plant biology

Plant Biology ISSN 1435-8603

RESEARCH ARTICLE

The consequences of flower colour polymorphism on the reproductive success of a neotropical deceptive orchid

B. L. Arida[1], F. Pinheiro[1], L. Laccetti[2], M. G. G. Camargo[3], A. V. L. Freitas[4] & G. Scopece[2]

1 Departamento de Biologia Vegetal, Instituto de Biologia, Universidade Estadual de Campinas, Campinas, Brazil
2 Department of Biology, University of Naples Federico II, Complesso Universitario MSA, Naples, Italy
3 Center for Research on Biodiversity Dynamics and Climate Change and Department of Biodiversity, Phenology Lab, São Paulo State University, Biosciences Institute, Rio Claro, Brazil
4 Departamento de Biologia Animal, Instituto de Biologia, Universidade Estadual de Campinas, Campinas, Brazil

Keywords
Epidendrum; floral traits; fluctuating selection; food deception; phenotypic polymorphism; pollinator-mediated selection.

Correspondence
F. Pinheiro, Departamento de Biologia Vegetal, Instituto de Biologia, Universidade Estadual de Campinas, Campinas, SP 13083-862, Brazil.
E-mail: biopin@unicamp.br

Editor
Z.-.X. Ren

Received: 29 October 2024;
Accepted: 24 February 2025

doi:10.1111/plb.70020

ABSTRACT

- Deceptive plants often exhibit elevated levels of polymorphism. The basis of the association between flower polymorphism and deceptive strategies, however, remains unclear. *Epidendrum fulgens*, a Neotropical deceptive orchid pollinated by butterflies, has an unexplored intrapopulation flower colour polymorphism. Here, we investigate the consequences of this polymorphism on its reproductive success.
- We performed field and common garden experiments, aiming to detect pollinator-mediated selection strength and direction over time, and test whether the presence of multiple colour morphs increases species' reproductive success. In the field, we monitored plant reproductive success and floral morphology on two populations over two flowering seasons and performed selection gradient analyses. In the common garden, we assembled plots of cultivated plants with same and different flower colour individuals (i.e., mono- and polymorphic plots), exposed them to pollinators and monitored their reproductive success. In both sites we also monitored the local pollinator community.
- In the field, colour morphs performed equally, but we found coherences between morphological differentiation and the direction of selection, which was very dynamic. In the common garden, mono- and polymorphic plots also performed equally, with highly variable reproductive success over time. We also found a highly diverse pollinator community.
- Our results suggest that flower polymorphism in *E. fulgens* is maintained by a combination of factors, including varying pollinator-mediated selection, assortative mating due to differential pollinator preferences and different phenotype heritability. Natural selection varied across time and space, indicating a dynamic interplay between pollinators and flower morphs.

INTRODUCTION

Intraspecific variation in phenotypic traits is widespread in nature. Darwin emphasised the general presence of this intraspecific variation, considering it as the raw material upon which natural selection might act (Darwin 1859). In general, phenotypic variation is fuelled by genetic recombination and mutation and is eroded by natural selection that eliminates all but the most favourable genetic combinations (Stebbins 1950). Therefore, intraspecific variation found in natural populations should result from an equilibrium between the genetic processes generating variation and the selecting forces leading to trait homogeneity (Ellegren & Galtier 2016). An extreme case of phenotypic variation occurs when individuals of a species fall into two or more categories, a condition referred to as phenotypic polymorphism (Westerband *et al.* 2021).

Flowers, i.e. the sexual organs of Angiosperms, are an ideal model to investigate the origin and the maintenance of phenotypic polymorphisms, as they are particularly variable within populations (e.g. Darwin's book *The different forms of flowers on plants of the same species*, Darwin 1877). Among different floral traits, colour is one of the most polymorphic (Sapir *et al.* 2021). This trait is typically used by pollinators as a signal to identify nectar or pollen sources (van der Kooi *et al.* 2021). In most Angiosperms, flowers within populations have weakly variable colours (i.e. monomorphic species), because plant individuals that pollinators are able to recognise as conspecific are more easily visited (e.g. *flower constancy*; Waser 1986). In these plant–pollinator relationships, plants are continuously exposed to a choice of pollinators, which imposes directional selection on flower traits (Schiestl & Johnson 2013), hence eroding intraspecific variation. This flower constancy is expected to be advantageous for both involved parties: as it increases plant reproductive success by more efficient pollen transfer between conspecific individuals, and it increases pollinator foraging efficiency as searching and learning to handle new floral types is costly (J.B. 1963; Chittka *et al.* 1999; Goulson 2000). This is the most common mechanism by which

pollinator-mediated natural selection on floral traits maintains intraspecific cohesion and reduces floral variation (Waser & Price 1981).

Despite this general situation, however, in many plant species flowers exhibit strong discrete or continuous colour variation (i.e. polymorphic species). Discrete polymorphism in flower colour is likely to result from genetic variation at a single or a few loci (Wu *et al.* 2013), whilst continuous variation is likely to result from a multigenic architecture with additive effects or from differential expression of genes involved in pigment biosynthesis (Davies *et al.* 2012; Scopece *et al.* 2020). Despite an intense research effort, the mechanisms that create and maintain flower colour polymorphisms within plant species are still not completely understood and are highly debated (Sapir *et al.* 2021). Competing hypotheses are that colour variation can be maintained due to balancing selection exerted by multiple selection regimes, fluctuating selection over time and space, heterozygote advantage and frequency-dependent selection (Kellenberger *et al.* 2019; Sapir *et al.* 2021 and references therein). A lack of selection may also maintain variation, although this currently remains almost untested (but see Jacquemyn & Brys 2020).

A peculiar situation is a category of plant species that offer no reward to their pollinators, known as deceptive plant species (Sprengel 1793). This pollination strategy evolved in several plant families but is particularly frequent in Orchidaceae, where an unusually high proportion of species employ deceptive pollination strategies (Shrestha *et al.* 2020). Unlike rewarding species, in deceptive species floral constancy is not expected because, after a few unrewarded visits, pollinators tend to avoid the rewardless phenotype (pollinator avoidance learning; Heinrich 1975; Dukas & Real 1993; Smithson & Macnair 1997; Raine & Chittka 2007). Thus, in these pollination strategies, pollinator-mediated natural selection is not expected to eliminate floral variation, potentially leading to high intraspecific flower polymorphisms. Accordingly, higher rates of intraspecific phenotypic variability in deceptive orchid species compared to rewarding ones have been reported (Heinrich 1975; Salzmann *et al.* 2007; Ackerman *et al.* 2011). The traditional hypothesis to explain this elevated phenotypic polymorphism in deceptive species is that it would slow down pollinator avoidance learning ability thus increasing plant reproductive success (Heinrich 1975; Nilsson 1992; Smithson & Macnair 1997; Ferdy *et al.* 1998). This hypothesis was recently tested in a manipulative experiment by Aguiar *et al.* (2020), who corroborated it with a bee pollinated species, showing how the colour variation of a deceptive orchid influences the pollinator's cognitive aspect of deception. However, in a meta-analysis of studies conducted to test this hypothesis, Juillet & Scopece (2010) found no relationship between flower polymorphism and increased reproductive success. Alternative explanations raised to justify this elevated polymorphism in deceptive orchids emphasise a negative frequency-dependent selection (Gigord *et al.* 2001), a weak selection on floral traits (Jacquemyn & Brys 2020), or a directional but fluctuating selection on floral traits in different flowering seasons (Scopece *et al.* 2017). The origin of flower colour polymorphism in deceptive species has also been associated with inaccurate colour discrimination by pollinators (Kagawa & Takimoto 2016). In fact, studies have indicated varied selection patterns as responsible for the maintenance of floral polymorphisms, such as the evident pattern found by Gigord *et al.* (2001) in *Dactylorhiza sambucina* (L.) Soò, an example of a deceptive orchid with colour morphs maintained by pollinator-mediated selection.

The lack of concordance in studies attempting to explain the reasons that form the basis of the elevated phenotypic polymorphism in deceptive orchids calls for additional case studies, particularly in under-investigated regions and groups. Here, using the colour polymorphic Neotropical orchid *Epidendrum fulgens* Brongn., we investigated the intensity and direction of pollinator-mediated natural selection over two consecutive flowering seasons in natural populations, and the relationship between flower colour polymorphism and reproductive success components in common garden conditions and in natural populations. Using these data, we specifically asked the following questions:

1 Is flower colour variation discrete in *E. fulgens*?
2 If yes, do different flower colour morphs experience different reproductive success during their flowering season in natural populations?
3 Are different colour morphs exposed to different pollinator-mediated selection?
4 If yes, does this reflect among-morph morphological differentiation?
5 Does the presence of different colour morphs affect reproductive success?

MATERIAL AND METHODS

Study species, experimental design and characterisation of colour polymorphism

Epidendrum fulgens is a Neotropical orchid species in the subtribe Laeliinae. This species is found on open sand dune vegetation, growing directly on sandy soils along the south and southeastern Brazilian coast (de Mattos *et al.* 2023). It has a long flowering season during the time of the year with the highest rainfall, with peak flowering between October and April. This species does not have floral nectaries and there are no records of floral fragrance, so that there is no correlation between floral colour and fragrance, as observed in other deceptive orchids, such as by Aguiar *et al.* (2021). It employs a generalised food-deceptive strategy to attract pollinators, which are exclusively butterflies (Moreira *et al.* 2008; Fuhro *et al.* 2010; Pinheiro *et al.* 2010) that are primarily visually oriented and able to discriminate yellow-to-red colours (Swihart & Swihart 1970; Arikawa 2003; Blackiston *et al.* 2011). The pollinators of *E. fulgens* are also essential for pollen transfer (Pinheiro *et al.* 2015) and fruit development, with no evidence of spontaneous autogamy (Fuhro *et al.* 2010). *E. fulgens* has evident intrapopulation variation in flower colour, with petals and sepals ranging from yellow to red, with orange intermediates (Fig. 1).

