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Can phenotypic selection on floral traits explain the presence of enigmatic intermediate individuals in sympatric populations of *Platanthera bifolia* and *P. chlorantha* (Orchidaceae)?

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Abstract

Pollinators represent one of the main agents of selection on floral traits. Here, we estimated phenotypic selection on floral morphology and phenology in a sympatric population of two orchid species, *Platanthera bifolia* and *P. chlorantha*, including enigmatic individuals with intermediate column morphology (as reflected by the distance between viscidia and caudicle length, two traits involved in assortative mating and reproductive isolation among *Platanthera* species), but genetically indistinguishable from *P. bifolia*. Our aim was to clarify whether the occurrence of intermediate phenotypes could be explained by the presence of selective pressures exerted by pollinators. Simple linear and quadratic regressions together with univariate and multivariate analyses were used to evaluate the strength of directional, disruptive and stabilizing selection. We found that selection on phenotypic traits varied between groups and sex functions. Contrary to our hypothesis, selection on the viscidia distance and caudicle length appeared to be consistent in the two *P. bifolia* groups. Interestingly, the viscidia distance was under significant stabilizing selection through female reproductive success in intermediate individuals. Based on these results, we conclude that, despite a significant selective pressure on some phenotypic traits, the presence of individuals with intermediate phenotype is not due to selection. Stabilizing selection on distance between viscidia in intermediate individuals may suggest that assortative mating play a role in the maintenance of this phenotypic polymorphism.

KEYWORDS

intermediate individuals, Orchidaceae, phenotypic selection, *Platanthera*, pollination

1 | INTRODUCTION

Plant–pollinator interactions are thought to have shaped the evolution and differentiation of flowering plants (Baker, 1961; Darwin, 1862; Grant, 1971; Stebbins, 1950, 1970). Darwin (1862) was among the first to investigate this relationship in the Orchidaceae, building on previous pioneering observations of C. K. Sprengel (1793). Thereafter, a number of studies conducted on this plant group have supported the idea that selection driven by

pollinators represents the fundamental force driving the evolution of floral traits in orchids (Fenster, Armbruster, Wilson, Dudash, & Thomson, 2004; Maad, 2000; Nilsson, 1992; Schiestl & Johnson, 2013; Schiestl & Schlüter, 2009; Tremblay, Ackerman, Zimmerman, & Calvo, 2005).

Plant adaptations to pollinators are generally studied at the species level, but they are expected to evolve at the population level and to be reflected in fine-scale patterns of phenotypic trait variation (Anderson & Johnson, 2009). The development of experimental approaches to estimate selection differentials in natural populations (Lande & Arnold, 1983) resulted in the common discovery of directional selection on floral traits in many plant systems (Hereford, Hansen, & Houle, 2004; Kingsolver et al., 2001). Among orchids, an unusually high number of species produce no nectar and are pollinated through deceptive strategies (Jersáková, Johnson, & Kindlmann, 2006). Due to pollinator avoidance learning (Juillet, Salzmann, & Scopece, 2011), these species experience low levels of pollination success and, consequently, high levels of pollen limitation (Tremblay et al., 2005). Pollen limitation is likely to increase the opportunity for pollinator-mediated selection (Trunschke, Sletvold, & Ågren, 2017). However, most studies conducted on these severely pollen-limited deceptive species showed a weak selection that is likely to explain the elevated phenotypic polymorphism of this group if compared with closely related rewarding species (Juillet & Scopece, 2010; but see Trunschke et al., 2017). By contrast, previous studies on rewarding orchids, such as *Gymnadenia conopsea* (Chapurlat, Ågren, & Sletvold, 2015; Sletvold & Ågren, 2010) or *Platanthera bifolia* (Maad, 2000), have reported evidence for significant directional selection on floral traits among populations attributed to adaptation to local pollinators. This is also confirmed by the general stability of floral traits in rewarding species if compared with deceptive ones (Ackerman, Cuevas, & Hof, 2011).

Two rewarding orchid species, *Platanthera bifolia* and *P. chlorantha*, have already been considered as a model for understanding the role of floral specialization in the adaptive radiation of the Orchidaceae (Durka, Baum, Michalski, & Baum, 2017; Esposito et al., 2018; Hapeman & Inoue, 1997). The choice of *Platanthera* as a model for selection studies is due to the fact that components of both female and male fitness are easily estimated (Bateman, James, & Rudal, 2012; Bateman & Sexton, 2008; Efimov, 2011), and the column (i.e., in orchids, the organ uniting the stigma and the pollinaria) shows a high morphological variation within the genus, which can easily shift when it is subject to selection (Hapeman & Inoue, 1997).

Within the framework of a series of research devoted to *Platanthera* in Belgium (Esposito et al., 2018; Esposito, Jacquemyn, Waud, & Tyteca, 2016), we surveyed a sympatric population of *P. bifolia* and *P. chlorantha*, where individuals with a morphologically intermediate column were observed. Neutral genetic marker analysis (AFLPs) and scent composition profiles demonstrated that most of these individuals were attributable to *P. bifolia*, whereas field researchers and naturalists formerly used to identify them as hybrids (Esposito, Merckx, & Tyteca, 2017). Interestingly, in these mixed populations encompassing intermediate individuals, *P. chlorantha* has consistently been found to have a higher reproductive success compared to *P. bifolia* (Esposito et al., 2018), suggesting a higher attractivity of *P. chlorantha* phenotypic traits to the local pollinator community. This finding allowed us to hypothesize that morphologically intermediate individuals could be the result of a positive selection of *P. chlorantha*-like phenotypes in *P. bifolia* individuals that would potentially allow these individuals to exploit the pollinator set of *P. chlorantha*, thus increasing their reproductive success. This hypothesis would explain the presence of intermediate individuals with the genetic profile of *P. bifolia*.

To test this hypothesis, we quantified patterns of directional selection on column morphology in a sympatric population where *P. bifolia*, *P. chlorantha* and intermediate morphotypes grow intermixed, in order to understand whether *P. chlorantha*–like phenotypes in *P. bifolia* individuals are linked to a higher fitness. Specifically, as phenotypic traits in a population may evolve continuously and selection can act on several traits at once, we estimated selection differentials and coefficients in a multidimensional context (Hereford et al., 2004; Kingsolver et al., 2001; Lande & Arnold, 1983; Maad & Alexandersson, 2004). In addition, because the difference in flowering time among species may contribute to the maintenance of the phenotypic differences between species, the analysis of species phenology during the whole flowering season was also integrated.

