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Fluctuating selection across years and phenotypic polymorphism in food-deceptive orchids

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Pollinator-mediated selection is predicted to be among the most important mechanisms determining floral trait evolution in entomophilous species. Orchids are widely used as models to study asymmetric plant-insect interactions because they encompass several thousand species with nectarless flowers that deceive nectar-searching pollinators. High levels of intra-specific phenotypic polymorphism have been reported in deceptive orchids, suggesting a reduced ~~action~~ of pollinator-mediated selection on their floral traits. Nevertheless, several studies report on widespread directional selection mediated by pollinators even in these deceptive orchids. In this study we test the hypothesis that the observed selection can fluctuate across years in strength and direction thus ~~explaining~~ the phenotypic variability of this orchid group. We performed a three-year study estimating selection differentials and selection gradients for nine phenotypic traits involved in insect attraction in two Mediterranean orchid species, namely *Orchis mascula* and *O. pauciflora*, both relying on a well-described food-deceptive pollination strategy. We found overall weak directional selection and marginally significant selection gradients in the two investigated species with significant intra-specific differences in selection differentials across years. ~~Although our~~ data do not link this variation with a specific environmental cause, our results suggest that pollinator-mediated selection in food-deceptive orchids can change in strength and in direction over time. In perennial plants, such as orchids, different selection differentials in the same populations in different flowering seasons can contribute to the maintenance of phenotypic polymorphism often reported in deceptive orchids.

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Short title: Fluctuating selection in orchids

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ABSTRACT

Pollinator-mediated selection is predicted to be among the most important mechanisms determining floral trait evolution in entomophilous species. Orchids are widely used as models to study asymmetric plant-insect interactions because they encompass several thousand species with nectarless flowers that deceive nectar-searching pollinators. High levels of intra-specific phenotypic polymorphism have been reported in deceptive orchids, suggesting a reduced action of pollinator-mediated selection on their floral traits. Nevertheless, several studies report on widespread directional selection mediated by pollinators even in these deceptive orchids. In this study we test the hypothesis that the observed selection can fluctuate across years in strength and direction thus explaining the phenotypic variability of this orchid group.

We performed a three-year study estimating selection differentials and selection gradients for nine phenotypic traits involved in insect attraction in two Mediterranean orchid species, namely *Orchis mascula* and *O. pauciflora*, both relying on a well-described food-deceptive pollination strategy.

We found overall weak directional selection and marginally significant selection gradients in the two investigated species with significant intra-specific differences in selection differentials across years.

Although our data do not link this variation with a specific environmental cause, our results suggest that pollinator-mediated selection in food-deceptive orchids can change in strength and in direction over time. In perennial plants, such as orchids, different selection differentials in the same populations in different flowering seasons can contribute to the maintenance of phenotypic polymorphism often reported in deceptive orchids.

Keywords: food-deceptive orchids; floral traits; fluctuating selection; *Orchis mascula*; *Orchis pauciflora*; phenotypic polymorphism; selection differentials.

INTRODUCTION

Pollinator-mediated selection is one of the main ~~forces~~ driving the evolution of floral traits in entomophilous pollen-limited plant species (Fenster et al., 2004). The typical form of entomophilous pollination is based on a reciprocal advantage: the flower offers a reward (typically pollen or nectar) to the pollinator, which transports the pollen to conspecific individuals ensuring reproduction. However, pollinators are unable to directly assess the amount of nectar reward contained in the flowers and thus depend on the information provided by advertising floral traits (Benitez-Vieyra et al., 2010). Therefore, nectar-producing plants have an advantage in being easily recognisable by pollinators such that once a rewarding flower type is discovered, the pollinators usually concentrate on a single species (flower constancy; Waser, 1986). In these plant-pollinator relationships, plants are continuously exposed to the choice of pollinators, which imposes a selection on flower traits (Schiestl & Johnson, 2013). On the basis of this evidence, strong emphasis has traditionally been given to pollinator-mediated selection in shaping changes in phenotypic floral trait distributions (Fenster et al., 2004) and the development of easily applicable methods to estimate selection differentials in natural populations (Lande & Arnold, 1983), resulting in the common discovery of directional selection on floral traits in many plant systems (Kingsolver et al., 2001; Hereford et al., 2004).

Flower constancy is an important prerequisite for the establishment of strong selection (stabilising or directional); when insects concentrate on phenotypes that they can associate with a reward, they favour individuals that are ~~more recognisable~~ and potentially shape the displacement of the phenotypic distribution of floral traits via the elimination of abnormal phenotypes (Waser, 1986). However, plant-pollinator interactions can be more intricate, and patterns of pollinator-mediated selection may differ in the case of asymmetric and non-constant relationships.

