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The search for Pleiades in trait constellations: functional integration and phenotypic selection in the complex flowers of *Morrenia brachystephana* (Apocynaceae)

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correlational evolution;
floral integration;
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plant–pollinator interaction;
pollination.

Abstract

Pollinator-mediated natural selection on single traits, such as corolla tube or spur length, has been well documented. However, flower phenotypes are usually complex, and selection is expected to act on several traits that functionally interact rather than on a single isolated trait. Despite the fact that selection on complex phenotypes is expectedly widespread, multivariate selection modelling on such phenotypes still remains under-explored in plants. Species of the subfamily Asclepiadoideae (Apocynaceae) provide an opportunity to study such complex flower contrivances integrated by fine-scaled organs from disparate developmental origin. We studied the correlation structure among linear floral traits (i) by testing *a priori* morphological, functional or developmental hypotheses among traits and (ii) by exploring the organization of flower covariation, considering alternative expectations of modular organization or whole flower integration through conditional dependence analysis (CDA) and integration matrices. The phenotypic selection approach was applied to determine whether floral traits involved in the functioning of the pollination mechanism were affected by natural selection. Floral integration was low, suggesting that flowers are organized in more than just one correlation pleiad; our hypothetical functional correlation matrix was significantly correlated with the empirical matrix, and the CDA revealed three putative modules. Analyses of phenotypic selection showed significant linear and correlational gradients, lending support to expectations of functional interactions between floral traits. Significant correlational selection gradients found involved traits of different floral whorls, providing evidence for the existence of functional integration across developmental domains.

Introduction

Several studies have shown the extent and importance of the variation on individual flower traits and consequent effects on plant fitness (Nilsson, 1988; Herrera, 1993; Campbell *et al.*, 1996; Johnson & Steiner, 1997; Alexandersson & Johnson, 2002; Medel *et al.*, 2003; Moré *et al.*, 2012). Selective pressures exerted on floral traits have been primarily tested on trait sets that do not necessarily imply functional coordination. Such sets have included traits related to flower attractiveness, such as the magnitude of floral display, together with traits related to adjustments to pollinators, for example flower tube length (Gómez, 2000; Maad, 2000; Herrera, 2001; Maad & Alexandersson, 2004; Armbruster *et al.*, 2005; Sletvold & Agren, 2010). However, it is expected that natural selection also acts on more complex phenotypes involving several flower parts, which covary coordinately. Thus, selective pressures should be exerted not only on mean and variance of single flower traits (i.e. directional or stabilizing/disruptive selection)

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but also on the correlation between trait pairs that functionally cooperate for pollen receipt and export (i.e. correlational selection). Correlational selection persisting over several generations will result in a flower phenotype with one or more subsets of highly correlated flower traits associated with a particular function ('pleiad'; Berg, 1959, 1960; Conner, 2002; Herrera *et al.*, 2002; Armbruster *et al.*, 2004, 2005).

Although studies have lent support to the hypothesis that floral traits are targets of pollinator-mediated natural selection, the hypothesis of correlational selection has received weak support (O'Connell & Johnston, 1998; Benítez-Vieyra *et al.*, 2006, 2009; Cuartas-Domínguez & Medel, 2010; Benítez-Vieyra *et al.*, 2012). In fact, according to a recent review (Harder & Johnson, 2009), only 18 of 145 correlational selection estimations yielded significant gradients. According to a more recent review (Roff & Fairbairn, 2012), this proportion has been slightly improved where 21 of 70 estimations on correlational selection in plants yielded significant gradients. This proportion is puzzling because it is expected that correlational selection should be one of the most commonly applied modes of selection (Schluter & Nychka, 1994). The cause of the infrequent detection of significant correlational estimations could be based on the statistical difficulties emerging from the estimation of selection on combinations of traits (Cuartas-Domínguez & Medel, 2010; Roff & Fairbairn, 2012), methodological difficulties in quantifying complex flower structures (Benítez-Vieyra *et al.*, 2009), strong integration preventing the detection of correlational selection (Blows *et al.*, 2003) or the difficulties in recognizing functional interplay between traits.