2 | MATERIALS AND METHODS

2.1 | Studied species and population

Platanthera bifolia (L.) L. C. Rich. and *P. chlorantha* (Custer) Reichb. (Orchidaceae) are two terrestrial rewarding orchid species with a wide Eurasian distribution (Hultén & Fries, 1986). The flowering period of these species in central Europe ranges between May and July, with a partial overlap in the areas of sympatry (Delforge, 2005). Both species display 10–25 white flowers with nocturnal fragrance. Floral morphology, which is directly associated with pollination, is divergent between the two

species (Darwin, 1862; Nilsson, 1978, 1983, 1985). In order to identify *Platanthera* species, we followed the criteria firstly proposed by Nilsson (1983) based on several morphological characteristics of the floral column (Figure 1). In particular, the distance between the viscidia can be attributed to flower specialization for pollinator use and is associated with the mechanical fit between flowers and pollinators (Esposito et al., 2017; Nilsson, 1983, 1985). *P. bifolia* generally displays a small column with the viscidia close to and almost parallel to each other. The distance between the viscidia ranges between 0.2 and 1.1 mm, whereas the pollinium has a very short caudicle (0.2–0.5 mm). By contrast, the column of *P. chlorantha* is wider than that of *P. bifolia*, with a relatively long caudicle (1.2–2.2 mm), and the distance between the viscidia may vary between 2.3 and 4.9 mm. Individuals with an intermediate column morphology display a distance between the viscidia and a caudicle length that are, on average, larger than those in *P. bifolia* and smaller than those in *P. chlorantha* (Esposito et al., 2018). For short, we will call these individuals 'intermediates', 'intermediate individuals' or 'intermediate morphotypes'.

Hawk moths (Sphingidae) (Nilsson, 1983) are the predominant pollinators of *P. bifolia* and the pollinia are attached to the proboscis, whereas in *P. chlorantha* the greater distance between the viscidia leads to the attachment of the pollinia to the eyes of pollinators, which are mostly owlet moths (Noctuidae) (Nilsson, 1983). The intermediate morphotypes show an intermediate position of the pollinia compared to *P. bifolia* and *P. chlorantha* (Esposito et al., 2018; Nilsson, 1983). This position, due to the narrowly delimited surfaces on moths' heads (suitable for attachment of viscidia), may induce an inadequate pollinia attachment (Esposito et al., 2017; Nilsson, 1978, 1985), thus reducing the probability of hybridization or introgression in sympatry.

According to previous studies conducted by Nilsson (1983, 1985), the spur length of *P. bifolia* varies geographically (15–40 mm) in northern Europe and reflects the tongue lengths of the local moth pollinators. By contrast, the spur length of *P. chlorantha* varies less and often ranges between 20 and 30 mm. On the other hand, in the British Isles, the spur length varies similarly in both species, with *P. bifolia*'s spur length being on average two thirds of the length of a *P. chlorantha* spur, for a given latitude (Bateman & Sexton, 2008).

The studied sympatric population, Tienne de Botton, is located within a natural reserve in Ave-et-Auffe, southern Belgium. It consists of a total of 288 inflorescence-producing individuals and about 100 individuals that did not flower during the study. The major vegetation in the studied site is a calcareous grassland with some *Pinus sylvestris*, *Malus sylvestris* and *Prunus spinosa*. The calcareous grassland is managed by sheep grazing from mid-August to October.

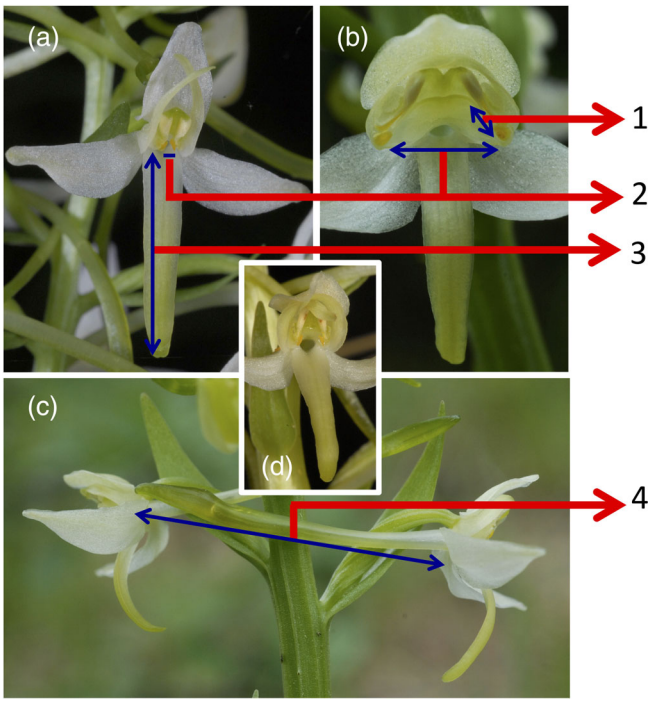

FIGURE 1 Column morphology in pure *Platanthera bifolia* (a), *P. chlorantha* (b and c) and intermediate *P. bifolia* (d): 1, caudicle length; 2, distance between the viscidia; 3, labellum length; 4, spur length (pictures D. Tyteca)

2.2 | Morphological traits, floral phenology and fitness: Choice of the investigated phenotypic traits

From May to the end of June in 2015, a total of 288 *Platanthera* individuals within the population were investigated. Species identification was based on the main distinctive traits (i.e., caudicle length and distance between viscidia). Intermediate morphotypes were assigned to *P. bifolia* based on molecular analyses carried out in the same population (Esposito et al., 2018). The population included 190 *P. chlorantha* individuals and 98 *P. bifolia* individuals (46 typical *P. bifolia*, hereafter referred as pure *P. bifolia*, and 52 intermediates) (Table 1).