Orchids are widely used as a model to study asymmetric plant-insect interactions because they include many species with nectarless flowers that deceive their pollinators (Schiestl, 2005; Jersàkovà et al., 2006). Among orchid deceptive pollination strategies, the most common is based on a generalised mimicry of floral traits that pollinators associate with the presence of nectar (generalised food-deception, Dafni, 1984). In these deceptively pollinated species, plant-pollinator interactions do not follow ~~the~~ flower constancy behaviour because insects learn to avoid deceptive flowers after a few rewardless visits (Gumbert, 2000; Smithson & Gigord, 2003). This pollinator behaviour can result in ~~a strong~~ selection for high phenotypic variability or in a relaxed selection on floral traits (Juillet & Scopece, 2010) of deceptive orchids, which is also suggested by the high intra-specific phenotypic variability in flower traits that has been observed in this plant group, including colour (Nilsson 1980), shape and size (Ackerman & Galarza-Pérez, 1991), and fragrance (Moya & Ackerman, 1993; Salzmann et al., 2007). In a survey, Ackerman et al. (2011) confirmed that such polymorphism is more common in deceptive compared to nectar-rewarding species, thus suggesting that the maintenance of a high phenotypic variability may be linked with exploitation in deceptive pollination systems (see also Salzmann et al., 2007).

The reasons that underlie the maintenance of high levels of phenotypic polymorphism in deceptive systems were investigated in several studies (see Juillet & Scopece, 2010 and references therein), particularly focussing on the idea, proposed by Heinrich (1975), that phenotypic variability decreases pollinator avoidance learning, thereby increasing orchid reproductive success. Such hypothesis has been recently suggested by Stejskal et al. (2015) for explaining the variation in labellum pattern of sexually-deceptive orchids. However, Juillet and Scopece (2010) showed that all attempts to identify a reproductive advantage linked with polymorphism in food-deceptive species were non-significant or indicated the opposite pattern, thus underlining the need of more studies to support or discard this hypothesis. Other potential causes that could account for higher phenotypic variation in non-rewarding species are negative frequency dependent selection (e.g. Gigord et al., 2001) or, indirectly, differences in demographic history through processes as gene flow, genetic drift and founder events (Knapp & Rice, 1998; Holderegger et al., 2006; Lawton-Rauh, 2008). However, the first was contradicted by several studies that excluded a reproductive advantage of the rarer phenotype (Juillet & Scopece, 2010 and references therein) and the second appears unlikely because polymorphism is a common characteristic of deceptive orchid populations and it is thus difficult to link with historic processes.

Nevertheless, despite high levels of phenotypic polymorphism, there is increasing evidence for directional selection on floral traits in food-deceptive orchids. For example, pollination success was found to be correlated with plant height in *Cypripedium acaule* (O’Connell & Johnston, 1998), to the number of flowers in *Anacamptis morio* (Johnson & Nilsson, 1999), to spur length in the *Disa draconis* species complex (Johnson & Steiner, 1997) and in hybrid zones between *Anacamptis morio* and *A. longicornu* (Zitari et al., 2012), to flowering time in some deceptive orchids (O’Connell & Johnston, 1998; Sun et al., 2009; but see Sletvold *et al.* 2010), to plant height, flower number and spur length in *Dactylorhiza lapponica* (Sletvold et al., 2010) and to flower brightness and contrast in *Anacamptis morio* (Sletvold et al., 2016). This evidence is unexpected, considering the high levels of phenotypic polymorphism seen in deceptive orchids and suggests that, even in this plant group, directional selection mediated by pollinators may be widespread and strong. However, these studies were performed in single flowering seasons and disregarded the fact that across years natural selection can vary in both the strength and direction (Darwin, 1859; Grant & Grant, 1989; Benitez-Vieyra et al., 2012; Sletvold & Ågren, 2014). Temporal variation in patterns of natural selection has been often documented in plants (Harder & Johnson, 2009) and has been linked to variation in the pollinator community (Conner et al., 2003), to the presence of herbivores (Sandring et al., 2007), and to abiotic factors (Maad, 2000; Caruso et al., 2003; Maad & Alexandersson, 2004). Despite this evidence, however, the incidence of seasonal variation on selection patterns in deceptive species has been poorly investigated. By estimating the covariance of pollination success with nine different phenotypic traits, we estimated selection differentials and gradients in two Mediterranean food-deceptive orchid species, *Orchis mascula* and *Orchis pauciflora*. In particular, we estimated the strength and direction of natural selection over three consecutive years in a sympatric population of these two species with the aim of specifically understanding whether, in the same populations, pollinator-mediated selection shows a concordant pattern over different years. We used two orchid species with similar flower morphology and a common set of pollinators (Van der Cingel, 1995; Cozzolino et al., 2006; Nilsson, 2008; Valterovà et al., 2007) as replicates to increase the power of our conclusions.