With the aims of understanding the mechanisms that maintain intrapopulation polymorphism and their consequences on reproductive success (questions *ii*, *iii* and *iv*), we performed field observations in natural populations of *E. fulgens* (hereafter, natural populations survey). Then, in order to understand whether the presence of different flower colour morphs has an effect on reproductive success (question *v*), we set up an experiment manipulating the presence of different colour morphs in a common garden setup (hereafter, plot experiment). In both

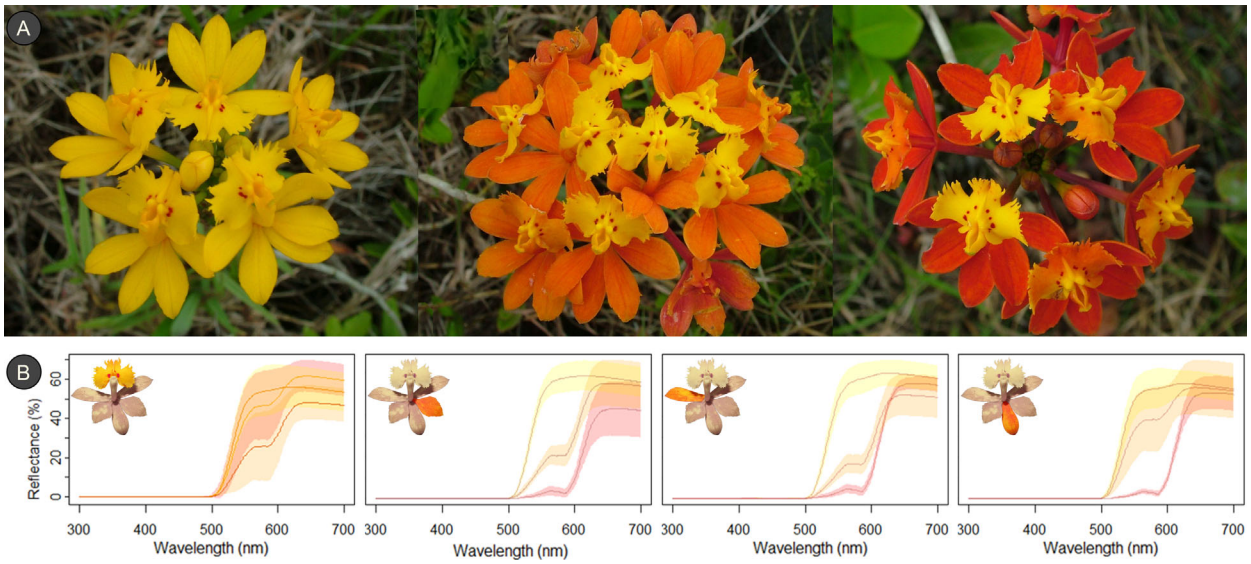

Fig. 1. Inflorescences of *Epidendrum fulgens* of the three colour morphs: yellow, orange and red (A). Reflectance average (line) and standard deviation (line shade) spectra of each measured floral part (from left to right: labellum, petal, lateral sepal and dorsal sepal) of the three morphs (represented by their respective colour) (B).

activities, reproductive success was then estimated. Following Scopece *et al.* (2015), pollinia removal was used as a proxy for male reproductive success (hereafter MRS) and proportion of fruit formation was used as a proxy for female reproductive success (hereafter FRS). Here, we focus on the visual aspect of *E. fulgens* and choose not to add an analysis regarding the importance of its floral scent in attracting pollinators. Although there is evidence that floral scent serves as a signal for attraction of butterflies in some cases, as pointed out by Andersson *et al.* (2002) and Chen *et al.* (2021), there is no evidence of floral scent in *E. fulgens* and the main known pollinators of the species are mainly visually oriented.

To characterise flower colour polymorphism, we collected reflectance data of the labellum, petal, lateral sepal and dorsal sepal of 21 flowers from different individuals, seven of each extreme colour (yellow and red) and seven intermediates (orange), with an Ocean Optics USB4000 Fibre Optic Spectrometer (Jaz Modular Optical Sensing Suite), allowing detection of visible and ultraviolet wavelengths and covering the sensitivity of the photoreceptors of different species of butterfly (van der Kooi *et al.* 2021). Using these data, we validated the three colour morph categories of *E. fulgens*: yellow, orange and red (hereafter, Y, O and R) (e.g. Dalrymple *et al.* 2015) and its polymorphism as discrete. The results supporting this conclusion are detailed in the Results, subsection Flower phenotype polymorphism.

Natural populations survey

Field activities were carried out in two natural populations with similar climate (de Mattos *et al.* 2023) and soil (de Lima *et al.* 2024) conditions (Bertioga and Ilha do Cardoso, both in the coastal region of São Paulo State, Brazil, hereafter BE and CA, respectively). To estimate frequency of the three flower colour morphs, we used the transect method proposed by Cottam & Curtis (1956) and took note of the number of individuals of each morph in an area of 400 m^2. This estimation was carried six times along the flowering phenology of the investigated species (four times in BE and twice in CA). In both populations, we labelled 15 individuals from each colour morph. Between December 2021 and April 2023, a total of 14 field expeditions in the natural populations were carried out (ten to BE and four to CA). On the different expeditions, we followed the same labelled individuals. Over time, however, if some of the labelled individuals were lost, we added new individuals to retain the original sample size. By doing so, a total of 70 individuals were monitored in BE and 68 in CA. For each labelled individual, we recorded eight morphological traits potentially involved in pollinator attraction: petal length (PetL), petal width (PetW), labellum area (LabA), labellum shape (LabS), lateral sepal length (LatSepL), lateral sepal width (LatSepW), dorsal sepal length (DorSepL), dorsal sepal width (DorSepW). For these measurements, we collected one flower from each labelled individual in each flowering season, dissected floral part and distended them between transparent sheets to avoid 3D deformation. We then took a digital scan of each flower using a tabletop scanner (HP Scanjet 4670), at a resolution of 1200 × 1200 dpi with a reference scale on the back. Calibrated images were thus used to extract flower trait measurements using ImageJ 1.33 (https://imagej.nih.gov/ij/). LabS was obtained instead by performing a landmark-based geometric morphometric analysis. For this, we selected a set of 23 landmarks and semi-landmarks (e.g. Bookstein 1997; Tyteca 2000) using TpsUtil and TpsDig 2 (Rohlf 2015). The coordinates of the landmarks and semi-landmarks of the labellum were superimposed using a Generalised Procrustes Analysis (GPA) (Rohlf & Slice 1990). Then, we performed a Principal Components Analysis (PCA) and PC values were mapped back onto labellum morphology using *eigenvectors* (i.e. a set of vectors associated with the set of landmarks and semi-landmarks and their variation in direction and strength). Finally, on each field expedition, data on MRS and FRS were recorded on each labelled individual.

To obtain an overview of Lepidoptera characterising the *E. fulgens* flower visitors and to assess possible changes in its

composition and abundance over time, we performed direct observations (for a total of 80 h) using the approach described in Fuhro *et al.* (2010). Observations were conducted during daytime (from 06:00 to 16:00 h) since *E. fulgens* is pollinated only in daylight (Fuhro *et al.* 2010). All butterflies found were recorded and classified into one of the three following categories: (a) pollinators (butterflies that were seen removing pollinia of *E. fulgens* or with pollinia attached to the proboscis), (b) potential pollinators (not seen removing pollinia of *E. fulgens* but with the potential of being pollinators according to their characteristics of behaviour, size, vision system and feeding habits), and (c) not pollinators (not seen removing pollinia of *E. fulgens* and no potential of being pollinators due to incompatible characteristics). Some of the butterflies interacted with individuals of *E. fulgens* but did not remove pollinia (e.g. oviposited), in which case they were not classified as flower visitors, but we indicate the presence of interaction in Fig. S5.

Plot experiment

The plot experiment was conducted using 60 *E. fulgens* individuals cultivated in common garden conditions at the experimental area of the Department of Plant Biology, Campus of the Universidade Estadual de Campinas (Campinas – SP, Brazil). To test whether the presence of different colour morphs affects reproductive success, we assembled different plots of cultivated individuals and exposed them to local wild pollinators. Each plot contained ca. 20 inflorescences. Mono- and polymorphic plots were placed far from each other (>20 m) in an open area, where naturally occurring butterflies are present, including those species already described as pollinators of *E. fulgens*. We repeated the experiment 12 times, randomising individuals and plot positions. Each round of exposure lasted 5 days, with a 9-day break between them. After each round, MRS and FRS were estimated, as explained above.

In these plots, we estimated percentage MRS (related to pollinia removal; PR) and FRS (related to fruit formation; FF) over time. We assembled three monomorphic plots (i.e. plots with only Y, only O and only R colour morphs; hereafter, MY, MO and MR) and one polymorphic plot (with Y, O and R morphs in the same proportion; hereafter, *P*, and morphs inside them PY, PO and PR). Percentage MRS and FRS were calculated for each of the four plots ($MRS_{MY/MO/MR/P}$ and $FRS_{MY/MO/MR/P}$) and for each of the three colour morphs within the polymorphic plot ($MRS_{PY/PO/PR}$ and $FRS_{PY/PO/PR}$) using the following formulae (here referring to the Y morph, but similarly applied for all three morphs):

$$MRS_{MY} = PR_{MY}/(PR_{MY} + PR_{MO} + PR_{MR} + PR_{P}) \times 100;$$

$$FRS_{MY} = FF_{MY}/(FF_{MY} + FF_{MO} + FF_{MR} + FF_{P}) \times 100;$$

$$MRS_{PY} = PR_{PY}/(PR_{PY} + PR_{PO} + PR_{PR}) \times 100;$$

$$FRS_{PY} = FF_{PY}/(FF_{PY} + FF_{PO} + FF_{PR}) \times 100$$

To understand which species of Lepidoptera characterise *E. fulgens* pollinator community at the experimental suburban area, we performed direct observations (for a total of 10 h) using the approach used for the natural populations survey.

Pollen limitation

To test for pollen limitation, in the BE population we performed manual crosses in the field, saturating 142 flowers from 16 individuals with pollinia collected from other individuals of the same population. On these individuals, we thus estimated the number of flowers pollinated and the number of fruits produced. We then compared these data with data collected in all ten field expeditions on open-pollinated individuals from the same BE population. We calculated a pollen limitation index for fruit formation using the following equation (PL; Larson & Barrett 2000):

$$PL = 1 - (P_o/P_h)$$

where P_o is the proportion of fruits in open-pollinated plants, and P_h is the proportion of fruits in pollen-saturated plants. The index ranges from 0 (no pollen limitation) to 1 (high pollen limitation).