In order to estimate the phenotypic selection, a number of potentially significant morphological, phenological and fitness measures were assessed from the studied *Platanthera*. For the morphological analysis, seven phenotypic traits thought to be important for pollination success were monitored. Three traits were related to plant

TABLE 1 Means and standard deviations (*SD*) of floral and fitness traits in pure and intermediate *P. bifolia* and *P. chlorantha*

Traits	*P. bifolia (pure)* Mean	*SD*	*P. bifolia* (intermediate) Mean	*SD*	*P. chlorantha* Mean	*SD*	*P. Bif.* Tuckey-Kramer	Inter.	*P. Chl.*
Size									
Plant height (cm) +	36.68	9.05	38.20	8.58	31.37	7.29	A	A	B
Inflorescence length (cm)	7.23	2.43	7.77	2.62	9.47	2.98	A	A	B
Number of flowers +	14.28	4.13	14.44	5.72	13.57	4.24	A	A	B
Phenology (days after May 1)									
Beginning flowering period +	10th June	3 days	9th June	4 days	27th May	3.5 days	A	A	B
Midflowering date	15th June	3.5 days	13th June	4 days	2nd June	3.5 days	A	A	B
Opening date of last flower +	19th June	4.5 days	17th June	5 days	7th June	4 days	A	A	B
Flower									
Spur length (mm)	29.34	3.31	29.45	3.13	27.01	2.61	A	A	B
Labellum length (mm)	11.73	1.91	11.39	1.60	12.78	1.77	A	A	B
Viscidia distance (mm)	1.22	0.16	1.67	0.27	3.55	0.45	A	B	C
Caudicle length (mm)	0.67	0.10	0.83	0.21	1.94	0.23	A	B	C
Reproductive success									
Male									
Number of pollinia removed	7.08	4.55	7.46	5.94	8.06	3.98	A	A	B
Female									
Number of fruits produced	6.10	5.29	6.52	6.00	8.45	5.52	A	A	B
Number of individuals	46		52		190				

Note: The last three columns indicate significantly different characters with the Tuckey-Kramer statistic ($p < .05$). Traits marked with + are not taken into account in the selection analyses.

vigor and size (plant height, inflorescence height and number of flowers) and four were linked to flower morphology (spur length, labellum length, caudicle length and distance between the viscidia) (Claessens & Kleynen, 2006; Nilsson, 1983, 1985) (Figure 1).

To investigate the flowering phenology, we recorded the beginning of the flowering period (first flower opened), the end of the flowering period (when the last flower opened) and the midflowering date (the day in the middle of an individual's flowering time) (Maad, 2002). We also monitored pollen uptake, which was measured as the number of pollinia removed per flower, divided by 2 (two pollinia per flower). Flowering phenology and pollinia uptake were both recorded at intervals of 1 to 3 days. Differences in flowering phenology and pollinia uptake between the two *Platanthera* species and intermediate morphotypes were analyzed. Two-sample Kolmogorov–Smirnov tests were used to find if the three groups were significantly different.

Fruit production and pollen removal were used to determine female and male reproductive success, respectively. To quantify female reproductive success, we recorded the number of mature fruits between 7 and 20 days after the end of the flowering season (July–August). For each plant, we estimated male relative fitness (w_m) as W_m /mean population W_m, where W_m is the male absolute fitness (absolute number of pollinia removed/2). Similarly, we calculated female relative fitness (w_f) as W_f /mean population W_f, with W_f being the female absolute fitness (absolute number of fruits produced) (Maad, 2000).

In order to estimate differences in morphological traits and to characterize the three morphotypes, we calculated character mean values and standard deviations, and detected differences between means with the analysis of variance (ANOVA) followed by the Tuckey-Kramer post-hoc test.

2.3 | Estimation of phenotypic selection

To estimate phenotypic selection, we used the regression-based methods first developed by Lande and Arnold (1983). To avoid using intercorrelated characters and to

TABLE 2 Correlation matrix among characters of pure *P. bifolia* (above the diagonal), intermediate *P. bifolia* (below the diagonal) and *P. chlorantha* (separate)

	Plant height	Inflorescence length	Number of flowers	Beginning flowering	Mid flowering	End flowering	Spur length	Labellum length	Caudicle length	Viscidia distance
Plant height		**0.844**	0.571	−0.300	−0.133	−0.008	0.191	0.185	0.253	0.186
Inflorescence length	**0.790**		0.567	−0.276	−0.102	0.021	0.162	0.162	0.253	0.122
Number of flowers	**0.702**	**0.801**		−0.059	0.232	0.378	0.143	0.320	0.182	−0.122
Beginning flowering	−0.226	−0.328	−0.236		**0.846**	**0.621**	0.215	−0.099	−0.024	−0.306
Midflowering	−0.203	−0.213	−0.078	**0.876**		**0.943**	0.221	0.081	0.004	−0.353
End flowering	−0.154	−0.092	0.054	**0.643**	**0.932**		0.191	0.181	0.021	−0.328
Spur length	0.333	0.177	0.140	−0.165	−0.210	−0.211		0.225	0.434	−0.138
Labellum length	0.453	0.419	0.358	−0.336	−0.343	−0.292	0.517		0.267	0.076
Caudicle length	−0.159	−0.052	−0.130	−0.278	−0.358	−0.360	0.064	0.206		−0.323
Viscidia distance	−0.194	−0.064	−0.028	−0.212	−0.078	0.035	−0.180	0.052	0.192	
Plant height										
Inflorescence length	**0.821**									
Number of flowers	0.485	**0.620**								
Beginning flowering	−0.045	−0.128	−0.148							
Midflowering	0.069	0.046	0.154	**0.865**						
End flowering	0.159	0.195	0.397	0.532	**0.885**					
Spur length	0.237	0.164	0.022	−0.009	−0.077	−0.122				
Labellum length	0.429	0.377	0.244	−0.081	−0.062	−0.030	0.388			
Caudicle length	0.207	0.294	0.143	−0.091	−0.058	−0.014	0.196	0.387		
Viscidia distance	0.213	0.243	0.067	0.025	0.023	0.016	0.056	0.418	0.348	

Note: Values in bold type are above 60% correlation.

keep only the most informative characters (Endler, 1986; Lande & Arnold, 1983; Maad, 2000; Mitchell-Olds & Shaw, 1987; O'Connell & Johnston, 1998), we performed a stepwise forward and backward analysis based on the AIC (Akaike information criterion). From this, the following morphological characters were selected: inflorescence length, spur length, labellum length, distance between the viscidia and caudicle length. Although the number of flowers is generally chosen for plant selection studies (Maad, 2000), we decided to use inflorescence length, because the regression analysis showed a more significant coefficient and both traits are strongly correlated (as shown in Table 2). In order to add phenological information, the midflowering date was added.

