Pollinator visitation pattern and differential reproductive compatibilities contribute to the unequal morph abundances in a nocturnal ginger from Western Ghats, India

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Summary

- Polymorphism, the presence of discrete floral morphs within a population, is often maintained by pollinator-mediated or frequency-dependent selection. While pollinators often act on visual displays, this study investigates hawkmoth-mediated selection in the nocturnal *Curcuma caulina* (Zingiberaceae), where visual cues play a minimal role. We explore the impact of non-visual traits, such as morph abundance, nectar rewards, and reproductive compatibility, on the maintenance of bract color polymorphism.
- We measured floral traits, nectar rewards, and abundance of morphs in the natural population, and quantified reproductive compatibility using hand pollination treatments. We next used path analysis to test the effect of morph abundance on reproductive fitness via floral and ecological traits.
- We identified tri-modal morph abundance with two common morphs and one rare morph. Morphs differed in nectar traits and reproductive compatibility but not in floral morphologies. Rare morphs had high-energy nectar, higher pollinator revisits and self-pollination, and lower seed output, while common morphs had low-energy nectar, lower revisits, higher outcrossing and fitness.

- Morph abundance in *C. caulina* is shaped by an interplay between nectar rewards, pollinator attraction, and compatibility. We conclude that in perennial rhizomatic species understanding historical selection regimes and both abiotic and biotic factors are necessary to characterize floral polymorphism.

Keywords

Floral color polymorphism, Frequency-dependent selection, Hawkmoth pollination, Path analysis, Reproductive fitness, Self-incompatibility

Introduction

In plants, floral color polymorphism (henceforth 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, mating strategies, 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 color (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). Almost all of these studies note that pollinators show selective preference for specific traits that are associated with floral color, hence influencing selection differentials among the floral color morphs. For example, in *Lobularia maritima*, pollinators show preferential visitations towards one of the morphs, however, it is not the floral color, but floral display size associated with the white morph that is the target of pollinator-mediated selection (Gomez, 2000). Similarly, traits such as plant height and flower production in *Hesperis matronalis* (Majetic et al., 2007; 2009), and floral nectar and flower number in *Protea aurea* (Carlson and Holsinger, 2013) have been found to be associated with floral color and targets for pollinator-mediated selection of FCP.

In the above-mentioned studies, selection is measured on the trait in an individual and morph abundance is often the outcome of selection processes. However, despite the recognition that the relative abundance of morphs within a population can influence selection, morph abundance as a critical structural feature of a polymorphic species has rarely been investigated. Narbona *et al.* (2018) reported that nearly all of the Mediterranean species with FCP have unequal morph frequencies at the population-level, while some species exhibited temporal fluctuations in morph frequencies (Dormont *et al.*, 2010; Tang & Huang, 2010; Tang *et al*., 2016). Morph frequency influences pollinator discrimination (Smithson and Macnair, 1996; Eckhart et al., 2006; Dormont *et al.*, 2010; Ortiz *et al.*, 2015; Wenzell *et al.*, 2023) and can maintain FCP via frequency-dependent selection (FDS) through disassortative mating (i.e., negative frequency-dependent visitation; Gigord et al. 2001; Gegear and Laverty 2005; Benadi and Gegear 2018) and adaptive foraging influencing assortative mating (positive frequency-dependent visitation; Jones and Reithel, 2001; Eckhart et al., 2006). Therefore, 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 morphs (Gigord *et al.*, 2001; Lau & Galloway, 2004; Koski *et al*., 2020).

In contrast, when FCPs are not driven by pollinator-mediated selection, the floral color morphs are either associated 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 has been shown to 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, despite it being under pollination limitation as observed in *Ipomoea purpurea* (Fry & Rausher, 1997; Subramaniam & Rausher, 2000) and in *Lysimachia arvensis* (Arista *et al.*, 2013; Ortiz *et al.*, 2015). Thus, inter-morph differences in physiological compatibilities can play a critical role in the maintenance of rare morphs within a population, through fitness differences among the morphs.

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 color 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, 2024). In genera like *Alpinia* and *Roscoea*, floral polymorphism, regardless of the type, has been shown to be critical for plant-pollinator interactions and speciation (Takano *et al.*, 2009; Paudel *et al.*, 2019). Among the polymorphic gingers, the taxonomically diverse genus *Curcuma* is known for its floral color 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 colors, and pollination efficiency (Weiss, 2001; Goyret *et al.*, 2008). In a nocturnal ginger *C. caulina*, we noted both the presence of FCP and unequal morph abundance within a population in Western Ghats, India. In this study, we tested whether floral trait differences, pollinator visitation differences, and reproductive differences suggest that the morphs are under pollinator-mediated and frequency-dependent selection. To the best of our knowledge, the maintenance of FCP has not been studied in any exclusively nocturnally flowering plant species and we address the following questions:

1. Do morphs share similar floral traits and outcrossing rates?
2. Does morph abundance affect pollinator visitations and reproductive fitness?
3. How are rare morphs maintained in a polymorphic population?