Statistical analysis

To explore the level of phenotypic variability in *E. fulgens*, we calculated a coefficient of variation (CV) as the ratio between standard deviation and mean for each of the eight investigated floral traits in the two flowering seasons then averaged them to obtain a single value representative for the species.

Relative frequency and phenotypic differences across the three flower colour morphs were tested in the two populations using Dunn Kruskal-Wallis multiple comparisons (with Bonferroni correction) for pairwise comparisons. The same approach was used to detect differences across the three colour morphs MRS and FRS, both in the natural populations survey and in the plot experiment. To detect a putative temporal variation in reproductive success, these parameters were also investigated separately in the two flowering seasons and in three different periods within one flowering season. The three periods were identified according to average precipitation data from 1990 to 2022 for Campinas, provided by the Center for Meteorological and Climatic Research Applied to Agriculture of the Universidade Estadual de Campinas (https://www.cpa.unicamp.br/), as: increasing rainfall (October to early December, Period 1), elevated rainfall (mid-December to early February, Period 2) and decreasing rainfall (mid-February to end of March, Period 3).

To infer the strength and direction of selection in both populations, in each colour morph we estimated selection gradients in the two flowering seasons using data collected on each labelled individual, as described in the previous section. In BE we also assessed differences in selection separately in each of the three periods within the flowering season. To do this, first we tested for the presence of intercorrelated floral traits using a Spearman's rank correlation test in order to remove highly correlated traits (>0.8). Then, following Lande & Arnold (1983), we performed multiple single linear regressions using MRS and FRS as response variables and standardised floral traits (z-

scores) as predictors. All the regression models were performed using the *lme4* R package (Bates *et al.* 2015).

RESULTS

Flower phenotype polymorphism

Reflectance data showed different colour polymorphism patterns on different floral parts: labellum showed continuous variation, petal and lateral sepal showed discrete polymorphism, and dorsal sepal showed overlap between orange and yellow (Fig. 1B). Morphometric data revealed a moderately low CV in *E. fulgens*, however CVs of different morphological traits showed some differences (PetL = 6.59%, PetW = 13.76%, LabA = 14.46%, LatSepL = 7.38%, LatSepW = 7.50%, DorSepL = 7.30%, DorSepW = 8.68%). LabS was mainly explained by variation in PC1 (23.48% variance explained) which represents the distance between the upper lobes of the labellum (see Fig. S2). The three colour morphs showed significant differences in PetW, PetL, LatSepL and DorSepL in the BE population (Figs. 2 and 3), while in CA we found significant differences in LabS (Fig. 2, Fig. S3).

Natural populations survey and pollen limitation

The frequency of the three flower colour morphs in natural populations differed between BE and CA. In the BE population, Y and R had the same frequency, and both were different from O, which was the most frequent (Dunn's $test_{YvsO} = 4.56$, $P < 0.001$; Dunn's $test_{RvsO} = 4.52$, $P < 0.001$), while in CA, the three colour morphs differed from each other, with R being less frequent, followed by *Y*, and *O* being most frequent (Dunn's $test_{RvsY} = -1.92$, $P = 0.02$; Dunn's $test_{RvsO} = 3.90$, $P < 0.001$; Dunn's $test_{YvsO} = 1.98$, $P = 0.03$) (see Fig. S1).

In both populations, the three morphs showed no significant differences in MRS and FRS in either of the two flowering seasons (Fig. 3). The same result was observed in the BE population, by dividing the flowering seasons in three different periods (see Fig. S4).

Overall, the results obtained in the selection gradients analysis suggest few marginally significant values, indicating a weak directional selection, with floral traits being under different selection regimes in the two populations and in the two seasons. In particular, during the first season, in BE, there was a significant negative selection on PetW for MRS in the *Y* morph, a significant negative selection on LabS for MRS in the O morph, and a significant positive selection on PetL for FRS in the R morph. In the same population, in the second season, there was a significant positive selection on PetW for FRS, both in the O and R morph (Table 1; Fig. 4A). Instead, in CA, in the first season, we detected a significant positive selection on LabS for FRS in the R morph. In the second season, there was a significant positive selection on PetW for MRS in the Y morph, a significant positive selection both for MRS and FRS on PetW in the O morph, and a significant positive selection on PetW for FRS in the R morph (Table 2; Fig. 4B). In BE, by dividing each season in different periods, different selective patterns were found in the different periods (Table 3).

In the BE and CA populations, a total of 35 species of Lepidoptera were identified (see Fig. S5). Thirteen of these species were observed pollinating *E. fulgens* or with pollinia attached to their proboscis. In total, we observed 16 events of pollination (average 0.2 pollination events per hour of observation). Of the 35 observed species, eight were only seen during the first flowering season, 23 in the second season and four in both seasons. In the BE population, pollen limitation was 0.82, with an average proportion of fruits in open-pollinated individuals of 0.16 and in pollen-saturated plants of 0.91.

Plot experiment

In the plot experiment, there were no significant differences for any comparisons of FRS, possibly because of a very low fruiting rate (average 0.013 fruits developed per flower in the overall

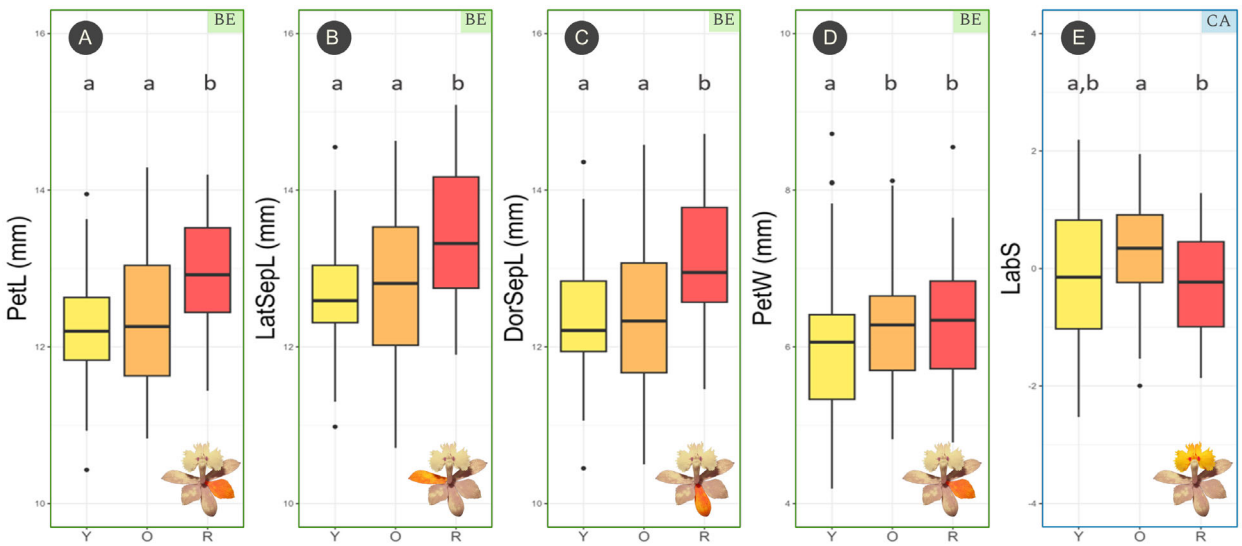

Fig. 2. Phenotypic traits showing significant differences among colour morphs of *Epidendrum fulgens* in Bertioga (A to D, green charts) and Cardoso (E, blue chart) populations: petal length (A), lateral sepal length (B), dorsal sepal length (C), petal width (D) and labellum shape (E). Different letters indicate significant differences ($P < 0.05$).