In detail, we calculated the selection differentials (S') as the regression coefficient from univariate linear regression for each investigated trait taken separately (Lande & Arnold, 1983; O'Connell & Johnston, 1998; Parachnowitsch, Raguso, & Kessler, 2012). As selection differentials only measure the difference in the mean of the investigated traits before and after selection, without taking traits' intercorrelation into account, we also measured selection gradients (β_i' and γ'_{ii}) to determine which traits were undergoing selection. To estimate directional/linear selection gradients (β_i'), following Lande and Arnold (1983), we performed a multivariate regression using relative fitness as the dependent variable and standardized traits (mean = 0, standard deviation = 1) as predictors. We also included quadratic (γ'_{ii}) terms to quantify non-linear selection. We estimated the linear selection gradients (β_i') and the quadratic selection gradients (γ'_{ii}) separately as both can be intercorrelated and linear coefficients' value depends on whether quadratic coefficients are included or not (Lande & Arnold, 1983; Maad, 2000). All the analyses were performed on the three phenotypic groups (i.e., *P. chloranta*, pure and intermediate *P. bifolia* morphotypes). All statistical analyses and graphics were performed in R version 3.5.1 (R Core Team 2015).

3 | RESULTS

3.1 | Characterization of *Platanthera* species and correlation among phenotypic traits

Although all the traits studied were significantly different between *P. chlorantha* individuals and both *P. bifolia* and

intermediate individuals, the only traits significantly different between *P. bifolia* and intermediate individuals were the distance separating the viscidia and the caudicle length (Table 1).

Mean values of male fitness (number of pollinia removed) and female fitness (number of fruits produced) differed significantly between *P. bifolia* morphotypes and *P. chlorantha* (Table 1). *Platanthera chlorantha* had a higher number of pollinia removed (and therefore was more visited) compared to both *P. bifolia* subgroups. Similarly, *P. chlorantha* had a higher female reproductive success as it produced more fruits than both *P. bifolia* morphotypes.

As the size traits plant height, inflorescence length and number of flowers were highly correlated in the three groups (Table 2), only inflorescence length was considered in the analyses and the two other traits were omitted. Similarly, only the midflowering date was further considered in the three measured phenological traits.

The flowering period of *P. chlorantha* started earlier, showing a difference of 6 and 12 days compared with intermediate and pure *P. bifolia* morphotypes, respectively (Figure 2). The flowering phenology is significantly different between *P. chlorantha* and pure *P. bifolia* morphotypes (Kolmogorov–Smirnov, $D = 0.5$, $p = .013$) and nearly significant between *P. chlorantha* and intermediate *P. bifolia* individuals (Kolmogorov–Smirnov, $D = 0.4$, $p = .082$). No significant differences were observed between the flowering periods of pure and intermediate *P. bifolia* morphotypes (Kolmogorov–Smirnov, $D = 0.15$, $p = .978$).

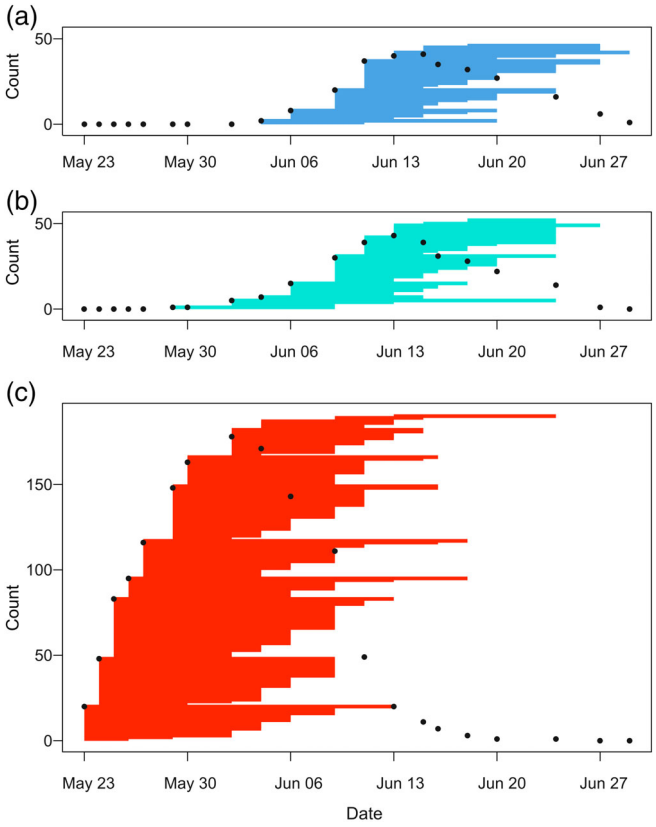

FIGURE 2 Flowering phenology of pure *Platanthera bifolia* individuals (a, blue), intermediate *P. bifolia* individuals (b, turquoise) and *P. chlorantha* (c, red) in 2015. Each horizontal line represents an individual plant and is drawn from the day the first flower opened to the day the last flower opened, sorted first by opening date of the first flower and then by flowering duration. Black dots represent the total number of inflorescences with at least one flower open for each recording time. Flowering phenology was recorded at intervals of 1 to 3 days

3.2 | Selection differentials

Results of selection differentials (Figure 3) showed that the length of the inflorescence is positively and significantly selected in the three investigated groups for both sex functions. Particularly, directional selection on this trait is stronger in the intermediate *P. bifolia* morphotype than in pure *P. bifolia* and in *P. chlorantha*.

We observed contrasting results for the impact of midflowering date on male and female fitness: whereas female *P. chlorantha* individuals flowering later tend to have a slight reproductive advantage (Table 3), the opposite is observed in the intermediate *P. bifolia* morphotype for both sex functions. No trend can be observed in the pure *P. bifolia* morphotype.

A strong and positive selection differential was observed on the length of the spur in intermediate *P. bifolia* individuals for both sex functions. A similar but not significant trend was observed in the pure *P. bifolia* morphotype and in *P. chlorantha*.