To explain the unequal morph abundance at the population level, we finally tested the significance of the four traits - plant abundance, nectar quality, pollinator visitation rate, and selfing/outcrossing rates, on the reproductive fitness of the morphs using path analysis.

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 born in cincinni (1–3 flowers per cincinnus), 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; 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).

Bract color polymorphism and variation in floral morphology and nectar rewards

color of the lateral bracts of *C. caulina* individuals varied within the population from predominantly green to predominantly red colored inflorescence (Fig. S2a). To determine the frequency of these variants, we used randomly placed ten transects, each measuring 10 x 4 m^2. Within each transect, frequency of all bract color variants were identified and counted. The extent of the reddish-pink coloration (0% to 100%) on the first or basal lateral bract was used to classify the variants and this was also measured using handheld spectrophotometer (Ocean Optics; Fig. S2a, b). This method identified six variants with continuous bract color variation within the population (Fig. S2c). However, we identified only three variants (0%, 60%, and 100%) as morphs based on Ford's criteria for polymorphic forms (Ford, 1945; Fig. S2). These three morphs were named based on the coloration of their lateral and coma bracts: the 0% variant as Green-White (GW), the 60% variant as Green-Red (GR), and the 100% variant as Red-White (RW; Supp Fig. 1a). The distribution of these morphs was unequal, with GR morphs being the most common (~ 44% occurrence), followed by GW (~ 29%) and RW (13%). The remaining three bract color variants (15%, 30%, and 45%) were extremely rare, each constituting less than 5% of the population (Fig. S2c).

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). 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). Differences between the morphs were tested using the Kruskal-Wallis test followed by Dunn's test as a post-hoc; the significance was 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 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 were manually scored, where the total number of visits by each pollinator and the total number of flowers present in the plot were measured. We 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. To compute the visitation rate to each morph within the entire population, we simulated visitation rates for each morph using observed data in a Monte Carlo simulation in R. We computed the visitation rate for each morph at the population level as follows: a) We generated 10,000 values (with replacement) for the two variables - pollinator visitation rates (PVR) and morph abundance using sampling with replacement from the observed data set (98 values for PVR and 30 values for morph abundance). b) We next calculated the product of the two variables (PVR x morph

abundance) and computed the mean and variance for this simulated data set (Fig. 1c, Z axis). This value represents the expected PVR for each morph in the population, given the recorded plot-level variation in morph abundance in the study area.

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). Temporal differences in visitation rates between the morphs were tested using the Mardia-Watson Wheeler test (or uniform score test; Zar, 1999). Since the pollinator observation plots were not always located within the morph-census transects, the pollinator visitation rates were standardized for morph frequency using Monte Carlo Simulation (n = 10,000). The morph frequency and pollinator visitation rates for each morph were utilized to estimate the distribution of their product for the respective morphs. To test for inter-morph differences in pollinator visitation rates we used 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 $p<0.05$. 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 color 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 categorized 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 study 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 cincinnus. 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 test traits effects on the reproductive fitness of the morphs

We used path analysis to understand the direct and indirect effects of abundance of a morph on its natural fitness as described by Mitchell (1993). Path analysis was tested for the entire Kaas population of *C. caulina* as well as for each morph independently (n = 300 individuals; i.e., 100 per morph) in order to assess the net effect and important selection regimes within the population and within each morph. Three indirect effects included in the path analyses were- total nectar reward, pollinator visitation rates, and physiological compatibilities. Values for the total nectar reward was normalized with morph abundance and of natural fitness with seed-set using Monte Carlo simulations (n = 10,000). Path analysis was performed in R (4.3.0; R Core Team, 2021) using the *lavaan* package (Rosseel, 2012) and path estimates were computed using 10,000 iterations in the bootstrapping procedure. Estimates of model fit were computed using Chi-squared goodness of fit, Comparative Fit Index (CFI) and Tucker-Lewis Index (TLI). A nonsignificant chi-squared value indicates that a model is not significantly different from the observed correlations in the data and is therefore a good fit whereas CFI and TLI indices above 0.9 are considered to be a good fit (Mitchell, 2001). Finally, we tested the hypothetical relationship between reproductive fitness and relative abundance using a regression model which was not part of the main path analysis.

Results

Floral (bract) color polymorphism and morphological variability

The abundance of the three morphs differed within the population. The GR morph (98.6 ± 7.2 individuals; ~44 %) and GW morph (75.7 ± 7.2 individuals; ~29 %) were the most common morphs; their frequencies were not significantly different from each other (Kruskal-Wallis $\chi 2$ = - 0.692, df = 1, p = 0.2446). The rarest of the three morphs was the Red-White morph (RW) with 24.2 ± 2.0 individuals (~13 %) in the population, and 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). All other bract color variants totaled to ~14% in the population and thus were extremely rare. The non-metric multidimensional scaling (nMDS) analysis of the 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. Since we detected polymorphism in bract colors and not floral colors, henceforth, the term color polymorphism refers to only bract color polymorphism in *C. caulina*.