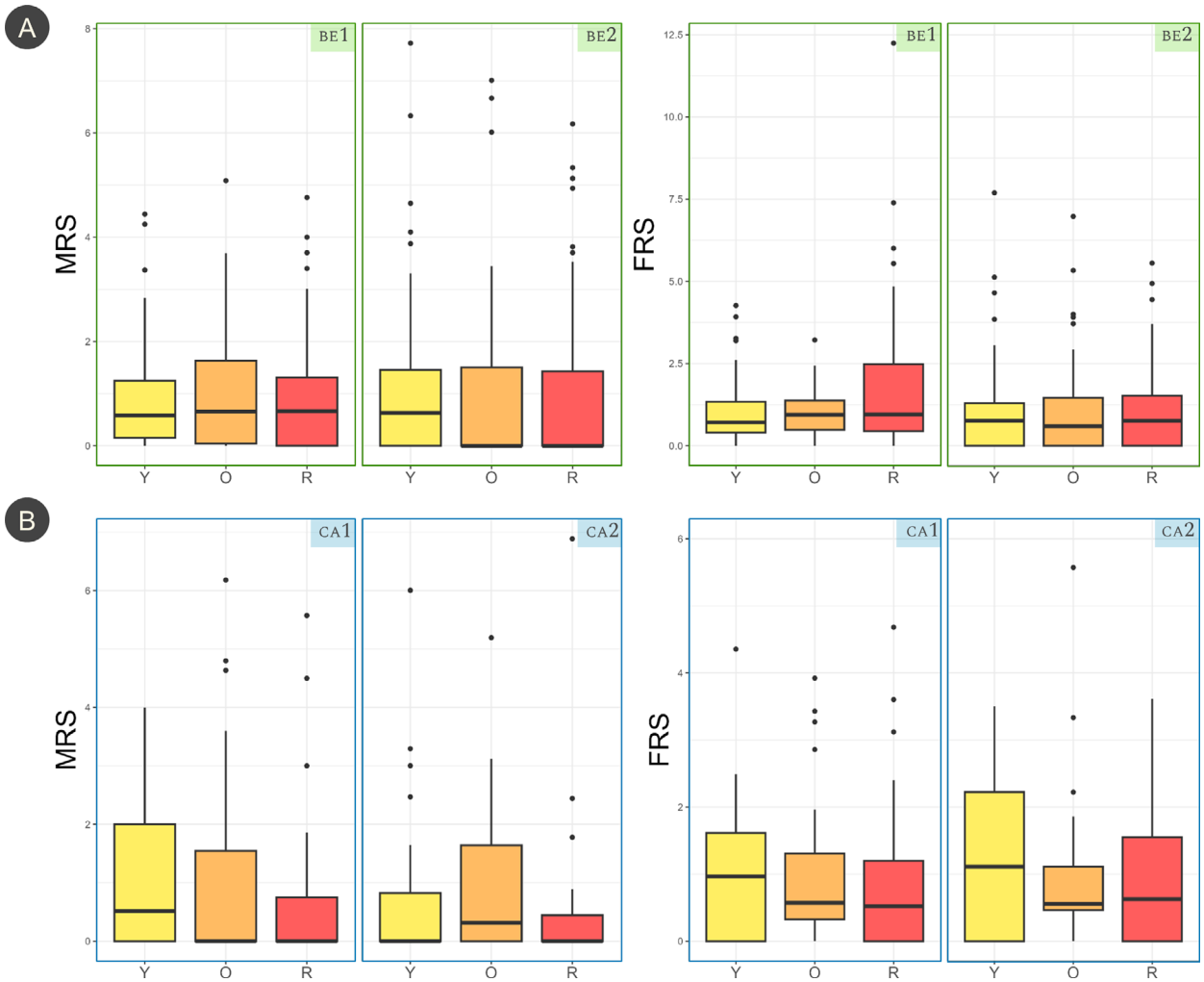

Fig. 3. Male reproductive success (MRS) and female reproductive success (FRS) of the three colour morphs (Y, O, R) in the two flowering seasons (1 and 2) in the two natural populations: Bertioga (A, green charts) and Ilha do Cardoso (B, blue charts). No significant differences ($P < 0.05$) were detected.

Table 1. Selection gradients in the Bertioga population across the two flowering seasons.

	yellow		orange		red	
	1st flowering season	2nd flowering season	1st flowering season	2nd flowering season	1st flowering season	2nd flowering season
MRS						
Petal length (mm)	−0.019	0.006	0.014	−0.016	0.008	0.049
Petal width (mm)	−0.149*	0.036	0.153	0.108	−0.094	0.181
PC1	−0.023	0.012	−0.185*	0.002	−0.066	0.034
Labellum area (mm^2)	−0.005	−0.005	−0.010	−0.005	0.006	−0.009
FRS						
Petal length (mm)	0.099	0.064	0.064	0.023	0.226*	0.015
Petal width (mm)	−0.103	0.175	0.175	0.348***	−0.051	0.270**
PC1	0.024	0.127	0.127	0.091	0.064	0.070
Labellum area (mm^2)	0.003	−0.006	−0.006	−0.010	0.003	−0.006

***$P \leq 0.001$, **$0.001 < P \leq 0.01$, *$0.01 < P \leq 0.05$.

plot experiment), which is common in food-deceptive species. Over the whole experiment, the average MRS of the monomorphic plots (M) was also not significantly different from that of the polymorphic plot (P) (Fig. 5A). However, when separately considering the monomorphic plots (MY, MO, MR) and morphs within the polymorphic plots (PY, PO, PR), plants in

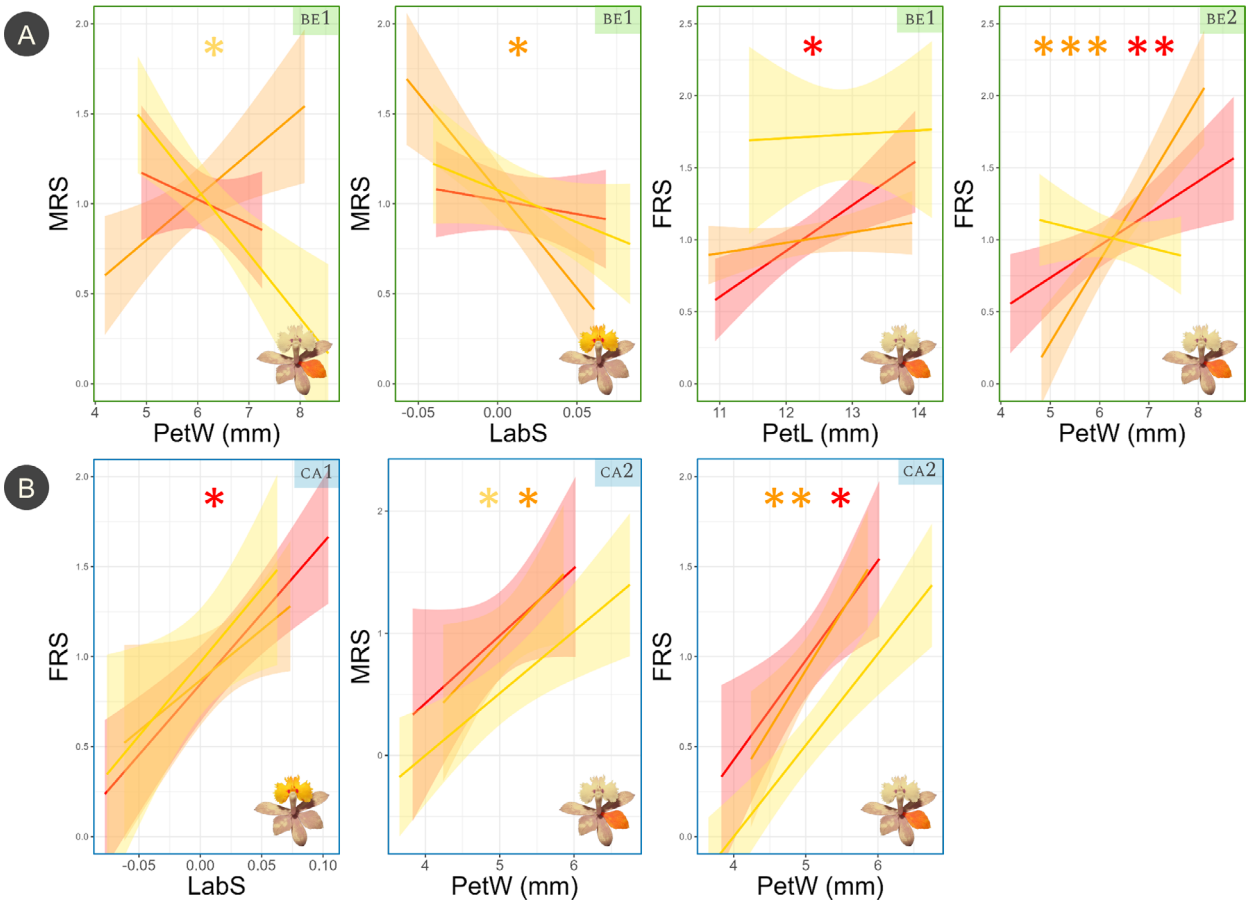

Fig. 4. Significant selection gradients, with mean of predicted values (line) and 95% confidence interval (line shade), of the morphological traits on each colour morph (Y, O, R, represented by their respective colour): petal width (PetW), labellum shape (LabS) and petal length (PetL) in both populations, Bertioga (A, green charts) and Ilha do Cardoso (B, blue charts) in the two flowering seasons (1 and 2). Asterisks and their respective colours indicate a significant difference in the selection gradient of the corresponding flower colour morph. ***$P \leq 0.001$, **$0.001 < P \leq 0.01$, *$0.01 < P \leq 0.05$.

Table 2. Selection gradients in the Cardoso population across the two flowering seasons.

	yellow		orange		red	
	1st flowering season	2nd flowering season	1st flowering season	2nd flowering season	1st flowering season	2nd flowering season
MRS						
Petal length (mm)	−0.132	−0.092	−0.084	−0.104	−0.239	−0.151
Petal width (mm)	0.435	0.680*	0.429	0.437*	0.517	0.542
PC1	−0.005	−0.021	−0.091	−0.035	0.117	−0.005
Labellum area (mm^2)	−0.021	−0.018	−0.020	−0.003	−0.007	−0.004
FRS						
Petal length (mm)	−0.220	−0.128	−0.178	−0.084	−0.209	−0.097
Petal width (mm)	0.234	0.379	0.243	0.912**	0.109	0.421*
PC1	0.155	0.033	0.130	−0.023	0.253*	−0.006
Labellum area (mm^2)	−0.005	−0.005	−0.004	−0.017	0.009	−0.008

**$0.001 < P \leq 0.01$, *$0.01 < P \leq 0.05$.

the PY plot had a higher reproductive success than R morph plants in both treatments ($MRS_{PY} > MRS_{MR}$, MRS_{PR}) or O morph plants in the monomorphic plot ($MRS_{PY} > MRS_{MO}$) (Fig. 5B). By dividing the plot experiment in three different periods according to rainfall regime, we also found significant differences in MRS in the first two periods, while in Period 3

Table 3. Selection gradients in the Bertioga population across the three periods of each of the two flowering seasons.

