamous, selfing, and outcrossing treatments only 108 treatments set fruits (Fig. 2a). None of the autogamous treatments set fruits. Selfing treatments failed to set fruits exclusively in the GR morph, whereas the GW and RW morphs demonstrated leaky self-compatibility with success rates of 6.67% and 9.68%, respectively. Fruit-set success in selfed treatments was 5.8% which was significantly lower ($\chi 2 = 31.299$, $p < 0.0001$; Table S4) when compared with fruit-set success in the intra-morph cross (48.38%) and inter-morph cross (58.4%). The GR morph demonstrated the highest fruit-set in the intra-morph outcrossed treatment with a success rate of 46.67%, followed by the RW morph at 27.03% and the GW morph at 18.75% (Fig. 2a).

In the inter-morph crosses, we noted donor-specific differences in the GW and RW morphs. The donor specificity was related to whether a morph was used as a male parent or a female parent in the inter-morph cross-pollination treatments. In crosses where GW morph was the male parent (i.e., pollen donor) overall lower success rates (pair a: GW x GR,

24.24% and in pair b: GW x RW, 25%) were recorded, when compared to crosses where it was used as a female parent, that is pollen recipient (in pair a: GR x GW, 43.33% and in pair b: RW x GW, 44.44%, Fig. 2a). In the last inter-morph treatment (pair c) where RW and GR morphs were crossed, fruit set rates were 37.84% when RW was the female morph and 46.67% when RW was the male morph.

Among all hand-pollination treatments, self-pollination treatments exhibited the lowest seed count (mean number of seeds per matured fruit ± s.e.; 8.63 ± 3.15), followed by intra-morph crosses (16.93 ± 1.8) and then inter-morph crosses (18.63 ± 1.2). In the self-pollination treatment, the GR morph exhibited a significantly lower seed count (zero) compared to the GW morph (14.5 ± 6.5) and RW morph (13.33 ± 4.37; Kruskal-Willis $\chi 2$ = 5.2499, df = 2, p = 0.07244; Fig. 2b; Table S5 and S6). In the intra-morph cross-pollination treatment, the seed counts varied significantly among the morphs. The GW morph exhibited the lowest seed count (10.72 ± 2.7) compared to the GR morph (20.71 ± 2.8) and the RW morph (15.7 ± 2.7; Kruskal-Willis $\chi 2$ = 5.2499, df = 2, p = 0.07244; Fig. 2b; Table S6). Seed count in inter-morph cross-pollination treatments was not significantly different among the three pairs (Fig. 2b; Table S6). Finally, the mean total fruit set per plant in open pollination treatments was recorded to be equal in all three morphs (Fig. 3a). However, the mean seed count per fruit was significantly different among the morphs (Kruskal-Wallis $\chi 2$ = 36.3222, df = 2, $p < 0.0001$) with the GR morph being the highest (23.7 ± 1.5), followed by GW (20.3 ± 1.2) and RW (11.5 ± 1.4; Fig. 3b).