Variability in nectar traits and hawkmoth visitation rates

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 and concentration (Fig. 1b; 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 probosces: *Agrius convolvuli* (~12.4 cm) and *Hippotion rafflesii* (~4.6 cm, Fig. S3; Sphingidae). 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 observe the curling of the proboscis during foraging, as noted 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*, and the rupture in anther theca could be used as a visual feature of flowers 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 three morphs showed the highest hawkmoth activity during the first few hours of anthesis, between 19:00 hrs to 23:00 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. 2c; Kruskal-Wallis chi-square = 6.52; df = 2; p = 0.03839; Table S3). However, the visitation rate standardized for morph abundance showed a significantly lower visitation rate (Kruskal-Wallis chi-square = 3.77; df = 2; p = 0.01511) to the RW morph (mean number of visits per hour ± s.e.; 119.94 ± 10.75), than the common GR (175.15 ±17.29) and GW morphs (171.39 ± 18.72; Fig. 1d;).

From video recordings of pollinator visitations, we also observed that hawkmoths revisited the same flowers within an RW morph (~ 10-12 times), once they discovered this rare but 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.

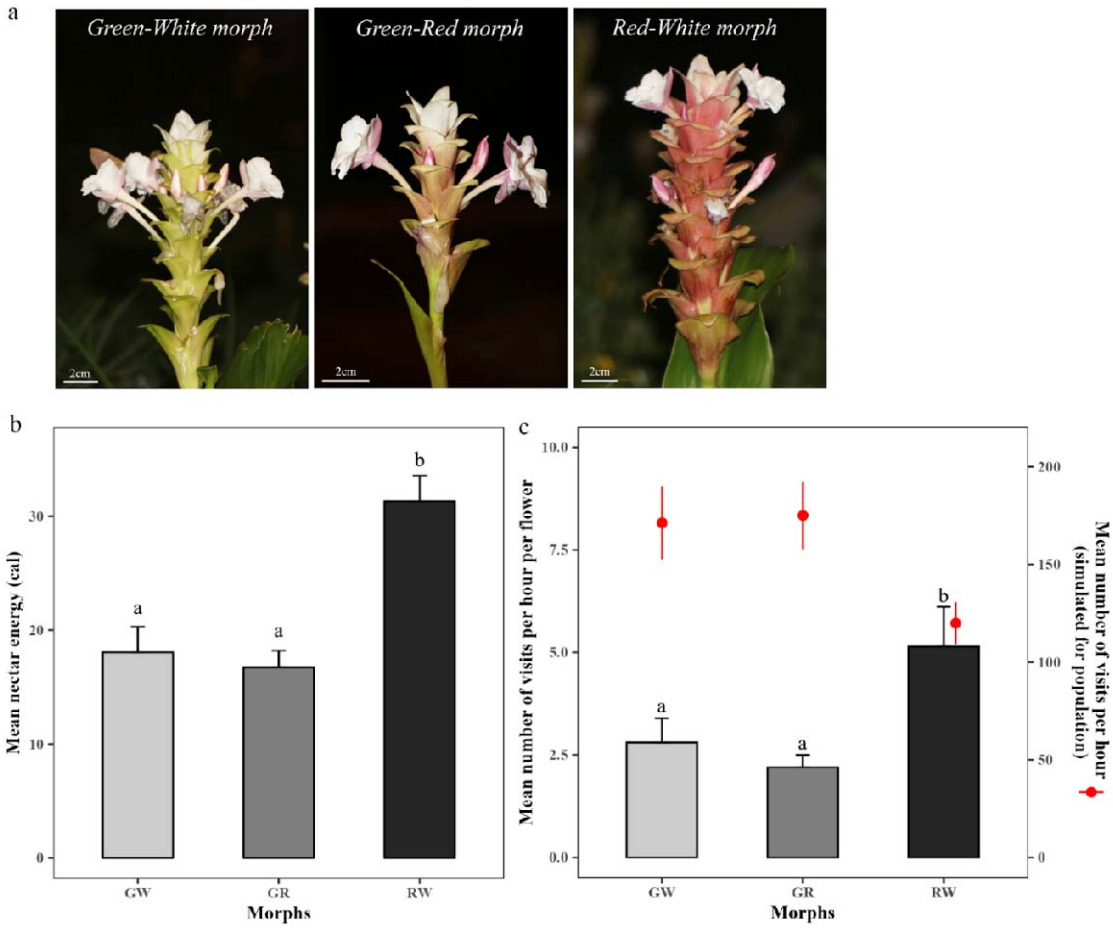

Fig. 1. Three bract color morphs of *C. caulina*, their nectar rewards energy, and natural pollinator visitation rates. a. From left, GW, GR, and RW morphs of *C. caulina*, b. The mean (± s.e.) nectar energy (cal), c. Bar plot represents pollinator visitation rates (mean ± s.e.) measured for each morph in the observation plots (y-axis) and simulated pollinator visitation rates (mean ± s.e.) calculated for the population shown by red dots. Different alphabets indicate significant differences between morphs ($p < 0.05$).

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). We also noted that the seed counts recorded in the open (natural) pollination and self-pollination treatments of RW were similar (Fig. 2a and 3a).