1st flowering season				2nd flowering season			
	yellow				yellow		
	period 1	period 2	period 3		period 1	period 2	period 3
MRS				MRS			
Petal length (mm)	−0.284	−0.795	0.282	Petal length (mm)	−0.411	−0.306	0.187
Petal width (mm)	−0.110	−0.600	−0.455	Petal width (mm)	−0.120	−0.107	−0.312
PC1	−0.245	0.480	−0.358	PC1	−0.286	0.249	0.562
Labellum area (mm^2)	0.000	−0.031	0.009	Labellum area (mm^2)	−0.002	−0.019	0.006
FRS				FRS			
Petal length (mm)	0.179	−0.091	0.073	Petal length (mm)	−0.156	−0.173	0.031
Petal width (mm)	−0.320	−0.260	−0.074	Petal width (mm)	−0.252	0.220	−0.34
PC1	0.145	−0.192	0.145	PC1	0.136	−0.047	0.243
Labellum area (mm^2)	0.018	0.013	−0.008	Labellum area (mm^2)	0.004	−0.002	−0.006
	orange				orange		
	period 1	period 2	period 3		period 1	period 2	period 3
MRS				MRS			
Petal length (mm)	−0.530	−0.025	−0.351	Petal length (mm)	0.919	−0.341	−0.346
Petal width (mm)	−0.481	−0.221	0.065	Petal width (mm)	0.738*	0.942**	0.171
PC1	−0.502	−0.395	−0.356	PC1	0.374	−0.060	−0.315
Labellum area (mm^2)	0.021	0.043	0.012	Labellum area (mm^2)	−0.038	−0.066*	−0.079
FRS				FRS			
Petal length (mm)	0.039	0.345	−0.160	Petal length (mm)	−0.065	−0.364	0.059
Petal width (mm)	0.684	−0.042	0.243	Petal width (mm)	0.981**	0.920**	0.455*
PC1	0.217	−0.039	−0.345*	PC1	−0.362	−0.225	−0.052
Labellum area (mm^2)	−0.026	−0.011	−0.003	Labellum area (mm^2)	−0.012	−0.069*	−0.009
	red				red		
	period 1	period 2	period 3		period 1	period 2	period 3
MRS				MRS			
Petal length (mm)	0.318	−0.209	0.492	Petal length (mm)	0.672	−0.063	−0.261
Petal width (mm)	0.324	−0.508	0.442*	Petal width (mm)	0.556	−0.104	0.063
PC1	−0.171	−0.349	0.000	PC1	−0.081	0.025	0.290
Labellum area (mm^2)	0.002	−0.015	0.023	Labellum area (mm^2)	0.025	0.000	0.000
FRS				FRS			
Petal length (mm)	0.982**	0.306	−0.032	Petal length (mm)	0.845	0.089	0.011
Petal width (mm)	0.601	0.200	−0.223	Petal width (mm)	0.932**	0.041	0.145
PC1	0.23	−0.093	−0.370*	PC1	0.202	0.165	0.240
Labellum area (mm^2)	0.019	0.002	0.015	Labellum area (mm^2)	0.018	0.003	0.006

**$0.001 < P \leq 0.01$, *$0.01 < P \leq 0.05$.

there were no differences for any of the comparisons (Fig. 6). In Period 1, PY was higher than R and O in both treatments ($MRS_{PY} > MRS_{MO}$, MRS_{MR}, MRS_{PO}, MRS_{PR}), and in Period 2, PY was higher than R in both treatments ($MRS_{PY} > MRS_{MR}$, MRS_{PR}) and higher than the O morph in the monomorphic plot ($MRS_{PY} > MRS_{MO}$) even without considering periods.

We identified 12 species of Lepidoptera in the experimental suburban area, seven of which are also present in the natural population of BE (Fig. S5). Of these, four taxa were identified as pollinators of *E. fulgens* and, in total, we observed four events of pollination (average 0.4 pollination events per hour of observation). Despite habitat differences, the potentially pollinating species in natural populations and in the experimental area were very similar, or equivalent species in terms of habitat preference (open and sunny areas).

DISCUSSION

The origin of flower colour polymorphism is highly debated (Sapir *et al.* 2021), ranging from weak selection or neutral processes (Wang *et al.* 2016; Jacquemyn & Brys 2020) to active, divergent selection (Eckhart *et al.* 2006; Newman *et al.* 2012; Bergamo *et al.* 2016). Whatever the mechanism of origin, the presence of polymorphism can have important consequences on pollinator behaviour, thus influencing plant reproductive success and selection patterns. Here, by surveying natural populations of the colour polymorphic deceptive orchid species *E. fulgens*, and by conducting a manipulative experiment, we demonstrated that pollinator preferences for different morphs changes in space, in flowering seasons and in rainfall periods within a season. We also demonstrated that selection

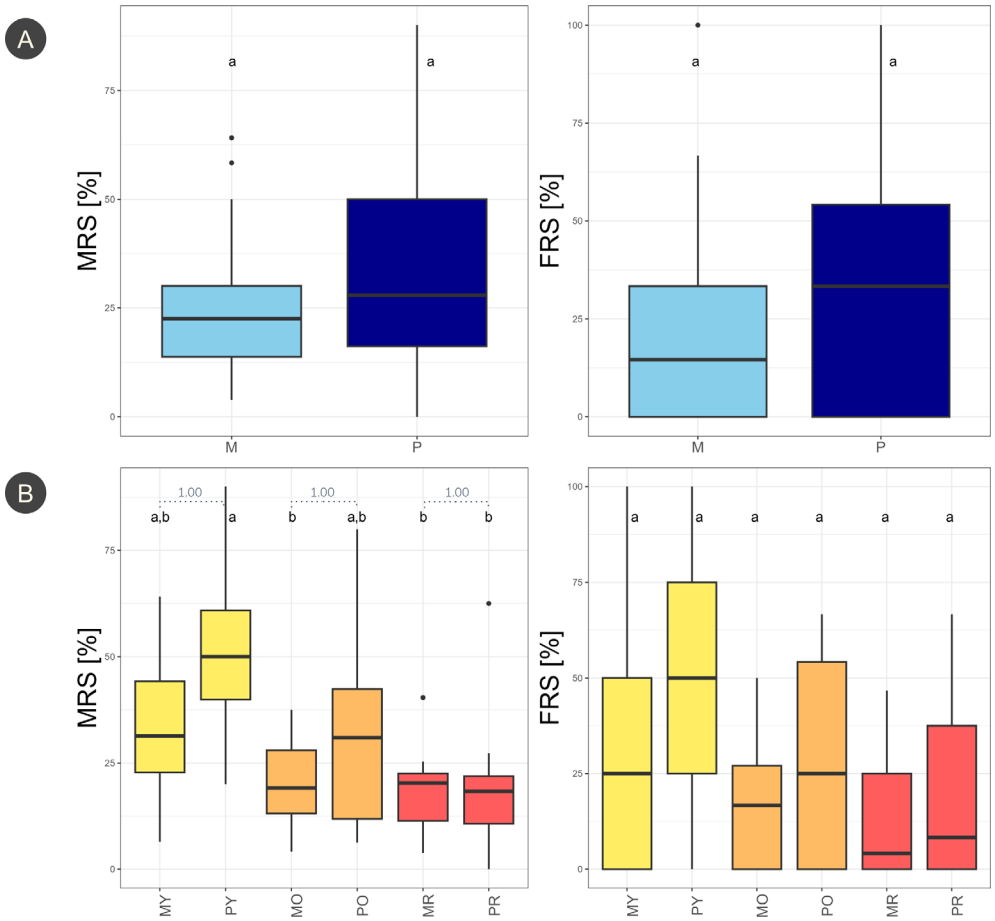

Fig. 5. Male reproductive success (MRS) and female reproductive success (FRS) of monomorphic and polymorphic plots (A); MRS and FRS of each monomorphic plot (MY, MO and MR) and each morph within the polymorphic plot (PY, PO and PR) (B). Different letters indicate significant differences ($P < 0.05$).

on floral traits involved in pollinator attraction is weak but followed different trajectories in different colour morphs and that pollinators prefer one colour morph over the others. Taken together, these results suggest that pollinators are able to detect different flower colour morphs, that different compositions of the pollinator community change pollination patterns, and that this ability has consequences for plant reproductive performance and on the evolution of floral polymorphic traits.

The presence of a polymorphism in flower colour in *E. fulgens* has been known since the first botanical explorations in the Neotropical region (Reichenbach 1878). Whether it was continuous or discrete variation was, however, not tested. Here, to resolve this issue, we analysed the reflectance spectrum of different flower parts of the extreme colour morphs (R and Y) and of the intermediate O morph. Our results show a clear discrete polymorphism in petals and lateral sepals, but not in the labellum (Fig. 1). Overall, a discrete polymorphism in a colour trait is often considered to result from genetic variation at a single or a few loci involved in pigment biosynthesis (e.g. Wu *et al.* 2013). Although determination of the genetic architecture of this phenotypic trait is beyond the scopes of our study, different phenotypic patterns in different floral parts might point to a role for expression rather than inherited genetic mutations. Alternatively, the presence of a modifier gene expressed in petals and lateral sepals (but not in the labellum) could also alter expression of flower colour genes through its action (e.g. Scopece *et al.* 2020).

Discrete flower colour polymorphism is infrequent but phylogenetically widespread and is particularly common in deceptive orchids (e.g. Gigord *et al.* 2001; Juillet *et al.* 2010; Jersáková *et al.* 2015; Narbona *et al.* 2018; Jiménez-López *et al.* 2020). This association between colour polymorphism and deceptive orchids has been considered a consequence of negative frequency-dependent selection, because the rarest morph should have an advantage, it in being less recognisable by pollinators (e.g. Gigord *et al.* 2001). However, by comparing MRS and FRS in the three colour morphs in natural populations we found no significant differences (Fig. 3), thus suggesting that no morph is advantaged in natural populations, contrary to the negative frequency-dependent selection hypothesis. This general finding was further confirmed after dividing the flowering season in three different periods defined by rainfall dynamics.