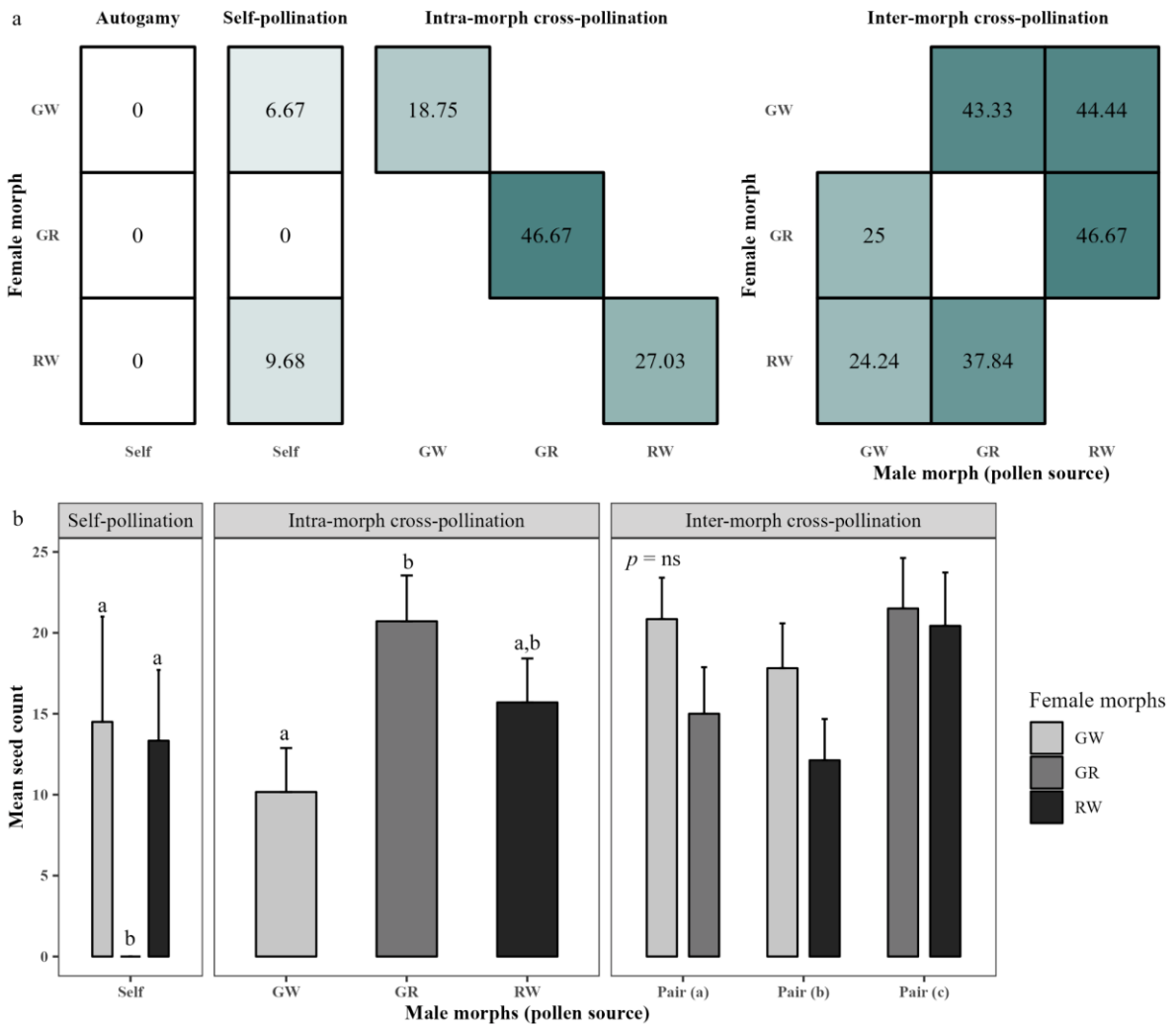

Fig. 2. Physiological compatibility between the three floral colour morphs of *C. caulina*. a. Heatmap illustrating the percent success rate (values inside box) from 478 hand-pollination from 15 treatments. The male morphs (pollen source) are displayed on the x-axis, while the female morphs (stigma identity) are shown on the y-axis. b. Mean (± s.e.) seed count from the pollination treatments in the three morphs of *C. caulina*. The male donors (pollen source) are displayed on the x-axis while the boxes represent female recipients (stigma identity). Significant differences are shown by different alphabets.