The labellum length was positively selected through both sex functions in the intermediate *P. bifolia* morphotype and in *P. chlorantha* (Table 3). This effect was stronger in intermediates than in *P. chlorantha* but no effect could be observed in pure *P. bifolia* individuals.

Selection differentials show a positive and significant effect of the distance between the viscidia on *P. chlorantha* male and female fitness. Similarly, the caudicle length was positively selected in the female *P. chlorantha*. However, selection differentials on the distance between the viscidia and the caudicle length were non-significant in both of the *P. bifolia* morphotypes.

Interestingly, when selection differentials were significant, they were always stronger in the *P. bifolia* intermediate morphotype than in the other two morphotypes.

3.3 | Directional and curvature selection

The directional and curvature (stabilizing/disruptive) selection gradients are reported in Table 4. The selection

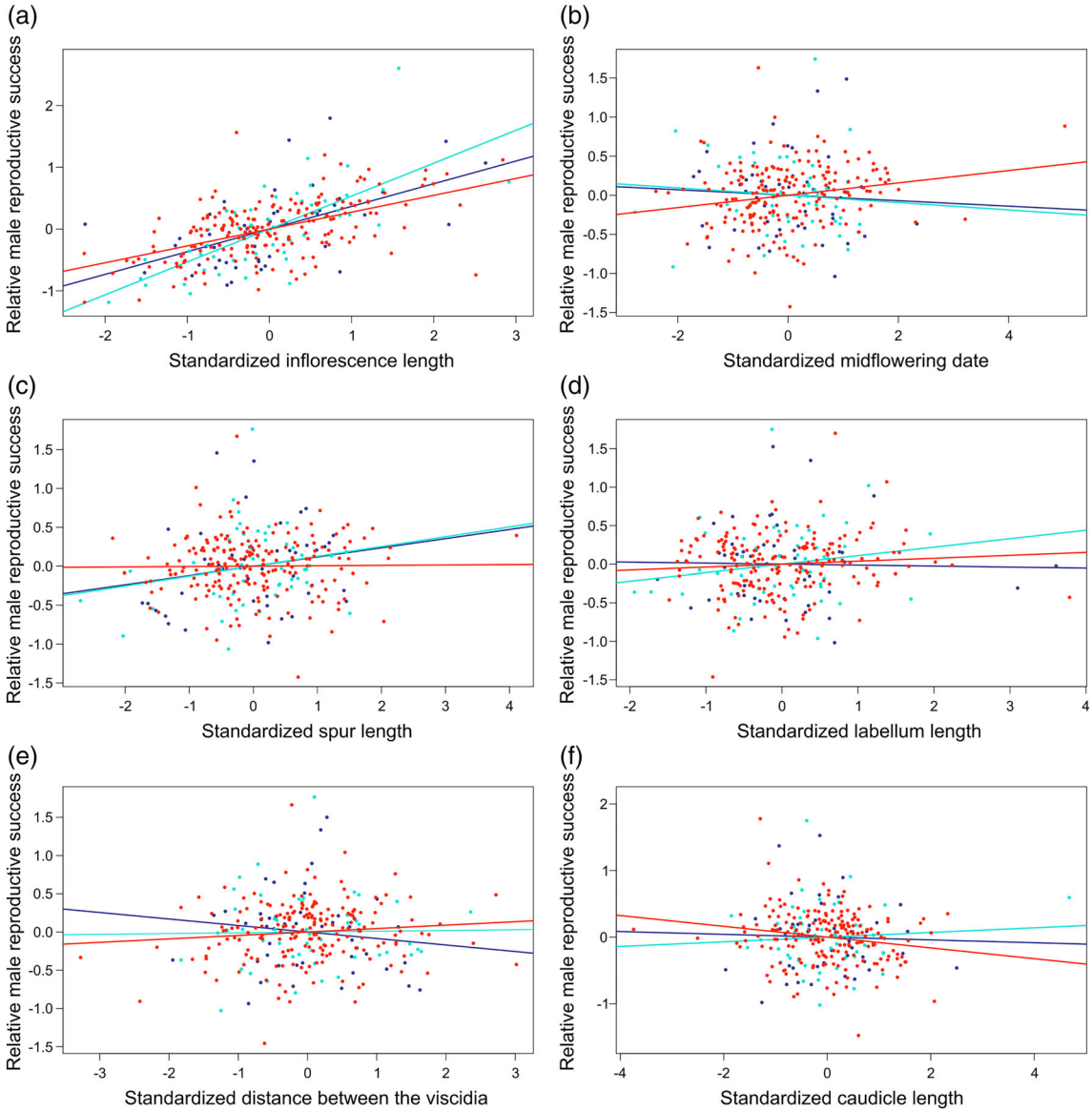

FIGURE 3 Standardized directional selection gradient for: (a) inflorescence length, (b) midflowering date, (c) spur length, (d) labellum length, (e) distance between the viscidia, (f) caudicle length in male pure (blue line and dots) and intermediate (turquoise line and dots) *Platanthera bifolia* and *P. chlorantha* (red line and dots) individuals. Selection gradients are illustrated with partial regression plots, in which the residuals from a linear regression of the relative fitness on all the standardized traits studied, except the focal trait, are plotted against the residual from the linear regression of the standardized focal trait on all the remaining standardized traits. Traits were standardized separately for each phenotype studied

of longer inflorescence was strong and significant for both male and female functions in all the three morphotypes studied. Interestingly, selection gradients on this trait are stronger in the intermediate *P. bifolia* morphotype then in the pure individuals and in *P. chlorantha*. A weak disruptive selection on inflorescence length was observed in both *P. chlorantha* sex functions and in male intermediate morphotypes and female pure *P. bifolia*.

Male and female *P. chlorantha* flowering later were positively selected. In contrast, female intermediate individuals flowering earlier were selected. Spur length and labellum length did not play any significant role in either male or female sex functions.