Different colour morphs can be subjected to different selection pressures if the pollinator set involved in their pollination is somewhat different and has different preferences. In natural populations, selection might be related to different factors, but in severely pollen-limited species the strength of selection is considered to be mainly related to action of the pollinators (Sletvold *et al.* 2010; Sletvold & Ågren 2014). In our study we

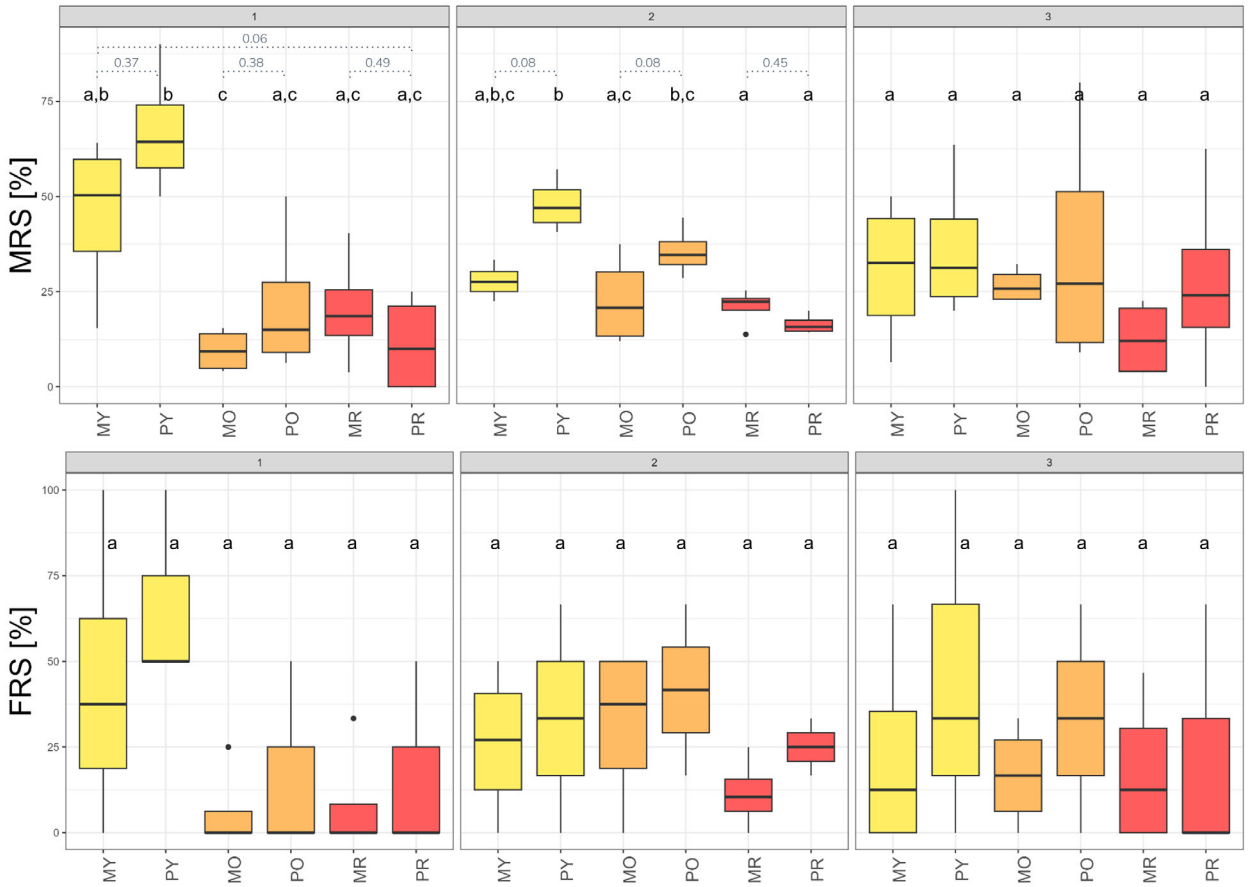

Fig. 6. Male reproductive success (MRS) and female reproductive success (FRS) of each monomorphic plot (MY, MO and MR) and each morph within the polymorphic plot (PY, PO and PR) in the three measured periods. Different letters indicate significant differences ($P < 0.05$).

found that reproductive success of *E. fulgens* is severely limited and that overall selection on floral traits is weak but varies in space, in seasons and in rainfall periods. This result, already reported for Mediterranean deceptive orchids (Scopece *et al.* 2017), is likely linked to variations in the pollinator community, a trend common in generalist plant species where local differences in prevalent pollinators can lead to local adaptation (Frachon *et al.* 2023). Accordingly, our pollinator observations found that the pollination strategy of *E. fulgens* is highly generalist, with at least 12 Lepidoptera species (i.e. 35.3% of the 34 reported Lepidoptera species; see Fig. S5) contributing to pollen transfer in the studied populations. This situation is typical of generalised food-deceptive species (reviewed by Fantinato *et al.* 2017), including several *Epidendrum* species (reviewed in Pinheiro & Cozzolino 2013).

If selection pressures are continuous and maintain the same direction, they can lead to morphological differentiation (Harder & Johnson 2009). Here, we found coherence between morphological differentiation and the direction of selection in some studied traits. In the population of BE, for instance, the trait PetL was positively selected (for FRS, Fig. 4A) only in the R morph, which also has significantly longer petals than the other two morphs (Fig. 2A); PetW was negatively selected (for MRS, Fig. 4A) only in the Y morph, which has significantly thinner petals than the other two morphs (Fig. 2D). These results suggest the presence of a somewhat different pollinator set among the different morphs that might lead to the observed morphological differentiation of colour morphs. Spatial and temporal habitat heterogeneity is one mechanism explaining the maintenance of polymorphisms within populations (Delph & Kelly 2014), and, in this context, the diversity of the pollinator assemblage might be interpreted as an important community feature enhancing species variations. In fact, de Mattos *et al.* (2023) showed that pollinator diversity and abundance not only contribute to morphological variation of flowers but also improve the habitat suitability for *E. fulgens*, partially shaping its geographic distribution.

We found evidence of variable direction of natural selection, with variation among years, among populations and acting on different floral traits (Tables 1, 2). Despite these variations, there was no evidence of any trait being under positive selection for one flowering season and negative for the next one, which suggests that different pollinators, indeed, have different preferences and their long-term consistency of choice affects the reproductive pattern of the colour morphs, resulting in assortative mating. Assortative mating is a known mechanism for maintenance of phenotypic variation (Kondrashov & Shpak 1998) and could explain the coherence between morphological trait variation in opposite directions among colour morphs. This could contribute to explaining maintenance of the observed polymorphism in

E. fulgens, regarding its rich and dynamic pollinator community (see Fig. S5). Also, we only detected this pattern in the multi-year study, thus we emphasise the importance of long-term studies for evolutionary questions and call for more investigations of seasonal variation on selection patterns in deceptive orchid species.

In deceptive orchids, phenotypic polymorphism is thought to be involved in decreased pollinator avoidance learning, thus leading to predictions of higher reproductive success in polymorphic populations (Heinrich 1975). To test this prediction, we performed a plot experiment, manipulating the presence of different colour morphs. Contrary to the prediction, monomorphic and polymorphic plots experienced similar levels of MRS and FRS. This finding confirms previous findings (reviewed by Juillet & Scopece (2010)) and challenges the role of polymorphism in decreasing pollinator avoidance learning.

The plot experiment also showed that the Y morph in the polymorphic plot (PY) was more attractive than the R morph, both in monomorphic and within polymorphic plots (MR and PR) (MRS in Fig. 5B). This could be linked to the perception ability of Neotropical butterflies, as documented in higher attraction in the yellow spectrum for several butterfly species (Weiss & Papaj 2003; Ômura & Honda 2005 and references therein). Recent studies have shown that yellow colour may be interpreted by butterflies as a positive sign of the presence of nectar, thus being preferred over other colours (Maharaj & Bourne 2017; Santana *et al.* 2022). This higher attractiveness of the Y morph was not observed in natural populations (likely because of variables such as relative abundance, heterogeneous distribution, sharing pollinators with other species of local flora, proximity to other *E. fulgens* populations, and/or elevated richness of pollinator community), but might contribute to explain why the Y morph is more abundant than the R morph in the CA population. However, why the higher attraction of the Y morph does not translate into overall higher frequency of plants with this phenotype in the natural populations is still an open question.

The three colour morphs have different abundances in natural populations, with a clear prevalence of the O morph over the R and Y morphs (see Fig. S1). The prevalence of the O colour morph in natural populations is likely linked to inheritability of this intermediate phenotype. Indeed, Pinheiro *et al.* (2010, 2016) report hybrids in the *Epidendrum* genus with orange flowers, which were generated by crosses between species with yellow and red/pink flowers. Alternatively, flower colour morphs can also show differential tolerances to abiotic factors (Warren & Mackenzie 2001; Arista *et al.* 2017) related to the association between floral pigments and protective flavonoids and anthocyanins, which can be selected either for or against in different habitats (Jiménez-López *et al.* 2020). Indeed, the O and R morphs also contain red pigments in their stems and leaves, contrasting with the pure green leaves and stems present in the Y morph (F Pinheiro pers. obs.). Future studies should investigate how flower pigments influence physiological responses of vegetative organs related to soil properties (Schemske & Bierzychudek 2007; Wu *et al.* 2022), drought, light or heat stress (Arista *et al.* 2017; Costa *et al.* 2023).

Flower colour polymorphism is a common phenomenon in nature, and its maintenance has been a topic of much debate (Sapir *et al.* 2021). Taken together, our results suggest that flower colour polymorphism is maintained by a combination of factors, including weak but varying pollinator-mediated selection, assortative mating due to different pollinator preferences, and heritability of the intermediate phenotype. We detected variations in pollinator preferences and selection intensities across space, seasons and rainfall periods, indicating a dynamic interplay between pollinators and flower morphs through space and time (Scopece *et al.* 2017; Frachon *et al.* 2023). Our manipulative experiment further demonstrated that the presence of different colour morphs did not significantly affect overall reproductive success, suggesting that the observed polymorphism is likely maintained by other mechanisms, excluding the occurrence of deterring pollinator avoidance learning. While the different colour morphs did not show differences in their overall reproductive success in the field, our plot experiment revealed that the Y morph was more attractive to butterflies than the R morph. The fact that the higher attractiveness of the Y morph does not translate into an overall higher frequency of plants carrying this phenotype in the field deserves further studies, to investigate the role of floral pigments in other plant physiological responses to abiotic variables, such as water, light and heat stress. Furthermore, other biotic interactions, such as mycorrhizal (Burkle & Zabinski 2023) and ant–plant associations (Ibarra-Isassi & Oliveira 2018) are essential data for studies seeking to understand patterns of adaptation at small geographic scales. Considering all contributing factors, a more comprehensive understanding of the selection pressures shaping floral variation in hyperdiverse regions (such as the Neotropics) can be achieved.

AUTHOR CONTRIBUTIONS

BLA, AVLF, GS and FP planned and designed the research. BLA performed the pollinator observations and the experimental approach. BLA and MGGC conducted the color analyses. BLA, GS and LL analyzed the selection signs in the data. AVLF identified pollinator species. BLA and LL performed morphological analyses. BLA wrote the manuscript. FP, LL, MGGC, AVLF and GS carefully reviewed the manuscript.