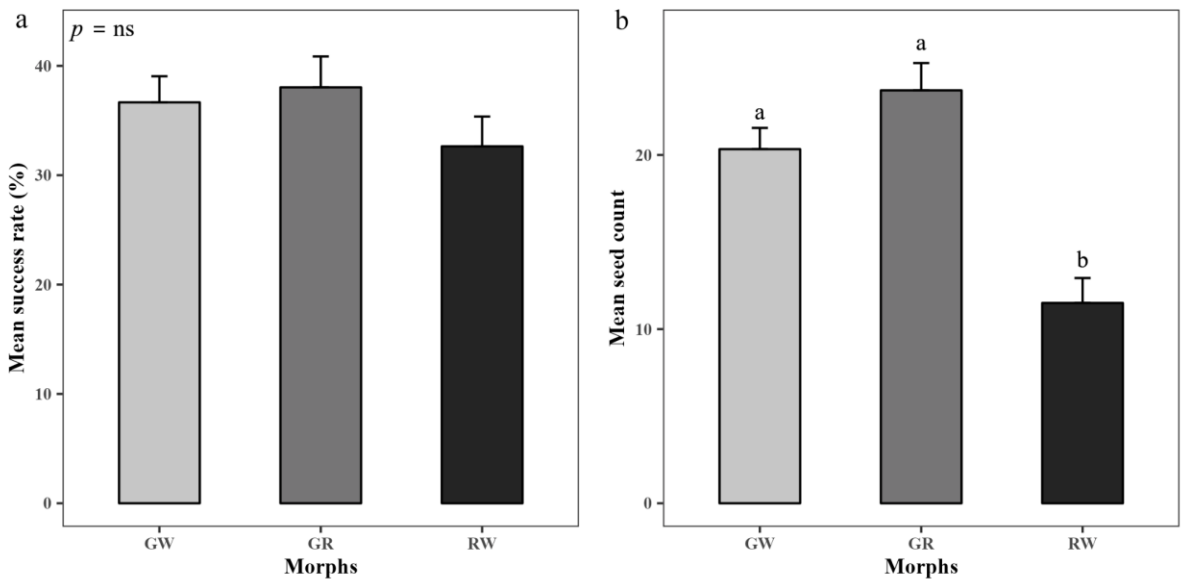

Fig. 3. Natural fitness of the three morphs of *C. caulina* represented as A. mean (± s.e.) success rates (%) of open pollination treatments and B. mean (± s.e.) seed count of the successful fruits from those open pollination treatments. Small letters indicate significant differences between female morphs. Results of the statistical test between morphs are given in Table S11.

Relationship between multiple floral traits and morph abundance

Across three paths, the path analysis detected six (total floral reward, pollinator visitation rate, abundance, self-compatibility, cross compatibility, and natural fruit-set) out of the seven variables to contribute towards a stable selection regime that can explain the presence of polymorphism in the KAS population. In path 1, the latent variable β1, that is the total floral reward was positively related with the pollinator visitation rate (Estimate = 3.371, z = 3.314, $p < 0.0001$; Fig. 4) and it was inversely related with the plant's abundance (Estimate = -3.481, z = -4.348, $p < 0.0001$). The natural fruit set was negatively related to the pollinator visitation rates (Path 2.1; Estimate = -2.573, z = -1.749, $p = 0.08$; Fig. 4), however higher natural fruit-sets were explained by outcrossing events (Path 2.2; Estimate = 0.235, z = 3.846, $p < 0.0001$) and not by selfing events (Path 2.1; Estimate = -0.122, z = -0.096, $p = 0.924$; Table S13). Finally, the second latent variable (β2) that is reproductive fitness increased with higher plant abundance (Estimate = 0.148, z = 2.250, $p = 0.024$) while it had no relationship with the total seed count. We found similar trends in the morph specific path analyses (Methods S1, Fig. S5, Table S7).