As opposed to plants, studies on animals recognize functional, often mechanical, interplay among traits to perform a certain function such as flight performance (Lande & Arnold, 1983), defence against predators (Calsbeek & Irschick, 2007) and fighting for or the attraction of mates (McGlothlin *et al.*, 2005). This may be due to our comparatively more limited knowledge of flower mechanical functions. Nevertheless, flower mechanisms can be elaborate in a way that is no less surprising than in animals. In most cases, selection of traits for the study of correlational selection in plants is not based on *a priori* knowledge of how selected traits contribute to a certain function. In other words, studies are not settled on the premise that traits expected to be under correlational selection are engaged in functional relationships.

The studies of mechanical interaction between flowers and pollinators often have focused on rather coarse adjustments between the body of the pollinator and the size of flowers. Whether there could also be fine-scaled interactions of flower parts with insect appendages has not been considered. The coarse-scaled scope may have also limited the search for functional interplay between flower parts and possibly has provided an additional explanation for the paucity of available information about selection of correlated traits.

Flowers of the subfamily Asclepiadoideae (Apocynaceae) are among the most complex of the Angiosperms (Kunze, 1991) and offer the opportunity to study complex phenotypes made up by organs from different developmental origins. Most Asclepiadoideae species show a relative uniform flower structure with a marked synorganization of flower parts and evolved organs. This includes remarkable functional designs as the corona, gynostegium and pollinaria, which interact in a complex mechanism operated by insects (Wiemer *et al.*, 2011). The corona is a sterile whorl additional to the corolla, covering the fertile structures (Fig. 1a). The gynostegium is an organ built up by the post-genital fusion of androecium and gynoecium, which is differentiated into five sectors, each representing a pollination unit, which is comprised of a guide rail, a stigmatic chamber and the receptive area (Kunze, 1996; Fig. 1b). In species such as the one studied here, this guide rail consists of a double guide rail system, with a basal inner section, the *inner guide rail*, which runs parallel and partially overlaps an apical external rail section, the *outer guide rail* (Wiemer *et al.*, 2011; Fig. 1b). Each guide rail section has an independent entrance defined by basally diverging edges. The pollinarium is the pollen dispersal unit. It is constituted by two pollinia from adjacent anthers, which are linked by a solid bridge of stigmatic secretions, the translator apparatus. This apparatus consists of a corpusculum and two caudicles (Fig 1c). The corpusculum is a rounded solid structure with a vertical groove continuous with the guide rail line (Fig. 1b,c). The caudicles are two slender flexible arms, which attach the pollinia laterally to the corpusculum (Fig 1c; Wiemer *et al.*, 2011).

Pollination takes place when an insect pursuing nectar (Fig. 1d) first gets caught by the outer guide rail, which forces the insect to move its trapped body part upwards, subsequently reaching the corpusculum. The corpusculum then clamps the pollinarium onto an insect, as its wedge-shaped median groove is able to become fastened to a pollinator's appendage or to a caudicle, similar to how a nautical clamp cleat would fasten a rope. When the same insect subsequently visits other flowers, one pollinium at a time is inserted into a new inner guide rail and becomes sliced by sharp edges at the end of the rail, completing the pollination process (Kunze, 1991; Liede, 1996; Ollerton & Liede, 1997; Wiemer *et al.*, 2011). During the process of insertion of a pollinium into an inner guide rail, the corresponding corpusculum of the recipient flower may become fastened onto the caudicle of the just-inserted pollinarium, thus building a chain (Fig. 1e). Up to two pollinaria may become attached to a previously picked up pollinarium, and several pollinaria may be successively linked dichotomously in branched tree-like complexes, a process called concatenation (Fig. 1e; Harder