ACKNOWLEDGEMENTS

We are grateful to many co-workers and friends who helped with the fieldwork: Celso Perez dos Santos, Edlley Max Pessoa da Silva, Gabriel Pavan Sabino, Giovana Narezi Trotta, Iris Dechiare Passos Ribeiro, Lucas Oliveira Mello, Raphael da Silva, Welington Luis Sachetti Jr. Thanks to Simeão de Souza Moraes and Gustavo Mattos Accacio for help in identifying butterfly species. We thank members of the Laboratory of Evolutionary Ecology and Genomics (LEEG), Universidade Estadual de Campinas (UNICAMP), for helpful discussions and comments regarding our results on the origins of flower colour polymorphisms. This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP; grant n° 2020/02150-3, 2021/03868–8) and FAPESP CBioClima (2021/10639-5), additional grants from Fundo de Apoio ao Ensino, Pesquisa e Extensão (FAEPEX – FUNCAMP) to FP, and fellowships to BLA (FAPESP grants no 2021/10798-6 and 2023/01736-2), AVLF (CNPq productivity grant no 304291/2020–0) and FP (CNPq productivity grant no 302849/2021-1). This study was also financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

– Brasil (CAPES) – Finance Code 001. GS was also funded under the National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 Investment 1.4 – Call for tender No. 3138 of 16 December 2021, rectified by Decree n. 3175 of 18 December 2021 of the Italian Ministry of University and Research funded by the European Union – NextGenerationEU. LL was funded in the framework of a Ph.D. scholarship in the field of sustainable development, financially supported by INPS (National Institute of Social Insurance).

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Boxplots of the overall number of individuals of each flower colour morph (Y, O and R) found in an area of 100 m^2 in both natural populations: Bertioga (A, green chart) and Ilha do Cardoso (B, blue chart). Different letters indicate significant differences ($P < 0.05$).

Fig. S2. (A) PC1 eigenvectors associated with each landmark or semi-landmark and their distortion. (B) Image of a labellum with positioned landmarks and semi-landmarks. (C) Percentage variance of labellum shape explained by each principal component (from PC1 to PC42). (D) PCA plot of labellum shape of all flowers collected from both populations (BE and CA) (y-axis is PC2 and x-axis is PC1; beside the x-axis is a representative image of the shape variation on each direction of PC1, according to its eigenvectors).

Fig. S3. Phenotypic traits with no significant differences among colour morphs of *E. fulgens* in Bertioga (A to D, green charts) and Ilha do Cardoso (E to K, blue charts) populations: labellum shape (A), lateral sepal width (B and I), dorsal sepal width (C and J), labellum area (D and H), petal length (E), lateral sepal length (F), dorsal sepal length (G) and petal width (K). Different letters indicate significant differences ($P < 0.05$).

Fig. S4. Male reproductive success (MRS) and female reproductive success (FRS) of the three colour morphs (Y, O, R) in the BE population after dividing the flowering seasons in three rainfall periods.

Fig. S5. Lepidoptera community in the two natural populations, Bertioga (BE) and Ilha do Cardoso (CA), and in the experimental suburban area. Dots represent presence of the respective butterfly species (empty dots for presence without interaction; filled dots for presence with interaction with *Epidendrum fulgens*), and line types represent their category: pollinators (full line), potential pollinators (dashed line), not pollinators (dotted line).

REFERENCES

Ackerman J.D., Cuevas A.A., Hof D. (2011) Are deception-pollinated species more variable than those offering a reward? *Plant Systematics and Evolution*, **293**, 91–99.

Aguiar J.M.R.B.V., de Souza Ferreira G., Sanches P.A., Bento J.M.S., Sazima M. (2021) What pollinators see does not match what they smell: Absence of color–fragrance association in the deceptive orchid *Ionopsis utricularioides*. *Phytochemistry*, **182**, 112591.

Aguiar J.M.R.B.V., Giurfa M., Sazima M. (2020) A cognitive analysis of deceptive pollination: Associative mechanisms underlying pollinators' choices in non-rewarding colour polymorphic scenarios. *Scientific Reports*, **10**, 9476.

Andersson S., Nilsson L.A., Groth I., Bergström G. (2002) Floral scents in butterfly-pollinated plants: Possible convergence in chemical composition. *Botanical Journal of the Linnean Society*, **140**, 129–153.

Arikawa K. (2003) Spectral organization of the eye of a butterfly, *Papilio*. *Journal of Comparative Physiology A*, **189**, 791–800.

Arista M., Berjano R., Viruel J., Ortiz M.Á., Talavera M., Ortiz P.L. (2017) Uncertain pollination environment promotes the evolution of a stable mixed reproductive system in the self-incompatible *Hypochaeris salzmanniana* (Asteraceae). *Annals of Botany*, **120**, 447–456.

Bates D., Mächler M., Bolker B., Walker S. (2015) Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, **67**, 1–48.

Bergamo P.J., Rech A.R., Brito V.L., Sazima M. (2016) Flower colour and visitation rates of *Costus arabicus* support the 'bee avoidance' hypothesis for red-reflecting hummingbird-pollinated flowers. *Functional Ecology*, **30**, 710–720.

Blackiston D., Briscoe A.D., Weiss M.R. (2011) Color vision and learning in the monarch butterfly, *Danaus plexippus* (Nymphalidae). *Journal of Experimental Biology*, **214**, 509–520.

Bookstein F.L. (1997) Landmark methods for forms without landmarks: Morphometrics of group differences in outline shape. *Medical Image Analysis*, **1**, 225–243.

Burkle L.A., Zabinski C.A. (2023) Mycorrhizae influence plant vegetative and floral traits and intraspecific trait variation. *American Journal of Botany*, **110**, 16099.

Chen S., Li M., Liu J., Feng Y., Yao J., Shi L., Chen X. (2021) Visual and olfactory sensory responses of the butterfly *Papilio maackii* during foraging and courtship. *Entomological Research*, **51**, 518–527.

Chittka L., Thomson J.D., Waser N.M. (1999) Flower constancy, insect psychology, and plant evolution. *Naturwissenschaften*, **86**, 361–377.

Costa A., Moré M., Sérsic A.N., Cocucci A.A., Drewniak M.E., Izquierdo J.V., Coetzee A., Pauw A., Traveset A., Paiaro V. (2023) Floral colour variation of *Nicotiana glauca* in native and non-native ranges: Testing the role of pollinators' perception and abiotic factors. *Plant Biology*, **25**, 403–410.

Cottam G., Curtis J.T. (1956) The use of distance measures in phytosociological sampling. *Ecology*, **37**, 451–460.

Dalrymple R.L., Hui F.K., Flores-Moreno H., Kemp D.J., Moles A.T. (2015) Roses are red, violets are blue – So how much replication should you do? An assessment of variation in the colour of flowers and birds. *Biological Journal of the Linnean Society*, **114**, 69–81.

Darwin C. (1859) *On the origin of species*. Collins, London, UK, pp 502.

Darwin C. (1877) *The different forms of flowers on plants of the same species*. D. Appleton, London, UK, pp 352.

Davies K.M., Albert N.W., Schwinn K.E. (2012) From landing lights to mimicry: The molecular regulation of flower colouration and mechanisms for pigmentation patterning. *Functional Plant Biology*, **39**, 619–638.

de Lima T.M., Silva S.F., Ribeiro R.V., Sánchez-Vilas J., Pinheiro F. (2024) Salt tolerance in a neotropical orchid in the absence of local adaptation to salt spray. *American Journal of Botany*, **111**, e16373.

de Mattos J.S., Pinheiro F., Luize B.G., Chaves C.J.N., de Lima T.M., Palma-Silva C., Leal B.S.S. (2023) The relative role of climate and biotic interactions in shaping the range limits of a neotropical orchid. *Journal of Biogeography*, **50**, 1315–1328.

Delph L.F., Kelly J.K. (2014) On the importance of balancing selection in plants. *New Phytologist*, **201**, 45–56.

Dukas R., Real L.A. (1993) Learning constraints and floral choice behaviour in bumble bees. *Animal Behaviour*, **46**, 637–644.

Eckhart V.M., Rushing N.S., Hart G.M., Hansen J.D. (2006) Frequency-dependent pollinator foraging in polymorphic *Clarkia xantiana* ssp. *xantiana* populations: Implications for flower colour evolution and pollinator interactions. *Oikos*, **112**, 412–421.

Ellegren H., Galtier N. (2016) Determinants of genetic diversity. *Nature Reviews Genetics*, **17**, 422–433.

Fantinato E., Del Vecchio S., Baltieri M., Fabris B., Buffa G. (2017) Are food-deceptive orchid species really functionally specialized for pollinators? *Ecological Research*, **32**, 951–959.

Ferdy J.B., Gouyon P.H., Moret J., Godelle B. (1998) Pollinator behavior and deceptive pollination: Learning process and floral evolution. *The American Naturalist*, **152**, 696–705.

Frachon L., Arrigo L., Rusman Q., Poveda L., Qi W., Scopece G., Schiestl F.P. (2023) Putative signals of generalist plant species adaptation to local pollinator communities and abiotic factors. *Molecular Biology and Evolution*, **40**, msad036.

Fuhro D., Araújo A.M.D., Irgang B.E. (2010) Are there evidences of a complex mimicry system among *Asclepias curassavica* (Apocynaceae), *Epidendrum*

fulgens (Orchidaceae), and *Lantana camara* (Verbenaceae) in southern Brazil? *Brazilian Journal of Botany*, **33**, 589–598.

Gigord L.D., Macnair M.R., Smithson A. (2001) Negative frequency-dependent selection maintains a dramatic flower color polymorphism in the rewardless orchid *Dactylorhiza sambucina* (L.) Soo. *Proceedings of the National Academy of Sciences*, **98**, 6253–6255.

Goulson D. (2000) Are insects flower constant because they use search images to find flowers? *Oikos*, **88**, 547–552.

Harder L.D., Johnson S.D. (2009) Darwin's beautiful contrivances: Evolutionary and functional evidence for floral adaptation. *New Phytologist*, **183**, 530–545.

Heinrich B. (1975) Bee flowers: A hypothesis on flower variety and blooming times. *Evolution*, **29**, 325–334.

Ibarra-Isassi J., Oliveira P.S. (2018) Indirect effects of mutualism: Ant–treehopper associations deter pollinators and reduce reproduction in a tropical shrub. *Oecologia*, **186**, 691–701.