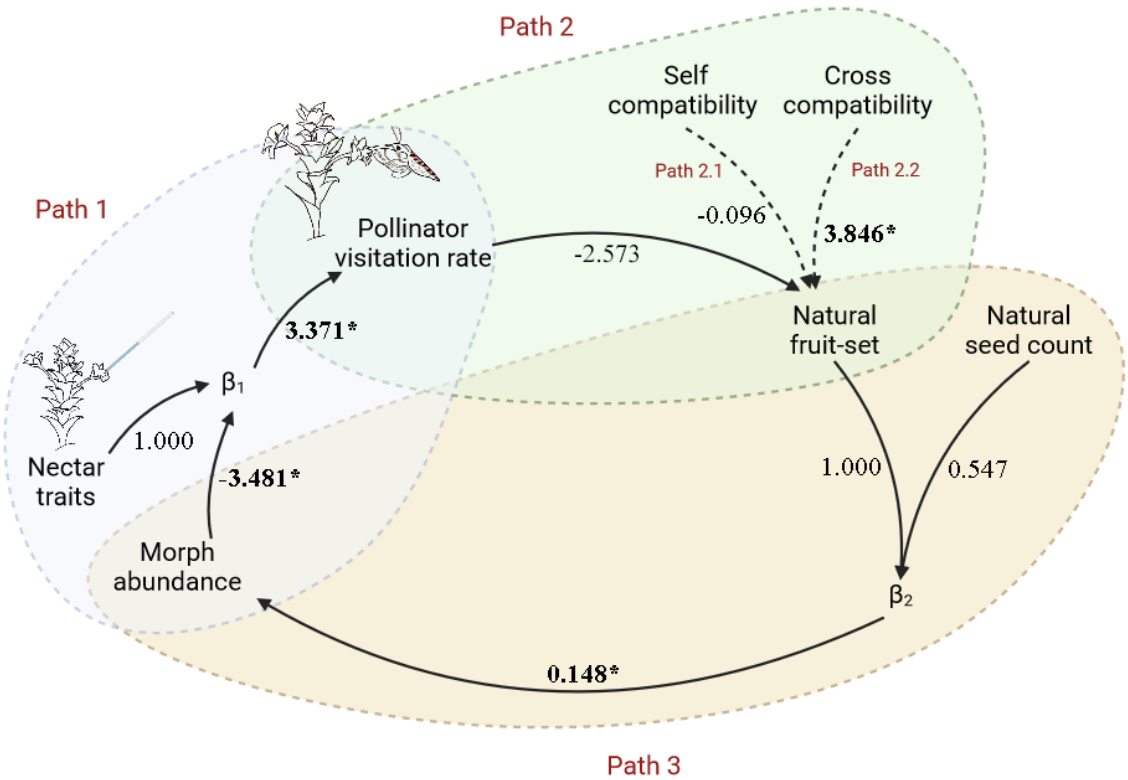

Fig. 4. Schematic representation of three paths (1, 2 and 3) that were used to test the selection model of bract colour polymorphism in *C. caulina*. The response variables for each path are shown in the bottom while the explanatory variables are on the top. Latent variables are mentioned in bold symbols (β_1, β_2, and β_3). Path 2 was tested using two separate explanatory variables self-pollination (Path 2.1) and cross-pollination (Path 2.2). Estimated values are provided for each path, with asterisks (*) indicating the significance of regression ($p < 0.05$).

Discussion

In the most comprehensive phylogenetic study of the genus *Curcuma* that is available to date, Leong-Škorničková *et al*. (2007) commented: "All of the *Curcuma*- like species have highly derived autapomorphic flower morphologies that misled previous authors into classifying them into separate genera. These distinctive morphological deviations are probably the result of specific ecological preferences and specific pollination systems." One of the *Curcuma*-like species referred to in this quote is *Curcuma caulina*, which taxonomically shuttled between the genus *Curcuma* and *Hitchenia* (Baker, 1894; Leong-Škorničková *et al.*, 2010) because of its unique morphological characters such as long floral tubes. In this study, we show that *C. caulina* is not only polymorphic in bract colours, but the polymorphism in traits extends to nectar characteristics as well as physiological

compatibilities that result in differential reproductive success among the morphs. Using selection models, we show that these traits are under balancing selection and maintain bract colour polymorphism in the species.

Floral colour polymorphism has been recorded extensively in plants, but there are very few studies that have quantified intra-population morph frequencies and characterised the floral morphs within a species (Elam & Linhart, 1988; Lau & Galloway, 2004; Caruso *et al.*, 2010; Berardi *et al.*, 2016; Koski *et al.*, 2020; Jacquemyn & Brys, 2020). While both the order Zingiberales (Costaceae, Heliconiaceae, Cannaceae and Marantaceae; (Temeles *et al.*, 2013; Gowda & Kress, 2013)) and the plant family Zingiberaceae (*Hedychium, Globba,* and *Curcuma*; (Leong-Skornicková *et al.*, 2007; Saryan *et al.*, 2020)) are known for their variation in floral or bract colour, very few studies have quantitatively identified polymorphisms in them. Therefore, we first identified and delineated three distinct morphs - GW, GR, and RW, based on their lateral bract colours (Fig. 1 inset) and their natural frequencies of occurrence, and we further characterised their polymorphic traits. We summarise below the morphological, ecological, and reproductive characteristics of the floral colour morphs of *C. caulina* which we eventually used to explain their structure and maintenance within the KAS population.

Floral colour polymorphism in C. caulina is defined by bract colour, nectar traits, and physiological compatibilities

Using Ford's (Ford, 1945) criteria for defining polymorphism, in the KAS population we identified three morphs (Fig. 1 inset) based on their bract colour. These three morphs were unequally distributed in the population with an almost tri-modal distribution wherein GR morphs are the most common (~ 44% occurrence), followed by GW (~ 29%), and RW (13%). A few bract colour variants (~ 14%) were extremely rare and infrequent across years and each variant was present in less than five percent of the population. Although none of the morphological characters showed significant association with bract colour, the unequal or rather skewed distribution of the morphs in the KAS population suggests that the morphs are under biotic selection (Ortiz *et al.*, 2015; Narbona *et al.*, 2018).