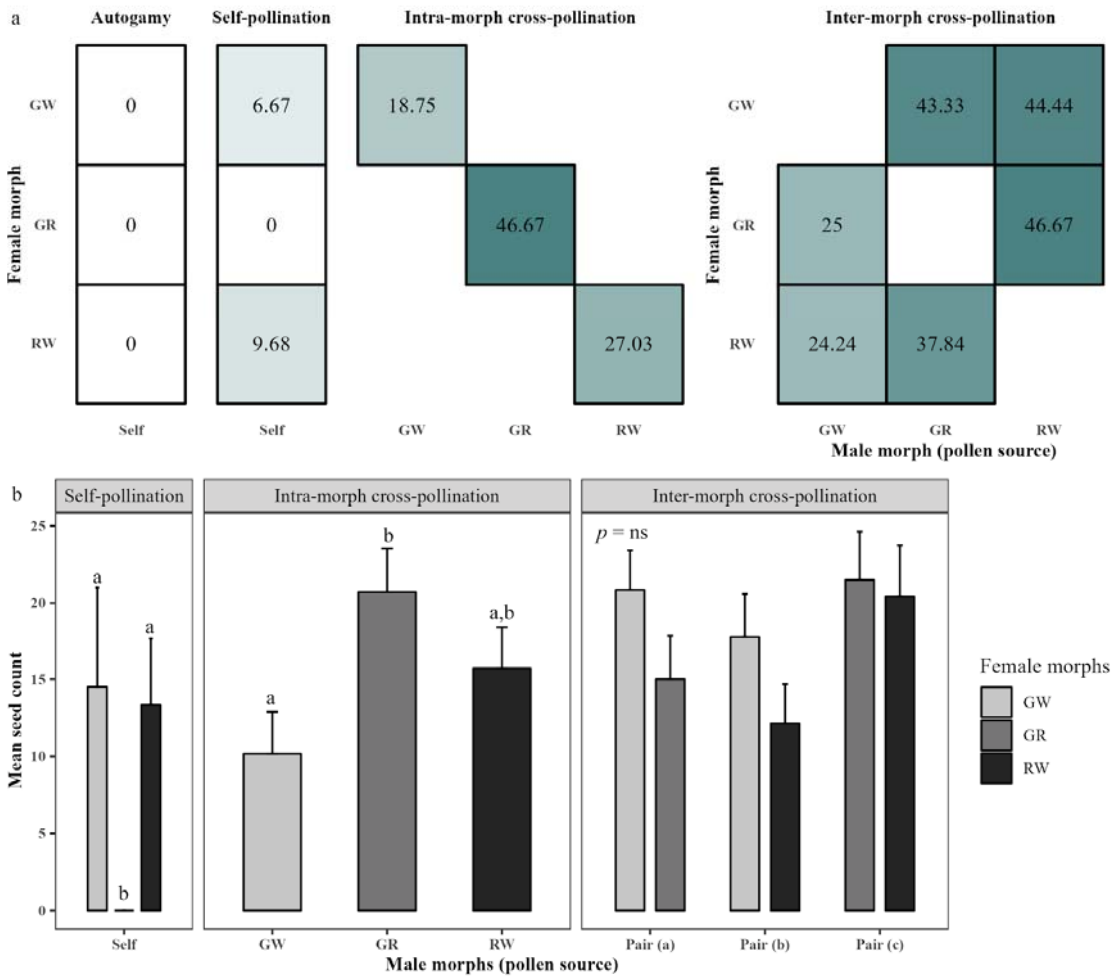

Fig. 2. Physiological compatibility between the three floral color morphs of *C. caulina*. a. Heatmap illustrates the percent success rate (values inside box) from 478 hand-pollination from 15 treatments. The male morphs (pollen source) are displayed on the x-axis, while the female morphs (stigma identity) 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.