Free J.B. (1963) The flower constancy of honeybees. *Journal of Animal Ecology*, **32**, 119–131.

Jacquemyn H., Brys R. (2020) Lack of strong selection pressures maintains wide variation in floral traits in a food-deceptive orchid. *Annals of Botany*, **126**, 445–453.

Jersáková J., Traxmandlová I., Ipser Z., Kropf M., Pellegrino G., Schatz B., Djordjević V., Kindlmann P., Renner S.S. (2015) Biological flora of Central Europe: *Dactylorhiza sambucina* (L.) Soó. *Perspectives in Plant Ecology, Evolution and Systematics*, **17**, 318–329.

Jiménez-López F.J., Ortiz P.L., Talavera M., Arista M. (2020) Reproductive assurance maintains red-flowered plants of *Lysimachia arvensis* in Mediterranean populations despite inbreeding depression. *Frontiers in Plant Science*, **11**, 563110.

Juillet N., Delle-Vedove R., Dormont L., Schatz B., Pailler T. (2010) Differentiation in a tropical deceptive orchid: Colour polymorphism and beyond. *Plant Systematics and Evolution*, **289**, 213–221.

Juillet N., Scopece G. (2010) Does floral trait variability enhance reproductive success in deceptive orchids? *Perspectives in Plant Ecology, Evolution and Systematics*, **12**, 317–322.

Kagawa K., Takimoto G. (2016) Inaccurate color discrimination by pollinators promotes evolution of discrete color polymorphism in food-deceptive flowers. *The American Naturalist*, **187**, 194–204.

Kellenberger R.T., Byers K.J., de Brito Francisco R.M., Staedler Y.M., LaFountain A.M., Schönenberger J., Schiestl F.P., Schlüter P.M. (2019) Emergence of a floral colour polymorphism by pollinator-mediated overdominance. *Nature Communications*, **10**, 63.

Kondrashov A.S., Shpak M. (1998) On the origin of species by means of assortative mating. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, **265**, 2273–2278.

Lande R., Arnold S.J. (1983) The measurement of selection on correlated characters. *Evolution*, **37**, 1210–1226.

Larson B.M., Barrett S.C. (2000) A comparative analysis of pollen limitation in flowering plants. *Biological Journal of the Linnean Society*, **69**, 503–520.

Maharaj G., Bourne G. (2017) Honest signalling and the billboard effect: How heliconiid pollinators respond to the trichromatic colour changing *Lantana camara* L. (Verbenaceae). *Journal of Pollination Ecology*, **20**, 40–50.

Moreira A.S.F.P., Fuhro D., dos Santos Isaias R.M. (2008) Anatomia floral de *Epidendrum fulgens* Brongn. (Orchidaceae-Epidendroideae) com ênfase no nectário e sua funcionalidade. *Journal of Neotropical Biology*, **5**, 23–29.

Narbona E., Wang H., Ortiz P.L., Arista M., Imbert E. (2018) Flower colour polymorphism in the Mediterranean Basin: Occurrence, maintenance and implications for speciation. *Plant Biology*, **20**, 8–20.

Newman E., Anderson B., Johnson S.D. (2012) Flower colour adaptation in a mimetic orchid. *Proceedings of the Royal Society B: Biological Sciences*, **279**, 2309–2313.

Nilsson L.A. (1992) Orchid pollination biology. *Trends in Ecology & Evolution*, **7**, 255–259.

Ômura H., Honda K. (2005) Priority of color over scent during flower visitation by adult *Vanessa indica* butterflies. *Oecologia*, **142**, 588–596.

Pinheiro F., Cardoso-Gustavson P., Suzuki R.M., Abrão M.C.R., Guimarães L.R., Draper D., Moraes A.P. (2015) Strong postzygotic isolation prevents introgression between two hybridizing neotropical orchids, *Epidendrum denticulatum* and *E. fulgens*. *Evolutionary Ecology*, **29**, 229–248.

Pinheiro F., Cozzolino S. (2013) *Epidendrum* (Orchidaceae) as a model system for ecological and evolutionary studies in the neotropics. *Taxon*, **62**, 77–88.

Pinheiro F., de Barros F., Palma-Silva C., Meyer D., Fay M.F., Suzuki R.M., Lexer C., Cozzolino S. (2010) Hybridization and introgression across different ploidy levels in the neotropical orchids *Epidendrum fulgens* and *E. puniceoluteum* (Orchidaceae). *Molecular Ecology*, **19**, 3981–3994.

Pinheiro F., De Melo e Gouveia T.M.Z., Cozzolino S., Cafasso D., Cardoso-Gustavson P., Suzuki R.M., Palma-Silva C. (2016) Strong but permeable barriers to gene exchange between sister species of *epidendrum*. *American Journal of Botany*, **103**, 1472–1482.

Raine N.E., Chittka L. (2007) Flower constancy and memory dynamics in bumblebees (Hymenoptera: Apidae: *Bombus*). *Entomologia Generalis*, **29**, 179–199.

Reichenbach H.G. (1878) *Otia botanica Hamburgensia*, Vol. **2**. Theodor Theophil Meissneri, Hamburg, DE, pp 119.

Rohlf F.J. (2015) The tps series of software. *Hystrix*, **26**, 9.

Rohlf F.J., Slice D. (1990) Extensions of the Procrustes method for the optimal superimposition of landmarks. *Systematic Zoology*, **39**, 40–59.

Salzmann C.C., Nardella A.M., Cozzolino S., Schiestl F.P. (2007) Variability in floral scent in rewarding and deceptive orchids: The signature of pollinator-imposed selection? *Annals of Botany*, **100**, 757–765.

Santana P.C., Raderschall C.A., Rodrigues R.M., Ellis A.G., de Brito V.L.G. (2022) Retention of colour-changed flowers increases pollinator attraction to *Lantana undulata* inflorescences. *Flora*, **296**, 152152.

Sapir Y., Gallagher M.K., Senden E. (2021) What maintains flower colour variation within populations? *Trends in Ecology & Evolution*, **36**, 507–519.

Schemske D.W., Bierzychudek P. (2007) Spatial differentiation for flower color in the desert annual *Linanthus parryae*: Was Wright right? *Evolution*, **61**, 2528–2543.

Schiestl F.P., Johnson S.D. (2013) Pollinator-mediated evolution of floral signals. *Trends in Ecology & Evolution*, **28**, 307–315.

Scopece G., Juillet N., Lexer C., Cozzolino S. (2017) Fluctuating selection across years and phenotypic variation in food-deceptive orchids. *PeerJ*, **5**, 3704.

Scopece G., Palma-Silva C., Cafasso D., Lexer C., Cozzolino S. (2020) Phenotypic expression of floral traits in hybrid zones provides insights into their genetic architecture. *New Phytologist*, **227**, 967–975.

Scopece G., Schiestl F.P., Cozzolino S. (2015) Pollen transfer efficiency and its effect on inflorescence size in deceptive pollination strategies. *Plant Biology*, **17**, 545–550.

Shrestha M., Dyer A.G., Dorin A., Ren Z.X., Burd M. (2020) Rewardlessness in orchids: How frequent and how rewardless? *Plant Biology*, **22**, 555–561.

Sletvold N., Ågren J. (2014) There is more to pollinator-mediated selection than pollen limitation. *Evolution*, **68**, 1907–1918.

Sletvold N., Grindeland J.M., Ågren J. (2010) Pollinator-mediated selection on floral display, spur length and flowering phenology in the deceptive orchid *Dactylorhiza lapponica*. *New Phytologist*, **188**, 385–392.

Smithson A., Macnair M.R. (1997) Negative frequency-dependent selection by pollinators on artificial flowers without rewards. *Evolution*, **51**, 715–723.

Sprengel C.K. (1793) *Das entdeckte Geheimniss der Natur im Bau und in der Befruchtung der Blumen*. Friedrich Vieweg der Ältere, Berlin, DE, pp 473.

Stebbins G.L. (1950) *Variation and evolution in plants*. Columbia University Press, New York, US, pp 643.

Swihart C.A., Swihart S.L. (1970) Colour selection and learned feeding preferences in the butterfly, *Heliconius charitonius* Linn. *Animal Behaviour*, **18**, 60–64.

Tyteca D. (2000) Morphometric analysis of the *Dactylorhiza majalis* group in France and western Europe, with a description of *Dactylorhiza parvimajalis* Tyteca et Gathoye, spec. Nov. *Journal Europäischer Orchideen*, **32**, 471–511.

van der Kooi C.J., Stavenga D.G., Arikawa K., Belušič G., Kelber A. (2021) Evolution of insect color vision: From spectral sensitivity to visual ecology. *Annual Review of Entomology*, **66**, 435–461.

Wang H., Talavera M., Min Y., Flaven E., Imbert E. (2016) Neutral processes contribute to patterns of spatial variation for flower colour in the Mediterranean *Iris lutescens* (Iridaceae). *Annals of Botany*, **117**, 995–1007.

Warren J., Mackenzie S. (2001) Why are all colour combinations not equally represented as flower-colour polymorphisms? *New Phytologist*, **151**, 237–241.

Waser N.M. (1986) Flower constancy: Definition, cause, and measurement. *The American Naturalist*, **127**, 593–603.

Waser N.M., Price M.V. (1981) Pollinator choice and stabilizing selection for flower color in *Delphinium nelsonii*. *Evolution*, **35**, 376–390.

Weiss M.R., Papaj D.R. (2003) Colour learning in two behavioural contexts: How much can a butterfly keep in mind? *Animal Behaviour*, **65**, 425–434.

Westerband A.C., Funk J.L., Barton K.E. (2021) Intraspecific trait variation in plants: A renewed focus on its role in ecological processes. *Annals of Botany*, **127**, 397–410.

Wu C.A., Streisfeld M.A., Nutter L.I., Cross K.A. (2013) The genetic basis of a rare flower color polymorphism in *Mimulus lewisii* provides insight into the repeatability of evolution. *PLoS One*, **8**, 81173.

Wu Y., Duan X.Y., Tong Z.L., Li Q.J. (2022) Pollinator-mediated selection on floral traits of *Primula tibetica* differs between sites with different soil water contents and among different levels of nutrient availability. *Frontiers in Plant Science*, **13**, 807689.