While morphological floral traits were not variable among the morphs, we noted morph-specific significant differences in the nectar traits which included sucrose concentration, nectar volume, and total nectar energy (Fig. 1a; Table S2) among the three morphs of *C. caulina*. Notably, we found the rare RW morph to have higher sugar concentration (32.5% Brix) and almost twice the nectar volume (21 µl) than the two common

floral morphs GR and GW (sugar concentrations 28-30% Brix and nectar volumes 11-15 µl). Similar cases where nectar traits show variations even when floral traits are invariable has been reported in plants such as *Ipomopsis aggregata* and *Ipomopsis tenuituba* (Melendez-Ackerman *et al.*, 1997; Melendez-Ackerman & Campbell, 1998). The discovery of morph-specific variation in the nectar traits highlights that polymorphism in *C. caulina* is not limited to visual floral traits and that rewards such as nectar can be defining-characters of the floral morphs.

It is known that plants can recognise self- and cross-pollen, and they sire more seeds using cross-pollen than self-pollen (Lloyd, 1992; Hiscock & McInnis, 2003). This was true in *C. caulina* as well where irrespective of the morph type, out-crossing treatments resulted in higher fruit set and seed count when compared to self-pollination treatments (Fig. 2). However, we noted the three morphs to have contrasting patterns in their physiological compatibility. We found the most common GR morph to be self-incompatible while the less common GW morph and the rare RW morph were self-compatible (Fig. 2a). Similar examples of differential self-compatibilities leading to mixed mating systems among morphs, although rare, have been reported in a few species. For example, in *Ipomea purpurea*, the less frequent white morph has higher selfing rates when compared to the two common morphs (Brown & Clegg, 1984; Fry & Rausher, 1997). In *Castilleja coccinea*, two of its morphs have similar fruit set and seed set in the self and outcrossed treatments, while one of the morphs (scarlet morph) has reduced fruit set and seed set in the autogamous and control treatment, but it produced more seed in the inter-morph crosses (Kim *et al.*, 2019). Finally, in *Lupius nanus* while visual floral polymorphism were known to be of only two types, based on the outcrossing rates along with few physiological characters such as self-compatibility, pollen production, ovule numbers, and stigmatic hair, Horovitz & Harding (1972) identified at least three morphs.

Morph-specific pollinator visitation patterns and outcrossing rates

C. caulina with its tubular, nocturnally opening, mildly fragrant, nectar rich, white flowers represent a classic moth-pollination syndrome (Johnson *et al.*, 2017). Based on pollinator visitation patterns and outcrossing rates we outline three key conclusions about the *C. caulina*-hawkmoth system: a) Pollinator visitation rates are driven by nectar characteristics of each morph rather than the abundance of the morph in the population. We conclude this from the observation that the rarest morph, RW which has the highest nectar rewards received almost twice the visitation rates (5.14 ± 0.96 visits per flower per hour) when

compared to the other two morphs (GW morph: 2.8 ± 0.59; GR morph: 2.19 ± 0.3 visits per flower per hour; Fig. 1b). Hawkmoths are known to perceive and readily discriminate against less-rewarding floral morphs (White *et al.*, 1994; Hodges, 1995; Kelber, 2003; Costa *et al.*, 2023). We present a similar pattern in *C. caulina* where visitation rates of the hawkmoths were significantly different for each morph and reflect the floral reward offered by those morphs. b) Revisitations by pollinators to a morph may facilitate higher within-inflorescence pollen movement resulting in high rates of geitonogamy and lower outcrossing rates (Harder *et al.*, 2004; M. Eckhart *et al.*, 2006; Ishii & Harder, 2006). We conclude this based on our observation of higher repeat visits to the same flowers by the moths in the rare and higher rewarding RW morph. In contrast the two common morphs GR and GW offer low nectar rewards and received lower visitation rates but with higher inter-individual switches (personal observations of SS and VG). They can be expected to have higher outcrossing rates in comparison to the RW morph. c) Pollen limitation plays an important role in determining the reproductive fitness of the morph, as suggested by the low seed-set (Fig. 3) in the rare and self-compatible RW morph which receives high pollinator visits but most are repeat-visits by a pollinator. In contrast the common GR morph's higher reproductive fitness (Fig. 3) can be attributed to higher outcrossed pollen from unique visits, and complete self-incompatibility. Similar cases where rare morphs experience pollen limitation and increased selfing due to repeat-visits has also been noted in *I. purpurea* (Epperson & Clegg, 1987) and *C. coccinea* (Kim *et al.*, 2019).