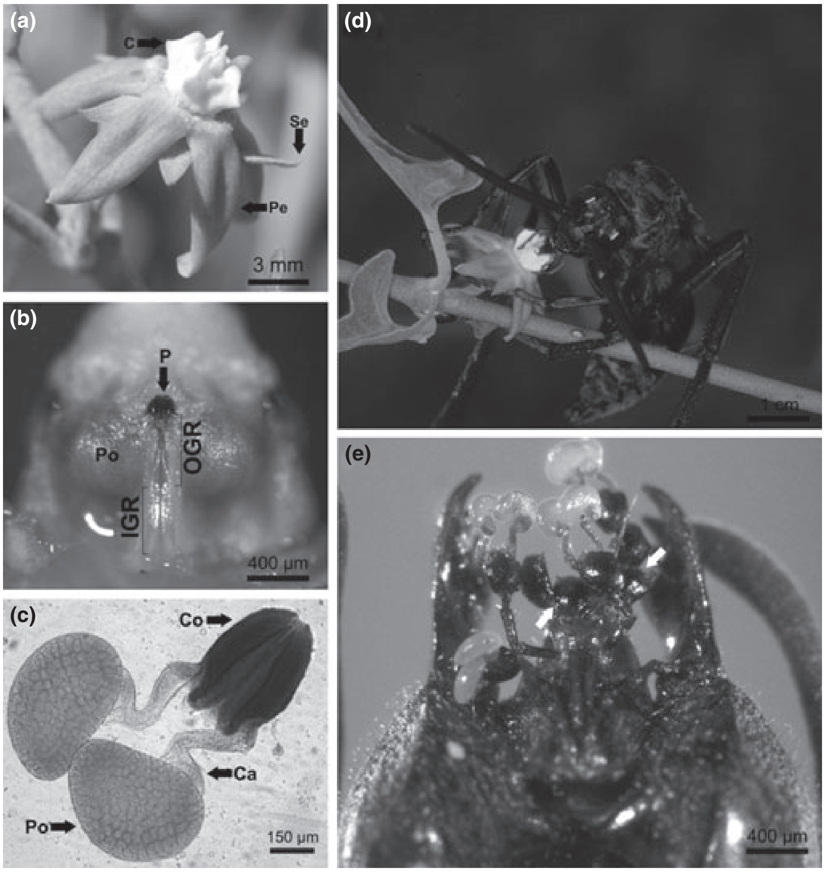

Fig. 1 Depiction of the studied system. (a) External lateral view of an undissected flower of *Morrenia brachystephana* showing the corolla, corona and the emerging tip of the gynostegium. (b) Lateral view of the partially removed corona exposing the gynostegium and showing one of five double guide rail system. (c) Pollinarium showing corpusculum, caudicle and pollinia. (d) The spiderhawk *Pepsis sp.* probing for nectar around the gynostegium. (e) Dorsal view of the mouth parts of *Brachygastra lecheguana* showing pollinaria of *M. brachystephana* on the glossa. Arrows indicate pollinaria chains formed by successive and dichotomous concatenations from one initial pollinarium. Abbreviations: petal (Pe), sepal (Se), corona (C), gynostegium (G), pollinarium (P), pollinia (Po), outer guide rail (OGR), inner guide rail (IGR), corpusculum (Co), Caudicule (Ca), pollinia (Po).

& Johnson, 2008; Wiemer *et al.*, 2011; Coombs *et al.*, 2012). Therefore, the guide rail in pollinarium transfer has two functions: first, the retention of the pollinarium by the inner guide rail; and second, the slicing of one caudicle. The function of the outer guide rail consists in gripping a pollinator appendage or the caudicle of a pollinarium transported by the pollinator and subsequently, guiding either of these structures to the corpusculum. Consequently, the inner guide rail is expected to influence female fitness by its ability to retain pollinia whereas the outer guide rail and the corpusculum, either independently or coordinately, are expected to impact male fitness by promoting the gripping of pollinaria to insect appendages or to other pollinaria. In addition, traits favouring pollinia retention should enhance male fitness by favouring pollinarium exportation through concatenation. It is expected, then, that a certain degree of correlation between those traits involved in the pollination mechanism would favour reproductive success (i.e. correlational selection). It should be stressed that pollinaria of different flowers may have the opportunity to be transported to other flowers in two instances: either directly attached to the body of an insect or attached to the caudicle of another pollinarium. Both these possible attachment locations suggest that the flower parts involved in the pollination mechanism may be subjected to selective pressure mediated by both the pollinators and by the adjustment between corpusculum and caudicle, which are involved in the concatenation process.