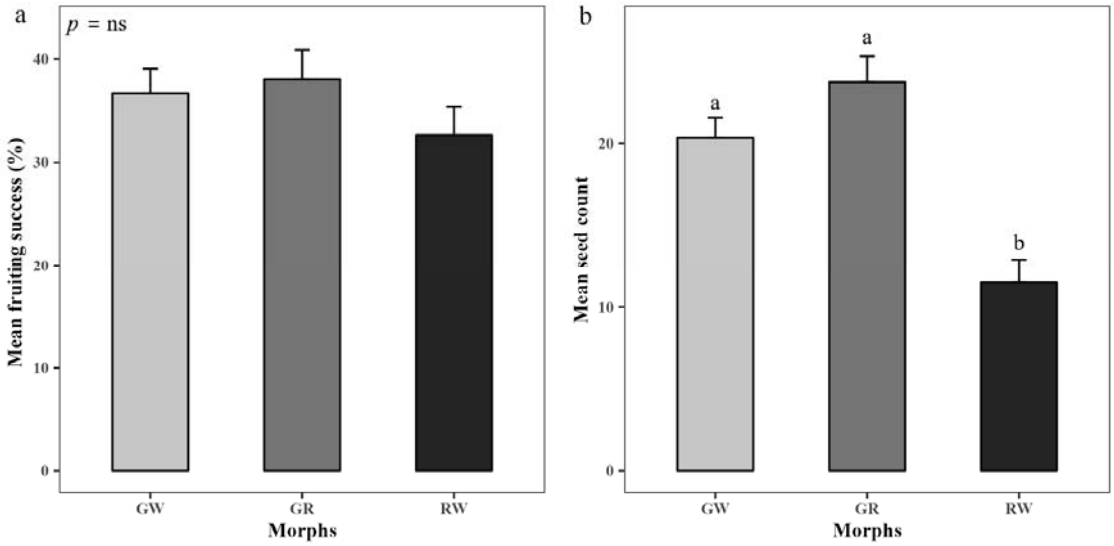

Fig. 3. Natural fitness of the three morphs of *C. caulina* measured as A. mean (± s.e.) reproductive success (% success) of open pollination treatments and B. mean (± s.e.) seed count of the successful fruits from those open pollination treatments. Small letters indicate significant differences between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

Associating morph abundance and floral traits

In the path analyses, the five variables resulted in one direct path between abundance and reproductive fitness (e), seven indirect paths (a to d and f to h), one correlation path between abundance and type of compatibility (i), and one path on the effect of reproductive fitness on relative abundance (j; Fig. 4). The Chi-squared goodness of fit test, CFI, and TLI scores identified a good fit for all four models (*C. caulina*, GR, GW and RW morph; Table 1). In the case of the GW morph, the model fit as estimated by TLI values was not good (TLI = -0.65), and we did not find any significant relationships between the traits as well as for the net effect. We found that both the direct effect (path e in Fig. 4) and the net effect of morph abundance on reproductive fitness were positive and statistically significant in three (*C. caulina*, GR morph, and RW morph) of the four path analyses models (Table 1).

In the species-specific model, *C. caulina* in KAS showed a positive and significant effect of only morph abundance on total nectar reward (a), self-compatibility on reproductive fitness (d'), and finally, reproductive fitness on relative morph abundance (j). The correlation between morph abundance and self-compatibility was also noted to be positive (i''). For the within-morph path analyses, significant effects among indirect effects were observed only in

the GR morph, although positive effects were present across several paths for the other two morph-specific models (GW and RW). In the GR morph, the following paths were significant - morph abundance and total nectar reward (a), total nectar reward and reproductive fitness (g), cross-compatibility and reproductive fitness (d''), and the regression of reproductive fitness on morph abundance (j). The direct effect between relative morph abundance and reproductive fitness (e), the regression of the reverse effect, that is, between reproductive fitness and abundance (j) was significant and positive in RW but negative and not significant for GW. We failed to detect any significant relationships between the other traits in the RW morph, although notably, the effect of self-compatibility on reproductive fitness was positive, whereas the effect of cross-compatibility on reproductive fitness was negative but not significant (path d; Table 1).