Interestingly, a strong and significant stabilizing selection was observed in the female intermediate morphotypes on the distance separating the viscidia. No significant selective gradients were observed in any of the three groups studied. A weak negative selective pressure was observed in male *P. chlorantha* on the caudicle length. As observed for the selection differentials, the selective pressures were stronger on the intermediate

TABLE 3 Standardized univariate selection coefficients represented as selection differentials (S′) ± standard deviation (*SD*) in pure and intermediate *P. bifolia* and *P. chlorantha*

Species	*Platanthera bifolia* (pure)	*Platanthera bifolia* (intermediate)	*Plantanthera chlorantha*
Male	S′ ± *SD*	S′ ± *SD*	S′ ± *SD*
Inflorescence length	**0.364 ± 0.080 *****	**0.665 ± 0.085 *****	**0.285 ± 0.029 *****
Midflowering date	−0.041 ± 0.097	−0.231 ± 0.108 *	0.071 ± 0.038
Spur length	0.194 ± 0.108	**0.350 ± 0.117 ****	0.065 ± 0.037
Labellum length	0.046 ± 0.097	**0.419 ± 0.096 *****	**0.122 ± 0.035 *****
Distance between viscidia	0.062 ± 0.097	0.037 ± 0.112	**0.114 ± 0.037 ****
Caudicle length	0.122 ± 0.109	−0.039 ± 0.168	0.012 ± 0.039
Female	S′ ± *SD*	S′ ± *SD*	S′ ± *SD*
Inflorescence length	**0.480 ± 0.111 *****	**0.767 ± 0.119 *****	**0.352 ± 0.041 *****
Midflowering date	−0.008 ± 0.132	**−0.401 ± 0.129 ****	0.131 ± 0.051 *
Spur length	0.216 ± 0.149	**0.405 ± 0.148 ****	0.060 ± 0.050
Labellum length	−0.047 ± 0.132	**0.521 ± 0.120 *****	**0.190 ± 0.046 *****
Distance between viscidia	0.104 ± 0.132	−0.010 ± 0.141	**0.177 ± 0.050 *****
Caudicle length	0.076 ± 0.153	−0.034 ± 0.208	**0.138 ± 0.051 ****

Note: Selection differentials in bold are significant even after Bonferroni adjustment. Significance levels: one star (*) means *p*-value less than .05; two stars (**) means *p*-value less than .01; three stars (***) means *p*-value less than .001.

P. bifolia individuals than on pure *P. bifolia* and *P. chlorantha* morphotypes.

4 | DISCUSSION

Understanding the strength, mode and frequency of selection in the wild is considered an important challenge for evolutionary biologists. It is frequently suggested that selection on floral traits should act primarily through the male function (e.g., Queller, 1983; Stanton, Snow, & Handel, 1986; Wilson, Thomson, Stanton, & Prigney, 1994). In this study, we estimated selection through both sexual functions, aiming to understand whether the presence of individuals with morphological traits intermediate between *P. bifolia* and *P. chlorantha* but genetically belonging to the former species could be explained by a positive selection of *P. chlorantha*-like traits in *P. bifolia* individuals. Overall, we found evidence of phenotypic selection in the two *Platanthera* species and in the intermediate individuals. However, contrary to our prediction, selection on the traits that are more differentiated between the two *P. bifolia* morphotypes (i.e., distance between viscidia and caudicle length) was consistent in the two groups, thus refuting our initial hypothesis.

Our data show a positive directional selection towards longer inflorescence for both *Platanthera* species (Table 4; Figures 3 & 4). Inflorescence length is a primary trait in floral display (Campbell, 1989; Conner, Rush, Kercher, & Jennetten, 1996; Donnelly, Lortie, & Aarssen, 1998; Ehrlén, Käck, & Ågren, 2002; Firmage & Cole, 1988; Schemske, 1980; Schmid-Hempel & Speiser, 1988) and together with plant height was already reported to be under positive selection in other populations of *P. bifolia* (Boberg et al., 2014; Maad, 2000, 2002; Maad & Alexandersson, 2004; Scopece, Schiestl, & Cozzolino, 2015; Sletvold, Grindeland, & Ågren, 2010). This directional selection occurred jointly with a weak disruptive selection for inflorescence length, for both sex functions in pure *P. bifolia* individuals and only for female function in *P. chlorantha*. Disruptive selection is a pattern in which extreme values are favored, potentially leading to a bimodal trait distribution, and in plants has been found to be linked to contrasting selective pressures exerted by

TABLE 4 Standardized multivariate selection coefficients in pure and intermediate *P. bifolia* and *P. chlorantha* represented as directional selection gradients (β_i') and non-linear selection gradients (γ'_{ij}) ± standard deviation (*SD*)

Species	*Platanthera bifolia* (pure)		*Platanthera bifolia* (intermediate)		*Plantanthera chlorantha*	
Male	$\beta'_i \pm SD$	$\gamma'_{ij} \pm SD$	$\beta'_i \pm SD$	$\gamma'_{ij} \pm SD$	$\beta'_i \pm SD$	$\gamma'_{ij} \pm SD$
Inflorescence length	**0.367 ± 0.089 *****	0.129 ± 0.067	**0.533 ± 0.079 *****	**0.118 ± 0.058 ***	**0.273 ± 0.032 *****	**0.050 ± 0.025 ***
Midflowering date	−0.035 ± 0.094	−0.071 ± 0.082	−0.047 ± 0.079	−0.114 ± 0.090	**0.079 ± 0.029 ****	0.031 ± 0.017
Spur length	0.118 ± 0.096	−0.040 ± 0.088	0.126 ± 0.084	−0.054 ± 0.071	0.005 ± 0.031	−0.010 ± 0.022
Labellum length	−0.013 ± 0.090	−0.025 ± 0.038	0.110 ± 0.093	0.147 ± 0.088	0.038 ± 0.036	−0.016 ± 0.021
Distance between viscidia	−0.085 ± 0.106	0.014 ± 0.083	0.010 ± 0.077	−0.117 ± 0.087	0.045 ± 0.032	0.004 ± 0.022
Caudicle length	−0.021 ± 0.099	−0.018 ± 0.059	0.035 ± 0.073	0.006 ± 0.029	**−0.081 ± 0.033 ***	−0.018 ± 0.019
Female	$\beta'_i \pm SD$	$\gamma'_{ij} \pm SD$	$\beta'_i \pm SD$	$\gamma'_{ij} \pm SD$	$\beta'_i \pm SD$	$\gamma'_{ij} \pm SD$
Inflorescence length	**0.489 ± 0.123 *****	**0.190 ± 0.087 ***	**0.541 ± 0.104 *****	0.124 ± 0.069	**0.310 ± 0.044 *****	**0.090 ± 0.033 ***
Midflowering date	0.067 ± 0.130	−0.082 ± 0.107	**−0.241 ± 0.104 ***	−0.072 ± 0.106	**0.094 ± 0.041 ***	−0.003 ± 0.023
Spur length	0.059 ± 0.133	−0.047 ± 0.114	0.133 ± 0.111	−0.094 ± 0.083	−0.040 ± 0.044	−0.040 ± 0.029
Labellum length	−0.155 ± 0.124	−0.074 ± 0.049	0.166 ± 0.123	0.207 ± 0.104	0.074 ± 0.051	−0.034 ± 0.027
Distance between viscidia	0.018 ± 0.146	−0.003 ± 0.108	−0.103 ± 0.102	**−0.266 ± 0.103 ***	0.055 ± 0.045	0.020 ± 0.029
Caudicle length	0.071 ± 0.137	0.068 ± 0.077	−0.037 ± 0.097	−0.027 ± 0.034	−0.004 ± 0.046	−0.022 ± 0.026