From the previous statements, the following hypotheses can be drawn: as floral traits are concurrently involved in pollinarium transfer, they are integrated in one or more functional modules. The integration of floral traits in a functional module results from their covariation, being promoted through selection that is dependent from their function in the pollen transfer mechanism and independent from their developmental origin. If selective pressures have an impact on plant fitness, under these hypotheses, the following predictions can be made: (i) correlations will be higher within modules of traits associated by their function in pollen importation and exportation than across these modules and (ii) correlational selection gradients between floral traits taking part in a functional module should be stronger than those between trait pairs taking part in different modules. To test these predictions, a floral phenotypic study on *Morrenia brachystephana* Griseb., a wasp-pollinated South American asclepiad was carried out. We made linear measurements of floral traits with and without relevance in the mechanical interplay in the function of pollen exportation and importation to explore: first, their correlation structure and second, the phenotypic selection patterns in relation to female and male fitness.

Materials and methods

Samples and study site

Along a transect, we randomly selected 125 flowering plants, which were checked from September to December 2009, in a population located in Rafael Garcia, Córdoba, Argentina (31°37′5.51″S 64°16′41.61″W). From each plant, we randomly harvested three recently opened, unvisited flowers to measure the floral traits. We photographed each whole flower and its gynostegium using a Leica M420 stereomicroscope (Leica Microsystems Ltd., Heerbrugg, Switzerland); one pollinarium from each flower was mounted on glycerine jelly and photographed with a Zeiss Axiophot microscope (Karl Zeiss, Oberkochen, Germany).

Floral variables

Traits related to the visual attraction of the pollinators (floral display) were measured: length and width of the petals (At1 and At2) and height and width of the corona (At3 and At4, Fig. 2a). Traits related to the visual attraction of the pollinators (floral display) were measured: length and width of the petals (At1 and At2) and width and height of the corona (At3 and At4, Fig. 2a). Traits related to pollinaria transfer were measured; in the guide rail, the width and length of the outer guide rail (Gy1 and Gy5), width and length of the inner guide rail (Gy2 and Gy4), the width of the upper edge of the inner guide rail (Gy3; Fig. 2b). In the pollinarium, the maximum width of the groove of the corpusculum (Co1), the width and length of the corpusculum (Co2 and Co3), the width of the caudicules at proximal and distal positions (Ca1 and Ca2), the length of caudicles (Ca3), and the width and length of the pollinium (Po1 and Po2; Fig. 2c) were measured. Measurements were taken on the scaled images with IMAGETOOL v.1.27 (University of Texas Health Science Center).