Multiple traits explain the maintenance of floral polymorphism

Path 1

Reproductive fitness in polymorphic species like *C. caulina* is influenced by morph-specific traits such as distribution frequency of the morph, nectar traits, pollinator visitation patterns, and reproductive compatibility. Among the three paths in the SEM analysis (Fig. 4) the first path identified an overall strong association (3.371) between the abundance of a morph, floral rewards present in the morph, and pollinator visitation rates (PVR) to the morph. The positive estimate between β_1 and PVR in the Path 1 corroborates the well-established result that pollinators show higher visitation rates to flowers that offer higher energy rewards (Heinrich, 1975; Salguero-Faría & Ackerman, 1999; Makino & Sakai, 2007; Fisogni *et al.*, 2011; Schiestl & Johnson, 2013; Knauer & Schiestl, 2015; Brunet *et al.*, 2015; Akter *et al.*, 2017). However, the estimate between abundance and the lateral variable β_1 which represents total nectar energy available from a morph in a population was noted to be negative (β_1 = -3.481),

corroborating our observation that morphs with higher abundance were associated with lower nectar energy, while the rare morph offered significantly higher floral reward in the population. Our results from *C. caulina* are contrasting to the only study that has shown a positive association between nectar volume and morph abundance, that is in *Protea aurea* (Carlson & Holsinger, 2013) where the common morph had higher nectar volume than the rare morph.

Path 2

This path was designed to test if the reproductive fitness in *C. caulina* is defined by the pollinator visitation rate to a morph and the morph's physiological compatibilities. In both Path 2.1 and 2.2, morphs with higher self-compatibility showed reduced reproductive success than morphs with higher self-incompatibility. The relationship between self-incompatible rates and natural fruit-set was statistically significant ($p < 0.0001$) and positive (0.235), suggesting that higher self-incompatibility encourages cross-pollination resulting in higher fruit-set. Complimentarily, Path 2 also revealed that self-compatibility leads to lower fruit sets (estimate = -0.122). Therefore, in *C. caulina*, cross-pollen contributes more towards fruit-set than self-pollen, suggesting that cross-pollination is favoured in the population.

Path 3

Finally, Path 3 confirmed that the most abundant morphs also had higher seed count and fruit set within the population. Reproductive fitness as a reliable predictor of the abundance of the morphs within the population was shown by the statistically significant and moderately positive estimate value (0.148) between the latent variable β_2 which represents total reproductive fitness and morph abundance.

Bract colour polymorphism in C. caulina is maintained by multiple floral traits

Pollinator dependence is key for sexual reproduction in *C. caulina* since autogamy is absent among all its morphs (Fig 2a). Floral colour is a key visual trait for a pollinator and is an important functional trait in plants that are under pollinator selection (Harder & Barrett, 2006). However, in plant-pollinator interactions where visual traits may not be critical such as in nocturnal species, we show that selective advantage of a particular morph may operate indirectly through associated floral traits. This is known from diurnal plants as well where floral traits associated with floral colour have been shown to be under selection (Wolfe, 1993; GÓmez, 2000; Majetic *et al.*, 2007; Rausher, 2008; Arista et al, 2013; Carlson & Holsinger, 2013; Ortiz et al, 2015; Lebel *et al*., 2018). Here, we identified four plant traits (plant

abundance, floral nectar reward, pollinator visitation rate, and physiological compatibility) in *C. caulina* that were associated with a morph's bract colour and are intricately connected to each other in a feedback loop that maintains the different morphs in the KAS population. To the best of our knowledge, our study is the first to provide evidence of pollinator driven ecological and physiological selection regimes that act together to maintain bract colour morphs within a population of a nocturnal plant species.

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Competing interests

The authors have no conflicts of interest to declare.

Author contributions

SS and VG conceived and designed the study. SS conducted the experiments and collected the field data. SS performed the statistical analyses with inputs from VG. SS drafted the manuscript; VG provided conceptual advice and edited the manuscript. All authors gave final approval for publication.

Data availability

The entire dataset and analysis codes used in this study are accessible from the authors upon reasonable request.

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Supplementary information (brief legends)

The following Supporting Information is available for this article:

Fig. S1 to S5

Table S1 to S7

Methods S1