MATERIALS AND METHODS

Study System

Orchis mascula and *O. pauciflora* are closely related species in the orchid subtribe Orchidinae (Aceto et al., 1999). *O. mascula* is a widespread European species, ranging from Sweden to the northern borders of the Mediterranean basin (Sundermann, 1980). It is typically found in sunny meadows or calcareous grasslands up to 2400 m in altitude. *O. pauciflora* is generally found on poorer calcareous soils in the south-eastern and central part of the Mediterranean basin, up to 1500 m in altitude.

The two species are self compatible but non autogamous and rely on generalised food-deception for pollinator attraction (Van der Cingel, 1995). Hymenopterans are the most common pollinators of these two species (specifically *Bombus* sp., but also *Psithirus*, *Eucera*, *Andrena*, *Osmia*, *Anthophora*), and reproductive success is severely pollen-limited (e.g., Cozzolino et al., 2006). Clonal propagation is extremely rare in both species.

Our study was performed during Spring 2002, 2003 and 2004 in sympatric natural populations of *O. mascula* and *O. pauciflora* located in the Cilento and Vallo di Diano National Park (Southern Italy). For each species, and in each year, plants were randomly selected in subgroups in an area of approximately 4 km^2 where distribution was nearly continuous. We measured a total of 1188 individual[illegible]492 *O. mascula* and 696 *O. pauciflora*; for details see Raw data).

Morphological measurement and data collection

To investigate pollinator-mediated selection, we measured nine morphological traits that are potentially important for pollinator visual attraction. Morphological trait measurements were obtained on the same day and at a time when all the examined plants were at peak flowering. As flower traits have been reported to be slightly variable within orchid inflorescences with stronger deviations occurring most frequently in the lowermost and uppermost flowers (Bateman & Rudall, 2006), flowers were sampled from the mid point of the inflorescence. For each individual that was sampled in this study, we recorded two "inflorescence size" variables: 1) flower number and 2) plant height (from ground to uppermost flower, to the nearest cm). From the same set of individuals, we measured three "flower size" variables: 3) labellum width (distance between the edges of the two lateral lobes, to the nearest 0.1 mm), 4) labellum length (distance between the labellum tip and spur mouth, to the nearest 0.1 mm) and 5) spur length (distance between the spur mouth and the spur tip, to the nearest 0.1 mm). To obtain these measurements, sampled flowers were dissected and floral elements were placed between two transparent plastic film sheets (supplementary figure 1). These sheets were subsequently scanned to obtain digital images in a 300 dpi TIFF format with a coordinate millimetre paper on the back for reference; measures of floral traits were obtained using ImageJ 1.33 software (Rasband, National Institutes of Health, USA). Finally, as previously described by Bradshaw et al. (1998), we also measured the pigment content in flower elements using a spectrophotometric method on the same individuals used for morphometric analyses. Anthocyanin concentration (purple pigment) was estimated from tepals or labellum, extracting the anthocyanins with 0.5-ml methanol/0.1% HCl, and determining the absorbance at 510 nm; carotenoid concentration (yellow pigment) was estimated similarly, using methylene chloride for pigment extraction and measuring absorbance at 450 nm. We thus estimated four “flower colour” variables: 6) carotenoid content in tepals, 7) carotenoid content in labellum, 8) anthocyanins content in tepals and 9) anthocyanins content in labellum.

We estimated pollen limitation (PL) as 1 - (mean female fitness of open-pollinated plants/mean female fitness of hand pollinated plants), ranging from 0 to 1. Female fitness of open pollinated plants was recorded on the same individuals used for traits measurements and was defined as the number of fruits produced by an individual relative to its number of flowers; female fitness of hand pollinated plants was calculated similarly based on literature data reporting results of crossing experiments conducted in the same populations (Scopece et al., 2007).

Data analysis

All analyses were performed independently for the two species and were conducted using R 2.1.1 software (R Development Core Team 2005). To explore the relationship among floral traits, we performed Spearman’s rank correlation. We examined significant differences in floral traits across years using Kruskal-Wallis tests of difference and Tukey HSD posthoc comparison tests. To explore the level of variation of floral traits, for both investigated species, we calculated a coefficient of variation (CV) as the ratio between Standard deviation and mean. CVs were calculated for each trait in the three investigated years and then averaged to obtain a value for each of the two species.

We estimated selection differentials (Lande & Arnold, 1983; Brodie et al., 1995) as the covariance between relative reproductive success and each morphological trait. Plant reproductive success was measured at the end of the flowering period as fruit set, i.e., number of fruit / number of flower, ranging from 0 to 1. The relative reproductive success of an individual was defined as its fruit set divided by the mean population fruit set. Morphological traits were scaled by population mean and standard deviation (z-scores, Lynch & Walsh, 1998). To compare selection differentials over different years, we computed bootstrap bias-corrected confidence intervals (CIs: Maad & Alexandersson, 200[illegible]). Non-overlapping CIs indicated significantly different selection differentials, CIs including 0 were deemed be non-significant.