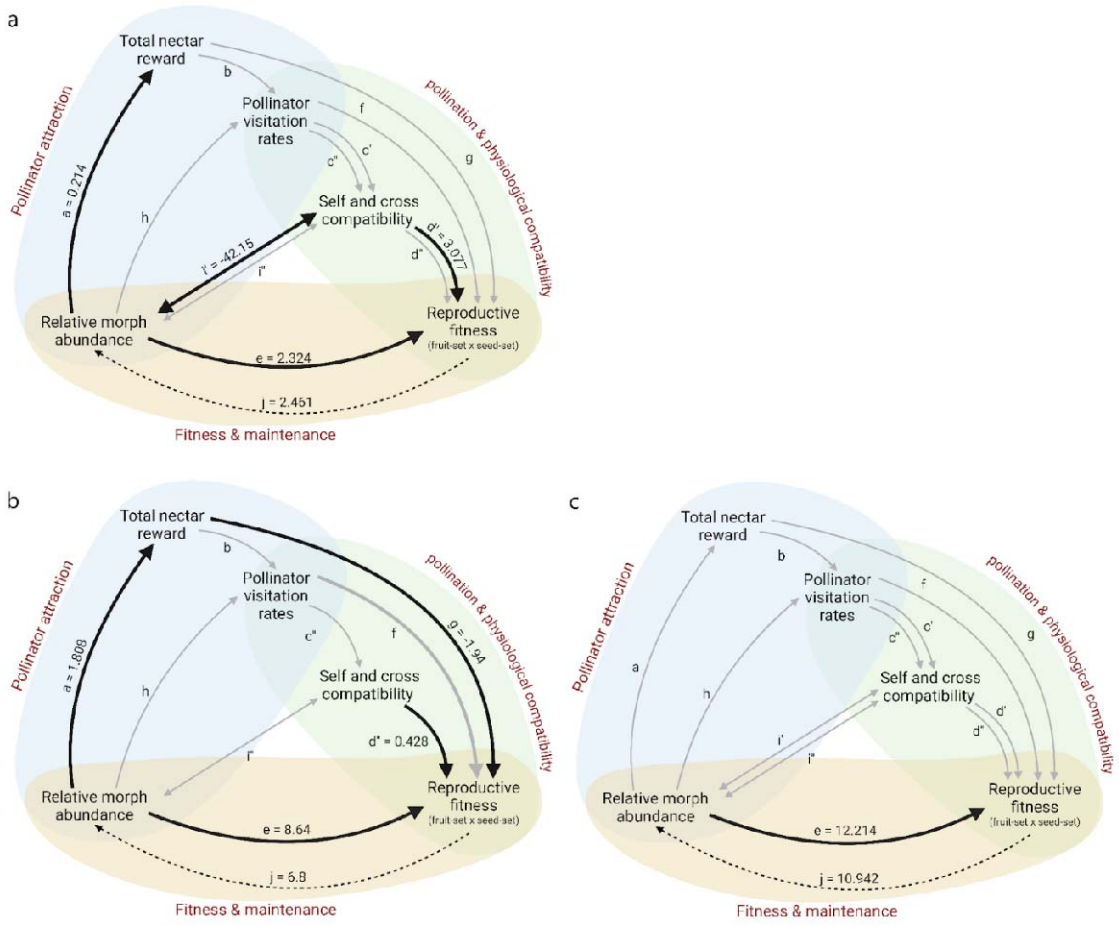

Fig. 4. Path diagrams illustrating the effect of morph abundance on reproductive fitness, analysed a) as species-specific model, b) for the common GR morph and c) for the rare RW

morph. One-headed arrows (paths a—h and j) indicate regression, while the two-headed arrow (path i) indicates correlation. Thick arrows denote significant effects (P < 0.05), with the strength of regression or correlation specified adjacent to them. Path IDs for all direct and indirect effects are provided, and their strengths can be referenced in Table 1.

Table 1: Results of path analysis for all four models. Bold values represent the statistical significance. Path IDs are the same as fig. 4.

			For *C. caulina*	**GR morph**	**GW morph**	**RW morph**
Model fit:	Chi-square statistics		0.052	0.037	0.006	0.664
	p-value (Chi-square)		**0.82**	**0.847**	**0.94**	**0.415**
	CFI value		**1**	**1**	1	**1**
	TLI value		**1.332**	**7.138**	-0.65	**1.494**
Regression:		**Path ID**				
Reproductive fitness ~ Relative abundance		e	**2.324**	**8.64**	-3.21	**12.241**
	Total nectar reward	g	-0.122	**-1.94**	0.416	-0.63
	Pollinator visitation rates	f	0.004	0.048	0.019	0.08
	Self-compatibility	d’	**3.077**	NA	1.922	8.672
	Cross-compatibility	d’’	0.092	**0.428**	0.121	-0.341
Pollinator visitation rates ~ Relative abundance		h	1.058	-8.15	-2.84	-3.509
	Total nectar reward	b	0.56	1.221	0.664	-1.327
Self-compatibility ~ Pollinator visitation rates		c’	-0.004	NA	-0.004	< 0.001
Cross-compatibility ~		c’’	-0.013	-0.038	-0.013	-0.024

Pollinator visitation rates					
Total nectar reward ~ Relative abundance	a	**0.214**	**1.808**	-0.156	-0.112
Relative abundance ~ Reproductive fitness	j	**2.461**	**6.800**	-2.871	**10.942**
Correlation					
Relative abundance ~~ Self-compatibility	i’	**-42.15**	NA	-5.488	-0.303
Relative abundance ~~ Cross-compatibility	i’’	214.939	-18.372	35.678	62.978
Net Total Effect		**2.302**	**4.814**	-3.219	**12.045**

Discussion

Floral color polymorphism is well-documented in various plant species, although the empirical assessment of the morphological, physiological, and ecological variations among floral color morphs in relation to their population densities remains underexplored (Elam & Linhart, 1988; Lau & Galloway, 2004; Caruso *et al.*, 2010; Berardi *et al.*, 2016; Koski *et al.*, 2020; Jacquemyn & Brys, 2020). In the tropics, species from the order Zingiberales (Costaceae, Heliconiaceae, Cannaceae and Marantaceae; Maas-Van De Kamer et al. 2016; Temeles *et al.*, 2000; Gowda & Kress, 2013) and the family Zingiberaceae (*Hedychium, Globba,* and *Curcuma*; Leong-Skornicková *et al.*, 2007; Saryan *et al.*, 2020) exhibit intra-specific floral and bract color polymorphisms along with pollinator-mediated selection of floral traits. This has prompted hypotheses regarding ‘one pollinator-one color morph’ specialization in several Zingiberales species (Temeles et al., 2013; Meléndez-Ackerman et al. 2005), though empirical validation has been limited to a few species, such as the Caribbean *H. caribaea* (Temeles et al. 2013) and the trans-Himalayan *Roscoea purpurea* and *R. tumjensis* (Paudel et al. 2018). This study reports findings on plant-pollinator interactions in the nocturnal color polymorphic *C. caulina*, endemic to the Western Ghats.