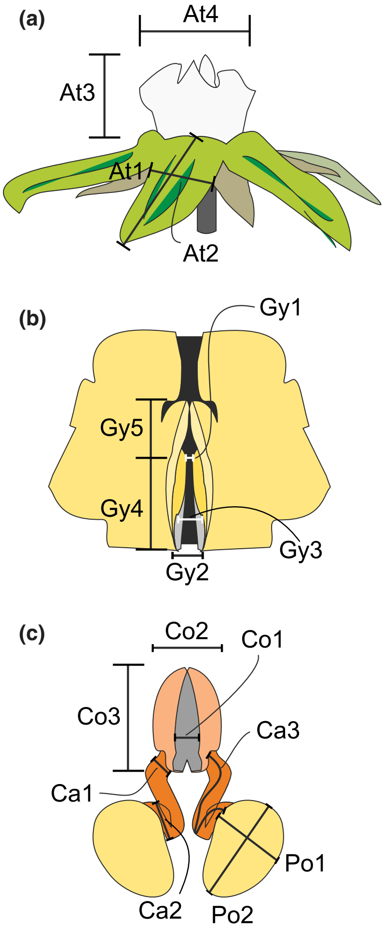

Fig. 2 Measured floral traits on a flower of *Morrenia brachystephana*. (a) Corolla and corona. (b) Gynostegium. (c) Pollinarium. The same colour indicates the same developmental origin. Abbreviations: length and width of the petals (At1 and At2), width and height of the corona (At3 and At4), width and length of the outer guide rail (Gy1 and Gy5), width and length of the inner guide rail (Gy2 and Gy4), width inner guide rail at level of upper edges (Gy3), width of the groove of the corpusculum (Co1), width and length of the corpusculum (Co2 and Co3), width of the caudicules at proximal and distal positions (Ca1 and Ca2), the length of caudicles (Ca3), and the width and length of the pollinium (Po1 and Po2).

Male and female reproductive success

To measure reproductive success, 15–35 senescent, open-pollinated flowers per individual (total $N = 3626$) were harvested. Fitness was quantified in two ways: (i) male fitness, as determined by the average of pollinaria exported per flower from the total number of flowers collected per individual and (ii) female fitness, as determined by the average number of pollinia receipt per flower from the total collected per individual.

Covariation structure analysis

First, the correlation phenotypic matrix for the population was calculated. Then, the magnitude and statistical significance of the whole flower integration (INT) was explored, using the eigenvalue variance of the phenotypic correlation matrix (Wagner, 1984; Cheverud *et al.*, 1989; Herrera *et al.*, 2002) for the mean value per plant of the 17 flower traits. This measure reports the global integration of a group of traits. Each eigenvalue represents the amount of variation accounted for by a given principal component. A low variance among eigenvalues indicates that the variation among all eigenvalues is equally divided between them (i.e. weak association among traits); on the other hand, a high variance among eigenvalues indicates high degrees of integration, as most of the phenotypic variation is accounted for by the first principal components (i.e. strong association among traits; Ordano *et al.*, 2008). ITN index ranges from 0% to 100%, at zero, traits are globally independent, and at 100, they cannot vary independently from each other. The standard deviation and confidence intervals of INT were obtained by bootstrapping 1000 times the dataset. The integration index was considered to reflect significant floral integration if its 95% confidence interval did not include zero. This analysis was performed using a routine programmed in R software v2.15.0 (R Development Core Team 2012).

Patterns of modularity were explored through two approaches: first, with the method proposed by Cheverud *et al.* (1989) whereby a hypothetical correlation matrix between traits is correlated with the empirical matrix. We developed three hypothetical matrices, filling the correlation matrix with values of 1 or 0 when there were or were not expectations of correlations between traits, respectively. The first matrix was built according to a morphological criterion, that is, considering units that could be distinguished as independent organs, filling with high correlations for those trait pairs that belong to distinct morphological organs of the pollination mechanism. Thus, this matrix presents expectations of correlation of traits within the corolla, the corona, the guide rail and the pollinarium. This matrix lacks expectations of correlations between traits across these flower organs (hypothetical matrix 1, see Appendix S1). The second matrix was built according to a functional criterion, placing high correlations between traits that take part in the function of attraction, pollen export or receipt and pollinium measures. Four functional modules are hypothesized for this matrix: (i) visual attraction, including all corona and corolla traits, (ii) pollen export, including all traits of the translator apparatus and outer guide rail system, (iii) pollen receipt, including all inner guide rail traits and (iv) pollinium, including the two pollinium traits. We expected these pollinium traits to not be integrated with the functions of export and receipt because they do not interact with these functions in the same flower and are rather targets of other nonmechanical instances of selection, such as the number of offspring (hypothetical matrix 2, see Appendix S1). Alternatively, a third matrix was proposed following developmental criterion, which assumed high correlations between traits of the same developmental domain (i.e. corolla, corona, androecium and gynoecium). This matrix can be seen as a null model in regard to functional hypothesis (Herrera *et al.*, 2002; hypothetical matrix 3, see Appendix S1). This analysis was performed using *vegan* package in R software v2.15.0.