To estimate selection gradients, following Lande & Arnold (1983), we performed a single multiple linear regression using relative fitness as dependent variable and standardized traits as predictors.

RESULTS

All floral traits were moderately positively correlated with the exception of carotenoid content in tepals and labellum, which were negatively correlated with other phenotypic traits (Table 1). In both species, several floral traits showed significant differences across different years (Fig. 1, Fig. 2).

In both investigated species, CVs were extremely high for all floral traits indicating a high variability of floral traits with a slight variation across years. Average CV was 0.35 in *Orchis mascula* and 0.42 in *Orchis pauciflora* (See Supporting information).

Data from Scopece *et al.* (2007) showed that, in both species, the ratio between hand-pollinated flowers and fruits developed was very high (1 for *Orchis mascula* and 0.92 for *O. pauciflora*), in contrast with fruit formation in open-pollinated individuals, which was low (0.11 in *O. mascula* and 0.10 in *O. pauciflora*). Thus, both species were severely pollen-limited (PL for *O. mascula* = 0.89; PL for *O. pauciflora* = 0.87).

All of the investigated floral traits in both species showed some amount of selection across years (Fig. 1, Fig. 2). A comparison of the selection differentials in three consecutive years revealed significant differences for 7 traits in both *O. mascula* (Fig. 1) and *O. pauciflora* (Fig. 2). Specifically, in *Orchis mascula*, selection differentials were significantly different for 6 out of 9 traits between 2002 and 2003, 3 out of 9 between 2003 and 2004, and 3 out of 9 between 2002 and 2004 (Fig. 1). In *Orchis pauciflora*, selection differentials were significantly different for 2 out of 9 traits between 2002 and 2003, 6 out of 9 between 2003 and 2004, and 2 out of 9 between 2002 and 2004 (Fig. 2).

Across the three years, the selection differential patterns were generally consistent with floral trait variation (i.e., decrease when floral traits increase). In *O. mascula*, the patterns were consistent in 7 out of 9 traits. In *O. pauciflora*, the patterns were consistent in 6 out of 9 traits.

We also found changes in the direction of selection (i.e., signs of selection differential) in 3 out of 9 floral traits in *O. mascula* (Fig. 1) and in 3 out of 9 floral traits in *O. pauciflora* (Fig. 2). Selection gradients for each investigated population are reported in Table 2. Results show only few marginally significant values indicating weak direct selection on floral traits.

DISCUSSION

The quantification and interpretation of the direction, strength and causes of natural selection have been at the centre of scientific debate since the formulation of Darwin's theory (Darwin, 1859). Among the main aims of studies on selection is the capture of snapshots and milestones in the process of phenotypic trait evolution mediated by natural selection. Thus far, many studies have shown selection ~~in action on~~ different animal or plant organisms (Kingsolver et al., 2001; Hereford et al., 2004), generating the idea that directional selection is widespread in natural populations. This idea is confirmed by the general stability of phenotypic traits observed in rewarding plant species, but it apparently conflicts with the elevated polymorphism observed in deceptive species (Ackerman et al., 2011). In this study, we estimated selection differentials and gradients for nine floral traits in two Mediterranean food-deceptive orchid species, *Orchis mascula* and *O. pauciflora* over three consecutive years. Overall, we found some degree of selection on all of the investigated traits but a strong variation in direction and intensity over different years (Fig. 1, Fig. 2). As expected in deceptive orchids (Tremblay et al., 2005), we found that the two investigated species showed high levels of pollinator limitation. Although pollen limitation is not predictive of pollinator-mediated selection, in severely pollen-limited species the strength of selection is mainly due to the action of pollinators (Sletvold et al., 2010; Sletvold & Agren, 2014). Despite increasing evidence showing pollinator-mediated selection in Mediterranean food-deceptive orchids (Johnson & Steiner, 1997; O'Connell & Johnston, 1998; Johnson & Nilsson, 1999; Sun et al., 2009; Sletvold et al., 2010; Zitari et al., 2012; Sletvold et al., 2016), our results showed a weak and variable selection on floral traits when analysed over different, consecutive seasons suggesting the absence of constant directional selection on these species. ~~This can~~ potentially be responsible for the elevated phenotypic polymorphism that we directly assessed in the investigated species through the calculation of coefficients of variation for morphological traits that were on average higher than those reported in previous literature surveys on deceptive orchid species (i.e. 0.35 in *O. mascula* and 0.42 in *O. pauciflora versus* an average of 15.2% in the deceptive species and of 11.1% for the rewarding species reported in Ackerman et al., 2011). Indeed, we found that selection differentials significantly varied at least once in direction or strength in 7 out of 9 traits in both *O. mascula* and *O. pauciflora* over different years (Fig. 1, Fig. 2) with a total of 12 changes out of 27 comparisons in *O. mascula* (Fig. 1) and 10 changes out of 27 in *O. pauciflora* (Fig. 2). In both investigated species, most of the studied floral traits were positively correlated (Table 1), which could in principle mask the action of natural selection in the selection differential analysis (Lande & Arnold, 1983). However, our analysis of selection gradients showed only a few marginally significant results, thus confirming the weak selection observed in selection differentials (see Table 2).