Based on our comprehensive characterization of the polymorphic *C. caulina* from Kaas plateau, we identified morph abundance to be tri-modal, with two common morphs (GR and GW) and one rare morph (RW) in the study population. Flowers of *C. caulina* displayed

classic moth-pollination syndromes (Johnson *et al.*, 2017), such as nocturnal opening, white color, long floral tube with copious nectar, and flowers exposed from the inflorescence. True to its syndrome, we discovered that *C. caulina* is exclusively pollinated by nocturnal hawkmoths. However, in this nocturnal ginger-moth interaction, the concept of color polymorphism is atypical. We found no morph-specific variations in floral morphologies, such as floral tube length, floral size, or floral or inflorescence color and size that are typically associated with pollinator-mediated selection in plants with floral color polymorphism (Meléndez Ackerman and Campbell 1998; Gomez 2000; Majetic et al. 2007; Milano et al. 2016). Instead, we found that the three morphs of *C. caulina* only differed in their nectar traits, reproductive compatibility, and fitness (question 1), and pollinator visitation was not shaped by morph abundance (question 2). We recorded an elaborate pollinator-mediated frequency-dependent selection (FDS) only in one trait, nectar rewards. We propose that the lack of variation in floral shape and size may be because these specific floral traits have already undergone a stabilizing selection (Waser and Price 1981) by the nocturnal hawk-moth pollinators, while nectar traits may still be under selection by pollinators (Carlson and Holsinger 2013) or some abiotic factors (Plos et al. 2023; Malovrh et al. 2024). Our failure to detect the selection of any morphs based on the color of their lateral bracts suggests that while color acts as a morphological identifier, other physiological or ecological traits linked to bract color may experience a direct pollinator-mediated or frequency-dependent selection (question 3).

While color may not be under direct pollinator-mediated selection, in the path analyses, we identified a few ecological and floral traits that showed a direct or indirect effect on the differential reproductive fitness of the morphs. The first trait we identified is morph abundance, which showed a strong direct effect on the morph's reproductive fitness (path e) in both species-specific and moth-species models (Fig. 4). We noted that while visitation rates were high for RW in our experimental setups, the simulated pollinator visitation estimates for all RW in the population showed lower visitation rates simply because RW is rare in the population (Fig. 1c Y axis vs Z axis). In contrast, we recorded fewer visits to the common GR morph in the experimental setups, but we noted higher visitation estimates in the simulation, since GR is common in the population. The contrasting pattern between the visitation rate recorded per morph (Fig. 1c, Y axis) in our experimental set ups and simulation results (Z axis) for the same morph shows that morph frequency does influence the total visits received by each morph in this population.

Physiological compatibility is the second trait affecting reproductive fitness (path d) with cross-compatibility having a significant effect only in the GR morph-specific model (Fig. 4b) and self-compatibility showing a significant effect in the overall species-specific model (Fig. 4a). The identification of self-incompatibility and enhanced reproductive fitness exclusively in the common GR morph, alongside the absence of high reproductive fitness in the self-compatible rare RW morph—despite higher pollinator visitation rates—further indicates that the contrasting morph-specific compatibility plays an important role in the differential reproductive assurance in the common GR and the rare RW morphs. Our discovery of self-compatibility in the rare morph also aligns with studies demonstrating that self-compatibility in floral color morphs is advantageous when the morph is rare in the population (Holsinger, 2000) and when the morph is under pollen or pollinator limitation (Subramaniam and Rusher 2000; Arista et al. 2013; Ortiz et al. 2015; Fry and Rausher 2017).

Physiological compatibility in *C. caulina* was noted to be labile, especially evident in the hand-pollination treatments. For instance, intra-morph crossing success was morph-specific, and inter-morph crossing success was donor-specific, meaning crossing success depended on which morph was used as a pollen donor or pollen recipient (Fig. 2a). Although such morph-specific compatibility differences are known only from a few studies, this pattern has been mostly reported from annual plants (Wolfe and Seller 1997; Jorgenson et al. 2006; Kosi and Galloway 2020) and rarely from perennials (Stanton et al. 1989). However, it is premature to assume that the origin and maintenance of this phenomenon are comparable between these contrasting life forms. While morph-specific compatibility can contribute to the maintenance of polymorphism under both negative or positive frequency-dependent selection in annuals, in perennial species like *C. caulina*, this is hard to determine (Jesson and Barrett 2002). This is because, in perennial species, it is possible that different morphs evolved under distinct evolutionary timeframes, resulting in an extant population composed of relic individuals from different selection regimes. Thus, we cannot entirely dismiss the fact that the different compatibility traits of the color morphs of *C. caulina* may have originated under different historical selection regimes and different ecological conditions.

Nectar reward is the third trait that is variable among morphs, has an indirect significant relationship with morph abundance, and affects reproductive fitness. Morph-specific variability in nectar traits within a population has been suggested to show a balancing selection in *I. purpurea* (Fry and Rausher, 1997; Subramaniam and Rausher, 2000). In *C. caulina*, morph-specific nectar variability may also result from balancing selection, although the mechanism is fairly complex and involves multiple traits. While high nectar energy in the

rare morph and low nectar energy in the common morph resembles negative FDS, the resulting reproductive fitness contradicts the expected outcomes. We observed lower reproductive fitness in the rare morph despite high pollinator visitation rates, whereas the common morph showed higher fruit and seed sets despite lower pollinator visitation rates. Therefore, although nectar quality predicts higher pollinator visitations, nectar alone did not determine reproductive fitness in *C. caulina*. However, it does ensure pollinator visitations, especially in the rare morph.