Note: Significant values are indicated in bold face: One star (*) means *p*-value less than .05; two stars (**) means *p*-value less than .01; three stars (***) means *p*-value less than .001.

different pollinator species or functional classes (sensu Fenster et al., 2004). However, the weak effect suggests that it can be explained by a weak difference in the distribution of pollinator species (i.e., Noctuid / Sphingid). This hypothesis should be further tested by performing field experiments.

The degree of synchronization between flowering period and pollinator availability may affect pollinator visitation rates (Waser, 1986). A positive directional selection gradient was observed for individuals that flower later in *P. chlorantha* (Table 4) for both sex functions as the capacity of *P. chlorantha* to export its pollinia and to produce fruits was positively correlated with the date of the end of its flowering period. In contrast, in accordance with the study of Maad (2000), early flowering individuals of intermediate *P. bifolia* phenotypes were positively selected for both sex functions (Table 4). Interestingly, pure *P. bifolia* morphotype did not have an advantage from either earlier or later flowering. Apparently, pollinator visitation rates were highest at the beginning of June in our population in 2015. These contrasting results suggest a selective pressure leading to an increase of the flowering overlap between *P. chlorantha* and the intermediate *P. bifolia* morphotype (Figure 2). Indeed, due to different mean flowering dates, the selection on late flowering *P. chlorantha* and early intermediate *P. bifolia* individuals coincides with the overlap of flowering time of the two species, and therefore with a high overall flower abundance, making this period an effective food source for pollinators. However, this pressure can be counterbalanced by the increased risk of waste of gametes in hybrid formation for individuals flowering together with heterospecific individuals (Ramsey, Bradshaw, & Schemske, 2003; e.g., Pegoraro, Cafasso, Rinaldi, Cozzolino, & Scopece, 2016). An estimation of pollinator abundance along the flowering time of the two species would help to disentangle the reasons for the observed selective patterns.

According to previous selection studies conducted on *P. bifolia* (Boberg & Ågren, 2009; Maad, 2000; Maad & Nilsson, 2004), we observed a positive selection differential on spur length in intermediate *P. bifolia* individuals (Table 3). A recent study conducted by Boberg et al. (2014) has shown that spur length within *P. bifolia* is responsive to selection exerted by local pollinator communities. Nonetheless, selection on a longer spur length in *P. bifolia* may be also explained as a consequence of a dry and hot season (as was the case in the year of our observations), as previously documented by Maad (2000) and Maad and Alexandersson (2004). Another trait important for insect attraction (i.e., labellum length) had a strong significant selection differential in both *P. chlorantha* and intermediate *P. bifolia* individuals for both sex functions.