Floral traits were not constant over different years and most of these traits showed significant differences in different reproductive seasons (Fig. 1, Fig. 2). In several cases, selection differentials were consistent with this variation; e.g., in *O. mascula*, plant height was under stronger selection when plants were less tall (Fig. 1), thus suggesting a weak directional selection over the years. However, for other traits, selection differentials were inconsistent with this variation (Fig. 1, Fig. 2), which suggests that fluctuations of natural selection for these traits are not an artefact but rather reflect the activities of different selective agents in different years.

Significant differences among floral traits in different years, within the same population, may be explained as a consequence of phenotypic plasticity. Indeed, in a variable environment, a single generalist genotype can potentially express a wide range of random phenotypes or show different responses to environmental cues via phenotypic plasticity (Kawecki & Ebert, 2004; Hill & Mulder, 2010). In our study, this can be accentuated by the extreme climatic conditions of the 2003 heat wave (Beniston, 2004). Interestingly, in 2003, measurements showed a quantitative reduction of most floral traits (Fig. 1, Fig. 2). However, despite the contribution of plasticity on the phenotypic expression of the investigated floral traits, their heritability was verified by a comparison of phenotypic expression of traits in hybrid zones (Scopece et al., ~~in prep.).~~ Fluctuating selection has been proposed to be common in natural populations (Siepielski et al., 2009; Kimball et al., 2012), but its potential role is still debated particularly due to the dearth of extensive multi-year studies. In our study, with a multi-year dataset, we showed that the change in the direction and intensity of selection on the same floral traits could determine a continuous but fluctuating pressure that favours different individuals in different years. Within the same population, this can potentially result in the maintenance of variable phenotypes. Indeed, although changes in selective pressures in annual plant species may generate a displacement of phenotypic traits, in perennial long-lived plants such as orchids, the individual's reproductive success is the result of its performance during its lifetime and thus only selection ~~pressures~~ that are constant over time can generate displacement or stabilisation of the distribution of phenotypic traits. In contrast, fluctuating ~~pressures~~ are more likely to explain the phenotypic polymorphism observed in the natural populations of deceptive orchids. The main source of this fluctuating selection is most likely the action of pollinators. In the nectar-rewarding orchid *Gymnadenia conopsea* spatio-temporal variation in interactions with pollinators contributes to among-years and among-populations variation in selection on floral traits but that several traits are also likely to be subject to different selective agents (Sletvold & Ågren, 2010; Chapurlat et al., 2015). In our study, we focused on severely pollen-limited food-deceptive species in which pollinators are likely to be the main selective agents (Tremblay et al., 2005; Sletvold et al., 2010; Sletvold & Ågren, 2014). Furthermore, direct estimation based on the comparison of reproductive performance between open-pollinated and hand-pollinated plants confirmed elevated levels of pollinator limitation for the two investigated species (PL for *O. mascula* = 0.89; PL for *O. pauciflora* = 0.87). Pollinator-mediated selection is a complex process that can be affected by a high number of environmental variables. For example, pollinators with different tongue lengths may exert different selection pressures that positively select flowers with shorter or longer nectar spurs (Johnson & Steiner, 1997). However, pollinator community varies over the flowering season and in different years depending on climatic differences that can alter both the phenology of plants and emergence of pollinators. Moreover, for similar reasons, the surrounding plant community may change in different years, thereby influencing local pollinator preference and abundance. The change in pollinators or surrounding plant community composition is particularly crucial for generalist deceptive species such as *O. mascula* and *O. pauciflora*, which attract a wide range of available pollinator species, and is the most likely source of the observed changes in selection differentials. Changes in pollinator-mediated selection have been widely documented in plant species, including orchids, and have been attributed to many environmental variables (e.g., Caruso et al., 2003; Conner et al., 2003; Gòmez et al., 2003; Toräng et al., 2008; Sletvold & Ågren, 2010). However, identifying the source of variation in selection differentials can only be achieved via detailed community ecological studies at the local scale. For instance, similar attempts have previously shown that vegetation height affects the strength of pollinator-mediated selection in the food-deceptive orchid *Dactyloriza lapponica* (Sletvold et al., 2013), thus suggesting that variation in selection also occurs within the same reproductive season at small geographic scales.

Future research avenues should address the basis of the elevated phenotypic polymorphism of food-deceptive species and confirm whether similar variation occurs even at a fine geographic scale. Such studies should include fine scale community ecological investigations, as for instance temporal and local variation in pollinator community, that aim to disentangle the factors affecting variation. Simultaneously, it would also be important to conduct more studies on nectar-rewarding orchids to test for the opposite pattern, i.e., lower variability of selection pressures in space and time.