So, what contributes to the observed disparity in morph abundances in *C. caulina* at Kaas? We propose a multi-trait model involving an interplay between nectar reward, pollinator attraction, and compatibility type (self vs. cross), which influences the reproductive success of different morphs and subsequently affects their population densities. For example, the common GR morph ensures greater reproductive success despite its lower nectar energy and lower pollinator visitation rate, since its higher abundance allows for a quicker detection by pollinators. This is complemented by self-incompatibility, which enhances fruit and seed set, promoting higher abundance of the common morph (Fig. 4b, Table 1). Conversely, the rare RW morph, while less likely to be discovered by pollinators, benefits from its high-energy nectar that encourages twice as many pollinator visits compared to the common morph. However, video recordings of individual visits revealed that RW flowers received repeated visits from the same moth, which can increase self-pollination (Harder *et al.*, 2004; M. Eckhart *et al.*, 2006; Ishii & Harder, 2006). While this ensures reproductive success in the self-compatible rare morph (Fig. 4c, Table 1), it comes at the cost of an overall reduced reproductive fitness in naturally pollinated RW individuals (Fig. 2a and 3a). In contrast, we observed a higher frequency of inter-individual switches (from video recordings, SS and VG) in the common GR and GW morphs, likely facilitating outcrossing, as confirmed by higher seed set in the open pollinated GR and GW morphs despite lower pollinator visits.

Population shifts in morph ratios are known in annual plants that are under FDS. While we elucidate ecological mechanisms for the maintenance of rare morphs in *C. caulina* in the Kaas population, we did not record transitions in morph ratios or elimination of the rare morph in at least 15 years of our ecological studies at the Kaas plateau (unpublished data, collected by authors). This may be because change in morph frequencies will often go undetected in perennial rhizomatous species that also propagate vegetatively (as reported in heterostylous species: Eckert and Barrett, 1993; Eckert et al., 1999; Barrett, 2015 and in dioecious species: Irish and Nelson, 1989; Petzold et al., 2013). Therefore, in the light of vegetative propagation whose importance cannot be discounted in gingers (Záveská et al.

2012; Skopalíková et al., 2023), we suggest that ecological factors identified in this study can only explain morph maintenance via sexual reproduction, and vegetative reproduction can reduce the intensity of frequency-dependent selection (Barrett and Harder 1996; Jesson and Barrett 2002).

Color morphs have often been associated with abiotic-stress-related anthocyanin production (Warren & Mackenzie, 2001; Tang & Huang, 2010; Berardi *et al.*, 2013, 2016; Narbona *et al.*, 2018; Sapir *et al.*, 2021). A question rarely addressed in perennial plants that show color polymorphism is whether the different color morphs represent historical stress regimes in the population. We reiterate that the different color morphs and traits linked with them, that is, morph abundance, physiological compatibility, and nectar traits, may represent morphs selected under different abiotic stress regimes. Therefore, while we elucidate three ecological mechanisms that maintain variable morph ratios in *C. caulina*, we do not discount the role of balancing selection via abiotic factors also shaping polymorphism in *C. caulina*. We aim to test this hypothesis under a population genetic framework since homologous mutations among morphs will be key in establishing the genetic identities of the morphs within and across populations.

In summary, *C. caulina* from the Kaas population demonstrates trait variations and differential selection among its color morphs, which also results in their differential reproductive fitness, which explains their highly skewed morph distribution. The role of environmental factors in the maintenance of FCPs has been discussed in a few perennial and rhizomatous plants (Twyford *et al.*, 2018; Vaidya *et al.*, 2018; Streinzer *et al.*, 2019; Tang *et al.*, 2016) and they suggest that FCPs are common in regions with higher climatic fluctuations and high diversity of herbaceous species. Interestingly, these are also habitat features of the sky-islands of the Western Ghats, where the Kaas plateau is located. Our results from *C. caulina* reveal physiological and ecological factors that differentiate morphs within a polymorphic perennial species and the interplay between them. Although some of these factors can be examined through an ecological lens, perennial plants carry the burden of the past, necessitating the consideration of both abiotic and biotic histories to fully comprehend polymorphism in plants.

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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)

Fig. S1 Plant habit, habitat, and key vegetative features of *Curcuma caulina*.

Fig. S2 Variation in bract color, floral reflectance, and morph frequency of *C. caulina*.

Fig. S3 Hawkmoth pollinators of *C. caulina*.

Fig. S4 Circular histogram and circular statistics values of pollinator visitation rates.

Table S1 List of floral and vegetative morphological characters used for the nMDS analysis.

Table S2 Within-group comparisons for nectar energy, volume, and concentration.

Table S3 Within-group comparisons for peak pollinator visitation rates.

Table S4 Results of the chi-square test for fruiting success rates of hand-pollination.

Table S5 Mean and standard error values for seed count for cross- and self-compatibility treatments.

Table S6 Between groups comparisons for seed counts for hand pollination treatments.