Cheverud's method employs statistical tests of *a priori* hypotheses when previous knowledge of trait interaction is available. Even if any of our hypothetical models are found to fit relatively well, caution in their interpretation is still required, given that other untested models may be equally parsimonious or significant (Magwene, 2001). For a second approach in the study of modularity patterns, the method of conditional independence developed by Magwene (2001) was used. Contrary to Cheverud's method, based on correlations that assume dependence among traits, this method uses a null hypothesis of independence of traits (Magwene, 2001). The graph shows the relationship between traits that remain linked after controlling for shared correlations between traits. To this end, the phenotypic correlation matrix was inverted and then scaled, resulting in a matrix of partial correlations for the dataset. The matrix of partial correlations was then tested for significance and strength of edges. This analysis was performed using a routine programmed in R software v2.15.0.

Phenotypic selection analysis

Floral traits from the detected intrafloral modules were selected taking the hypothetical correlation matrices that best fit the empirical matrix into consideration. We evaluated whether phenotypic selection was currently operating on these traits at the population level. It was of particular interest to explore whether the modular structure agreed with the floral functions that were expected to jointly or separately impact fitness through both male and female components. Prior to the phenotypic selection analysis, we determined how much of the total variation is attributed to variation within flowers, among flowers in an individual and among individuals in the population because only the latter is informative for phenotypic selection analysis. To this end, a subsample of 10 individuals was taken, for which traits in three pollinaria and three guide rails per flower in three flowers per individual were measured. A nested mixed model analysis was then performed separately for each trait, which tested the restricted maximum-likelihood estimation differences between hierarchical levels, that is, individuals, flowers within individuals and guide rail/pollinarium within flowers (Zuur *et al.*, 2009). For this analysis, the *lme* routine of *nlme* package of R software v2.15.0 was utilized. As within-flower variation was relatively low, subsequent measurements were performed on only one guide rail and one pollinarium per flower as representative of the whole flower, and in three flowers as representative of one individual.

The opportunity for selection for both sexual functions, *I*, and selection gradients acting in the population was estimated applying standard procedures (Lande & Arnold, 1983; Brodie *et al.*, 1995). Because residuals from regression analyses departed from normality, standard errors for selection gradients were calculated using bootstrap methods (Gross *et al.*, 1998). The 10 000 bootstrap samples from the original data set were generated. Selection gradients, estimated after each bootstrap, were used to obtain a frequency distribution. A selection gradient was considered significant if the bias-corrected confidence percentile intervals (90%, 95%, 99% and 99.9%) did not include zero (Moré *et al.*, 2012). We used the *boot* package of R software v2.15.0 (Canty & Ripley, 2011). For these analyses, we standardized the floral traits to zero mean and unit variance. The individual values of fitness measures were divided by the population mean. We performed cubic spline nonparametric regression to depict univariate associations with fitness for traits were shown to be significantly affected by phenotypic selection. The *gam* routine of *mgcv* package of R software v2.15.0 was used to estimate the cubic splines (Wood, 2006; Moré *et al.*, 2012). Smoothing parameters were obtained by minimizing the generalized cross-validation scores (Wood, 2008), and Bayesian standard errors were obtained according to Wood (2006). Finally, we visualized correlational selection acting on two traits plotting fitness surfaces using the correlational gradients in R software v2.15.0 with the corresponding parametric function of the Lande & Arnold (1983) model.

Results

Variation and covariation among traits and correlation structure

Among the 17 traits measured, those associated with the pollinarium transfer mechanism (i.e. pollinarium and guide rail) had higher coefficients of variation than those that were not associated with this mechanism (i.e. corona and corolla). Specifically, floral traits, such as the width and length of guide rail system, width of the caudicle, and the groove of the corpusculum showed the highest coefficients of variation among all traits (Table 1). Correlations among traits, though generally significant, were weak, with 93% of the coefficients lower than 0.60. The highest coefficient of correlation corresponded to the two pollinium traits, the length and width of pollinia (Po1 and Po2; $r = 0.86$), and the widths of the inner guide rail (Gy3 and Gy2; $r = 0.63$).