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Figure legends

Figure 1 Morphological traits (white bars) and selection differentials (grey bars) in *Orchis mascula*. Different letters indicate significant differences.

Figure 2 Morphological traits (white bars) and selection differentials (grey bars) in *O. pauciflora*. Different letters indicate significant differences.

Supplementary figure 1 Scanned digital images of A) *O. pauciflora* and B) *O. mascula* floral parts. From left to right: spur, labellum.

Figure 1(on next page)

Morphological traits and selection differentials in Orchis mascula

Morphological traits (white bars) and selection differentials (grey bars) in *Orchis mascula*. Different letters indicate significant differences.

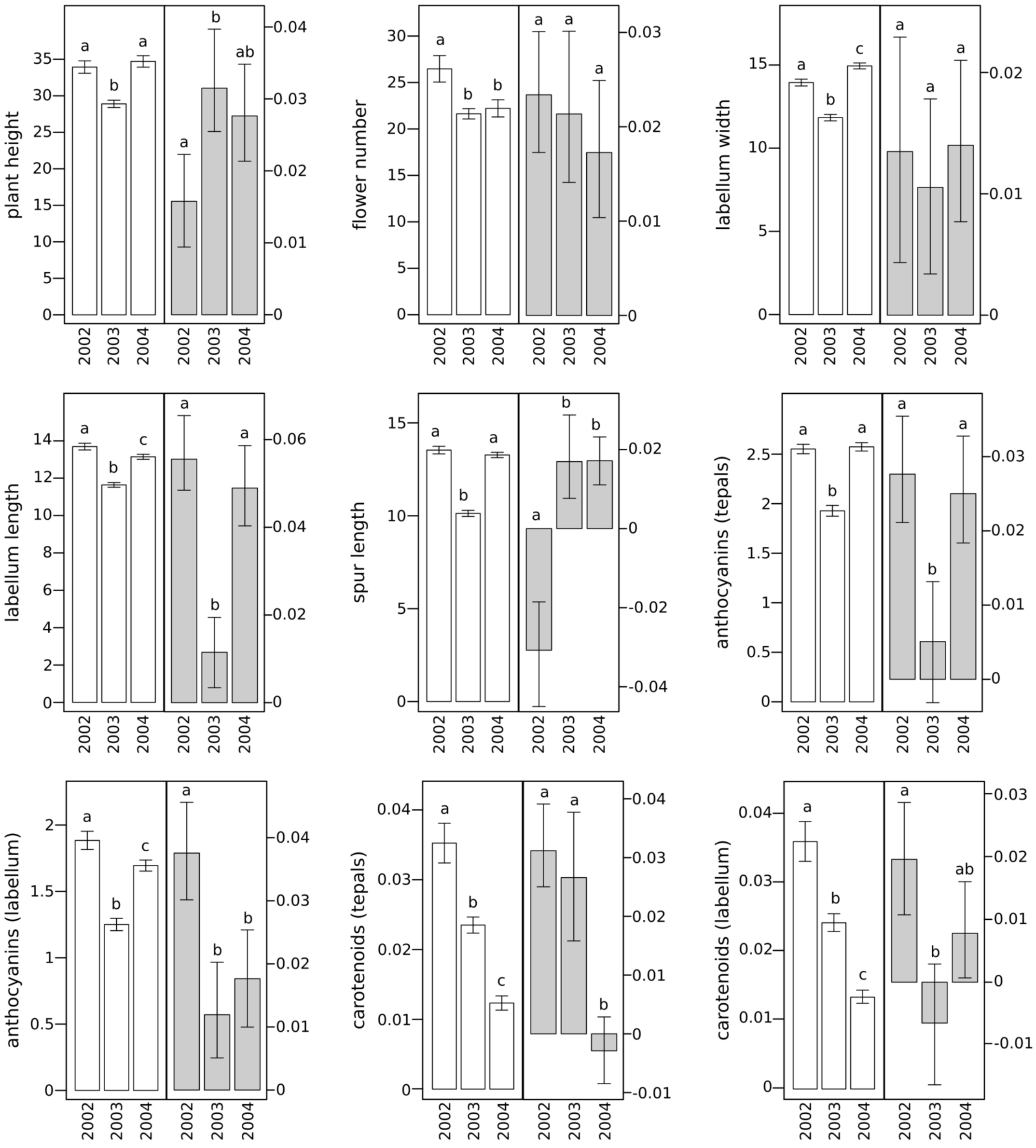
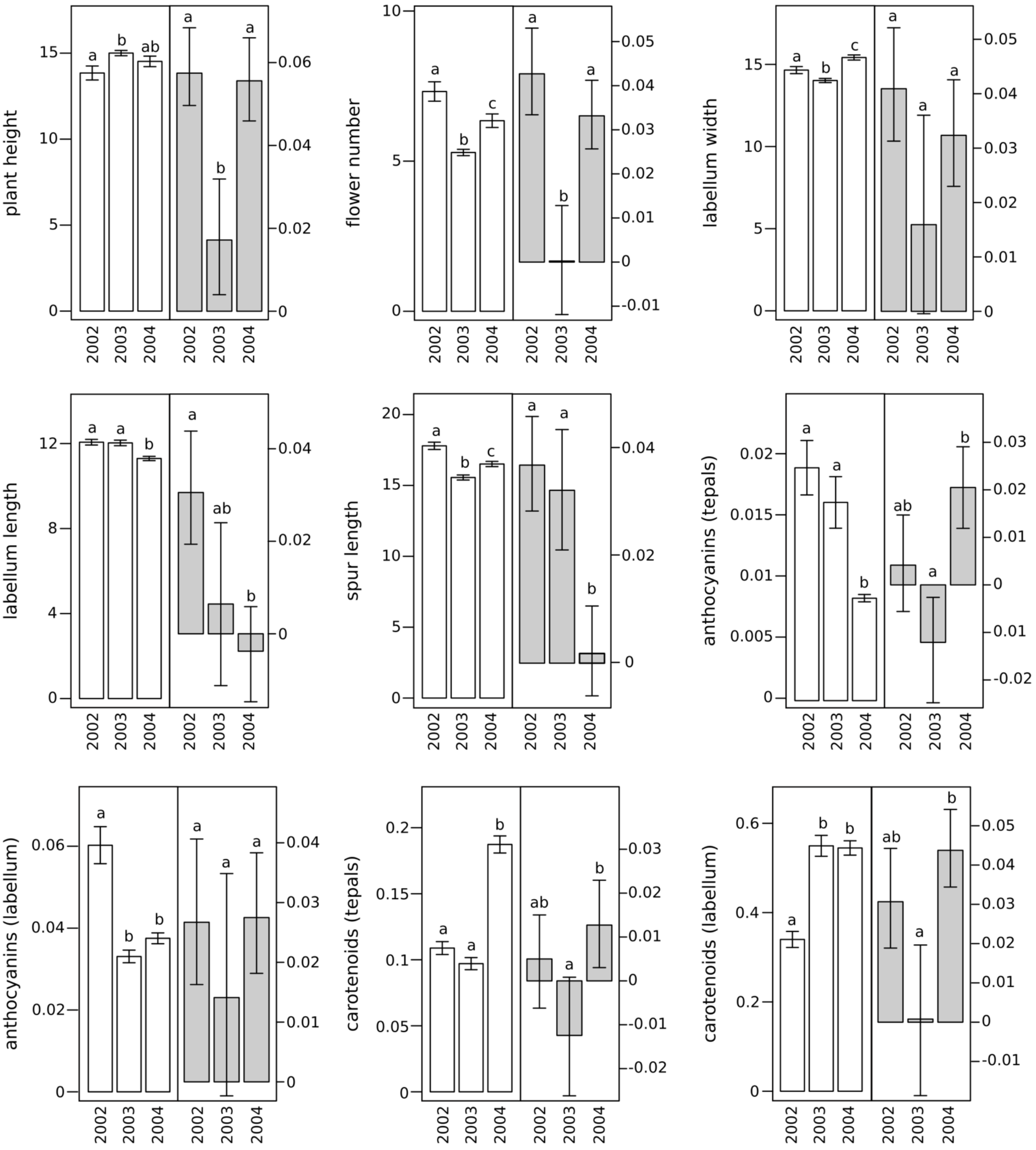