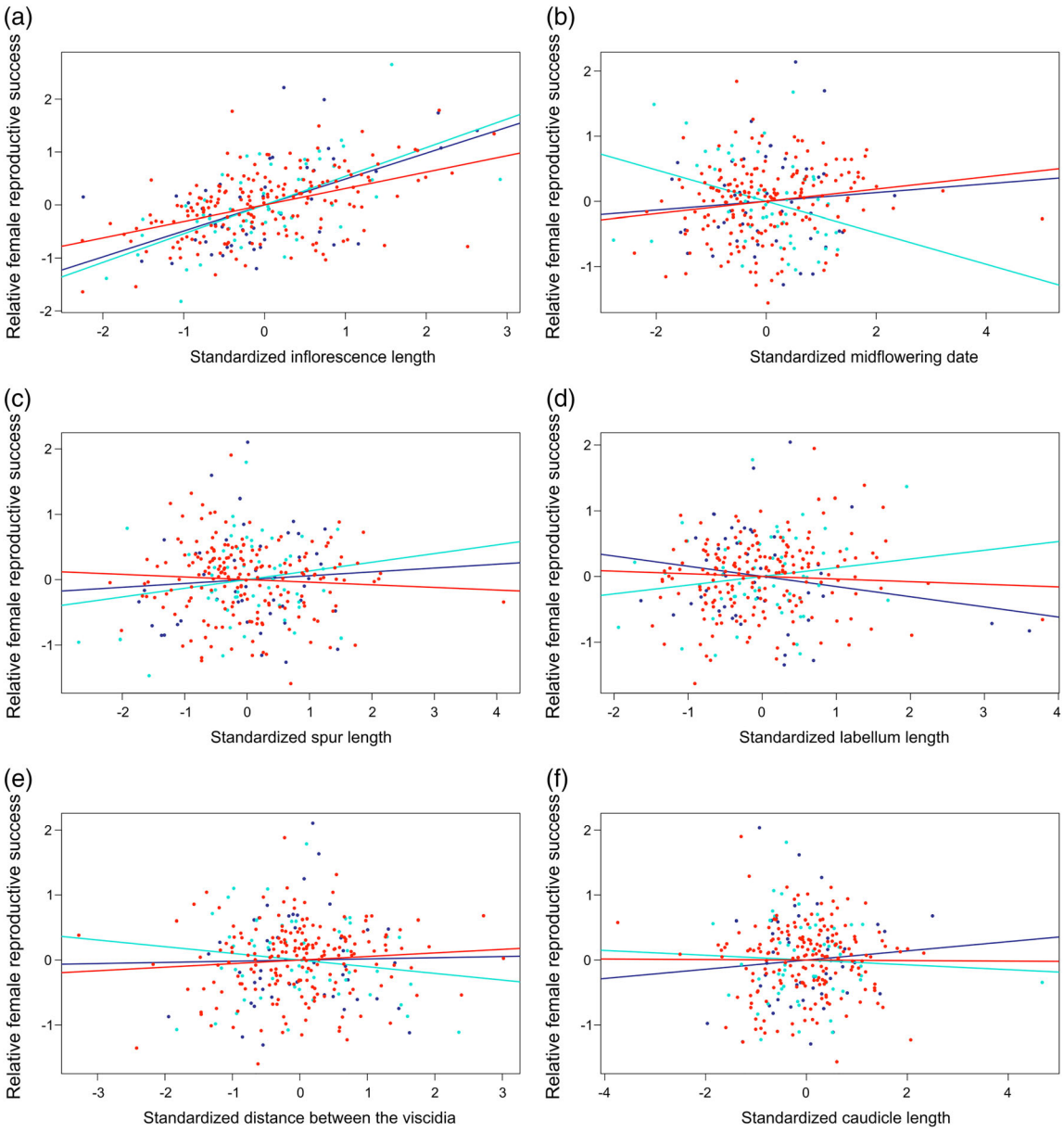

FIGURE 4 Standardized directional selection gradient for: (a) inflorescence length, (b) midflowering date, (c) spur length, (d) labellum length, (e) distance between the viscidia, (f) caudicle length in female pure (blue line and dots) and intermediate (turquoise line and dots) *Platanthera bifolia* and *P. chlorantha* (red line and dots) individuals. Selection gradients are illustrated with partial regression plots, in which the residuals from a linear regression of the relative fitness on all the standardized traits studied, except the focal trait, are plotted against the residual from the linear regression of the standardized focal trait on all the remaining standardized traits. Traits were standardized separately for each phenotype studied

A positive selection differential was also observed on viscidia distance in *P. chlorantha*. This floral trait is already known to affect foraging pollinators, as a bigger stigmatic surface was observed to be linked to an increase in pollen receipt (Maad & Nilsson, 2004). This result could also explain the fact that *P. chlorantha* showed a higher number of fruits compared to *P. bifolia*, because the larger distance between the viscidia in *P. chlorantha* will lead to a lower risk of interference between pollen import and export compared to *P. bifolia* (Boberg & Ågren, 2009; Maad & Nilsson, 2004). Interestingly, a strong and significant stabilizing selective pressure was observed on the distance between the viscidia in intermediate *P. bifolia* individuals for the female sex function and a concordant although not significant trend was observed for the male sex function. This finding suggests that an excessive distance between viscidia may lead to improper attachment of the pollinia on the head of the pollinator, leading to an increase of pollen loss, and

also that intermediate phenotypes experience assortative mating mediated by pollinators.

Despite the evidence of phenotypic selection on several floral traits, we cannot assert our initial hypothesis that the presence of intermediate morphotypes would be the result of an intraspecific adaptive divergence of *P. bifolia* individuals in response to the pressure exerted by local pollinators preferring *P. chlorantha*-like plants (Esposito et al., 2018). Indeed, despite their morphological differences, the two *P. bifolia* morphotypes share common selective trends for spur length and distance between the viscidia.

The basis for the presence of intermediate *P. bifolia* individuals could partially be explained by the pressures on some traits (i.e., labellum length and overlapping flowering time) shared with *P. chlorantha* individuals and different from intermediate *P. bifolia* individuals. However, the selective pressures on those two traits do not contribute to any significant differentiation between the pure and intermediate *P. bifolia* morphotypes.

Bateman et al. (2012) suggested that a single gene might be responsible for variation in column width at the origin of these intermediate individuals. According to this assumption, the expanded column, which characterizes the phenotypic shift from the sympatric *P. bifolia* to intermediate morphotypes, could be due to a limited number of genes. The presence of phenotypically divergent individuals can then be the result of mutations in a few genes. If the distance between viscidia does contribute to assortative mating mediated by the attachment of pollinia on the insect body (as could be suggested by our finding of stabilizing selection in intermediate individuals), in a population dominated by *P. bifolia*, these mutants should experience a low fitness due to the scarcity of potential mates. Under a similar scenario, traits that confer higher attractivity and increase pollination efficiency may be strongly advantageous for the mutants. Interestingly, this prediction is consistent with our results that show directional selection in traits is related to pollinator attraction and pollination efficiency tends to be greater in intermediate than in *P. bifolia* and *P. chlorantha* individuals. An increased attractivity of intermediates may thus compensate for the initial fitness decay, allowing intermediate phenotypes to spread in the population and explaining the observed phenotypic pattern. Another factor that can potentially compensate for the effect of the initial fitness decay may be the pollinator set exploited by the intermediate individuals. Indeed, although we have no data to support this idea, it is possible to hypothesize that intermediate phenotypes may exploit the pollinator sets of both *Platanthera* species (i.e., Sphingid and Noctuids), thus increasing the chances of pollination.

Interestingly, the results of a recent study by Durka et al. (2017) conducted in Germany, the Netherlands and Belgium, revealed the presence of three independent gene pools represented by *P. chlorantha*, *P. bifolia* and plants referred to as non-hybrid intermediates, which although phenotypically intermediate, were not of hybrid origin. In this context, the non-hybrid intermediates would represent the final step of a divergence process from *P. bifolia*, which could be still at an initial development state in our mixed populations, and detectable only through morphology. The existence of pure populations of these non-hybrid intermediates (Durka et al., 2017) would also suggest that they are the result of allopatric phenotypic divergence, either due to selection or to genetic drift. In this context, the sympatric populations investigated here would represent a secondary contact zone. Thus, the lack of genetic differentiation between *P. bifolia* and intermediate individuals observed using neutral markers in the sympatric populations can also be explained as the effect of genetic admixture among recently diverged lineages and can suggest that this taxon is not differentiated enough to overwhelm the challenge of sympatry.

Understanding which of the proposed hypotheses is more likely to explain the presence of intermediate individuals would require further molecular phylogenetic and ecologic investigations across larger spatial scales. Furthermore, the observed patterns may have a site-specific explanation, which was hardly investigated in this one-site research; this is another incentive to perform additional studies over several populations. Also, the study and quantification of reproductive isolation among the two species and the intermediate individuals would help in understanding this intriguing system.

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