Integration of the whole flower, while significant, was very low (INT% = 4.08, INT = 0.73 ± 0.12, $P < 0.0001$). The association test of the empirical matrix with the three hypothetical matrices was weak and not significant for either of the morphological and developmental hypotheses (rMorphological = 0.0769; $P = 0.193$; rDevelopmental = 0.0823; $P = 0.1824$). However, it was higher and significant for the functional hypotheses ($r_{\text{Functional}} = 0.2047$; $P = 0.021$). Results of the conditional dependence analysis revealed three modules (Fig. 3) that partially agree with the functional hypothesis of the previous analysis, stressing that modules are constructed by traits of different developmental origin. One of these modules included traits relevant to the functions of visual attraction and pollinia receipt, held by the relatively strong association among petals, corona and double guide rail. A second module included traits related to pollinarium export, mainly of the corpusculum, caudicle and inner guide rail. The third module was represented by the pollinium traits (Fig. 3).

Phenotypic selection analysis

For the pollinator-mediated phenotypic selection analysis, only traits that were consistent both with the detected modules and the functional hypothesis were included. Assuming that the pollinarium receipt and export functions have separate impacts on male and female fitness components and that the visual attraction function would impact both fitness components, we built two selection models. The first model (export model) included floral traits that interact during the process of pollinaria export and that we expected would be selected through male fitness, that is, corpuscle length (Co3), caudicle length (Ca1) and length of the outer guide rail (Gy4; Fig. 2). The second model (receipt model) included floral traits that interact during pollinia receipt and that we expected would be selected through female fitness, that is, length and width of the inner guide rail (Gy4 and Gy2, respectively) and width of the upper edge of inner guide rail (Gy3; Fig. 2). In both models, we included the corona width (At1) representing the visual attraction function, which is independent from mechanical interactions between flower and pollinator and is expected to promote both the export and receipt functions.

The nested analysis of variance showed significant variation among individuals (from 43% to 85% depending on the traits), indicating that the assumptions for natural selection have been met for these traits. From the total senescent flowers analysed to assess flower fitness, 58.19%, 29.31%, 10.02%, 1.97%, 0.32% and 0.02% exported none, one, two, three, four or five pollinaria, respectively. Regarding female fitness, pollinia receipt was low, because 88.10%, 10.25%, 1.4%, 0.22% and 0.02% receipt none, one, two, three or four pollinia, respectively. No flower showed pollinia in all five receptive sectors. The opportunity for selection was higher in male than in female functions, with $I = 0.06$ for the number of exported pollinaria and $I = 0.02$ for the number of pollinia receipt. These two fitness measures were highly correlated across the population ($r = 0.59$; $P < 0.001$).

Table 1 Mean values, standard deviations (SD), coefficients of variation (CV) and Pearson's correlation coefficients for 17 *Morrenia brachystephana* floral traits.