plant height
0 5 10 15 20 25 30 35
0 0.01 0.02 0.03 0.04
a b a a b ab
flower number
0 5 10 15 20 25 30
0 0.01 0.02 0.03
a b b a a a
labellum width
0 5 10 15
0 0.01 0.02
a b c a a a
labellum length
0 2 4 6 8 10 12 14
0 0.02 0.04 0.06
a b c a b a
spur length
0 5 10 15
-0.04 -0.02 0 0.02
a b a a b b
anthocyanins (tepals)
0 0.5 1 1.5 2 2.5
0 0.01 0.02 0.03
a b a a b a
anthocyanins (labellum)
0 0.5 1 1.5 2
0 0.01 0.02 0.03 0.04
a b c a b b
carotenoids (tepals)
0 0.01 0.02 0.03 0.04
-0.01 0 0.01 0.02 0.03 0.04
a b c a a b
carotenoids (labellum)
0 0.01 0.02 0.03 0.04
-0.01 0 0.01 0.02 0.03
a b c a b ab
2002 2003 2004 2002 2003 2004

Figure 2(on next page)

Morphological traits and selection differentials in O. pauciflora.

Morphological traits (white bars) and selection differentials (grey bars) in *O. pauciflora*. Different letters indicate significant differences.


plant height
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15
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a
b
ab
a
b
a
flower number
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5
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-0.01
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0.01
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a
b
c
a
b
a
labellum width
0
5
10
15
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0.01
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0.04
0.05
a
b
c
a
a
a
labellum length
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4
8
12
0
0.02
0.04
a
a
b
a
ab
b
spur length
0
5
10
15
20
0
0.02
0.04
a
b
c
a
a
b
anthocyanins (tepals)
0
0.005
0.01
0.015
0.02
-0.02
0.01
0
0.01
0.02
0.03
a
a
b
ab
a
b
anthocyanins (labellum)
0
0.02
0.04
0.06
0
0.01
0.02
0.03
0.04
a
b
b
a
a
a
carotenoids (tepals)
0
0.05
0.1
0.15
0.2
-0.02
-0.01
0
0.01
0.02
0.03
a
a
b
ab
a
b
carotenoids (labellum)
0
0.2
0.4
0.6
-0.01
0
0.01
0.02
0.03
0.04
0.05
a
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ab
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2002
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Table 1(on next page)

Phenotypic Correlations

Phenotypic Correlations (Spearman’s rank) among morphological traits in *Orchis mascula* (above diagonal) and *O. pauciflora* (below diagonal). All plants from the three years were pooled together.

Table 1 Phenotypic Correlations (Spearman’s rank) among morphological traits in *Orchis mascula* (above diagonal) and *O. pauciflora* (below diagonal). All plants from the three years were pooled together.

	Plant heigth	Flower number	Labellum width	Labellum length	Spur length	Anthocyanins content in tepals	Anthocyanins content in labellum	Carotenoid content in tepals	Carotenoid content in labellum
Plant heigth		0.76***	0.47***	0.48***	0.44***	0.29***	0.25***	0.07	-0.07
Flower number	0.51***		0.25***	0.30***	0.24***	0.15**	0.14**	0.15**	0.06
Lebellum width	0.32***	0.28***		0.71***	0.69***	0.39***	0.48***	-0.16**	-0.09
Labellum length	0.26***	0.22***	0.49***		0.63***	0.40***	0.50***	0.01	0.06
Spur length	0.22***	0.10*	0.44***	0.14**		0.47***	0.50***	-0.05	-0.05
Anthocyanins content in tepals	0.16**	0.19***	0.08	0.22***	0.01		0.66***	-0.01	-0.08
Anthocyanins content in labellum	0.18***	0.42***	0.31***	0.24***	0.08	0.45		-0.01	0.01
Carotenoid content in tepals	-0.09*	0.21***	0.19***	-0.03	-0.05	-0.05	0.18***		0.43***
Carotenoid content in labellum	0.24***	0.19***	0.30***	0.10*	0.00	0.18***	0.25***	0.32***	

***, P < 0.001; **, P < 0.01; *, P < 0.05.

Table 2(on next page)

Selection gradients

Selection gradients (ß) and their significance (p) calculated according to Lande and Arnold (1983).

Table 2 Selection gradients (ß) and their significance (p) calculated according to Lande and Arnold (1983)

	Flower number		Plant height		Labellum width		Labellum length		Spur length		Anthocyanins content in tepals		Anthocyanins content in labellum		Carotenoid content in tepals		Carotenoid content in labellum	
	ß	p	ß	p	ß	p	ß	p	ß	p	ß	p	ß	p	ß	p	ß	p
O. mascula 2002	0.440	0.020	-0.203	0.269	-0.231	0.140	0.462	0.002	-0.217	0.065	0.172	0.212	0.084	0.518	0.066	0.680	0.149	0,369
O. pauciflora 2002	0.030	0.853	0.281	0.138	-0.040	0.817	0.005	0.970	0.095	0.476	-0.038	0.734	0.184	0.118	0.030	0.793	0.005	0,969
O. mascula 2003	-0.119	0.389	0.173	0.248	0.018	0.865	0.070	0.545	0.065	0.590	-0.033	0.759	0.028	0.791	0.245	0.005	-0.062	0,477
O. pauciflora 2003	-0.132	0.157	0.135	0.152	0.017	0.850	0.101	0.198	0.041	0.646	-0.080	0.330	0.095	0.291	-0.016	0.841	-0.018	0,847
O. mascula 2004	-0.063	0.664	0.196	0.223	-0.258	0.032	0.538	0.000	-0.076	0.453	0.150	0.155	-0.102	0.359	-0.119	0.195	0.021	0,810
O. pauciflora 2004	-0.122	0.316	0.414	0.002	0.295	0.030	-0.411	0.001	-0.101	0.321	0.039	0.695	0.030	0.771	-0.054	0.586	0.187	0,087