				Pearson's correlation coefficients																
Floral traits	Mean	SD	CV	At1	At2	At3	At4	Gy1	Gy2	Gy3	Gy4	Gy5	Co1	Co2	Co3	Ca1	Ca2	Ca3	Po1	Po2
Petal length (At1)	6.84	0.62	9.03	–	0.35***	0.35***	0.43***	−0.09	−0.05	−0.08	0.31***	−0.10	0.21*	0.22**	0.27**	0.24*	0.26***	0.31***	0.17	0.21**
Petal length (At2)	2.35	0.14	5.92		–	0.38***	0.19*	−0.29**	−0.05	−0.09	0.25**	−0.24*	0.15	0.33***	0.18*	0.31***	0.21*	0.17	0.06	0.14
Corona width (At3)	2.73	0.12	4.36			–	0.26***	−0.14	−0.08	−0.07	0.26***	−0.01	0.23*	0.13	0.09	0.12	0.22*	0.27**	0.08	0.15
Corona height (At4)	1.67	0.16	9.86				–	0.03	−0.07	−0.11	0.40***	0.00	0.10	0.04	0.15	0.24*	0.57***	0.21*	0.22*	0.37***
OGR width (Gy1)	0.07	0.01	11.00					–	0.07	−0.02	−0.13	0.35***	−0.07	−0.16	−0.16	−0.12	−0.03	−0.17	0.10	0.07
IGR width (Gy2)	0.19	0.03	13.55						–	0.63***	−0.01	0.03	−0.14	−0.02	−0.03	−0.14	−0.11	0.10	0.00	0.02
Upper edge IGR width (Gy3)	0.09	0.02	19.32							–	−0.01	−0.02	−0.30***	0.01	0.10	−0.22*	−0.07	−0.08	−0.06	−0.09
IGR length (Gy4)	0.53	0.05	9.93								–	−0.48***	0.16	0.21*	0.22*	0.19*	0.32***	0.27**	0.19*	0.25**
OGR length (Gy5)	0.42	0.04	10.04									–	0.06	0.11	0.27**	0.11	0.46***	0.14	0.23*	0.29**
Groove corpusculum (Co1)	0.09	0.01	13.37										–	0.41***	0.10	0.10	0.05	0.32***	0.00	0.07
Corpusculum width (Co2)	0.19	0.01	7.59											–	0.48***	0.26**	0.12	0.23*	−0.12	−0.10
Corpusculum length (Co3)	0.26	0.02	7.08												–	0.24*	0.26**	0.18*	−0.01	0.01
Proximal caudicle width (Ca1)	0.06	0.01	11.08													–	0.29**	0.26	0.04	0.13
Distal caudicle width (Ca1)	0.04	0.01	10.91														–	0.15	0.21*	0.33***
Caudicle length (Ca3)	0.32	0.03	8.60															–	−0.01	0.07
Pollinium width (Po1)	0.21	0.02	8.08																–	0.86***
Pollinium length (Po2)	0.30	0.02	7.31																	–

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

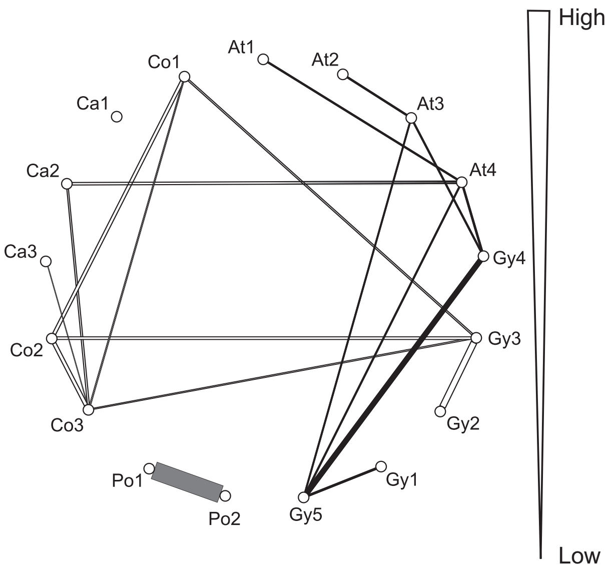

Fig. 3 Conditional independence graph showing the relationship between floral traits in *Morrenia brachystephana*. The width of the lines corresponds to the strength of the dependence. Attraction and pollen receipt module in black, pollen export module in white and pollen load in grey. Abbreviations are in Fig. 2.

For the export model, positive directional selection gradients were significant throughout both male and female fitness for floral display, corpuscle length and the outer guide rail length (Table 2). This means that selection favoured pollen export and receipt in plants that have large coronas, as well as corpuscula and long outer guide rails (Appendix S2). In this model, correlational selection was found acting through the two fitness measures (Table 2). In the male function, the combination of corpuscle length and outer guide rail length was favoured. The thin-plate spline fit indicated that individuals with high values of these traits exported more pollinaria (Fig. 4a). Also, the trait pair corpuscle and caudicle length revealed a marginally significant correlational gradient (Table 2). This trait combination revealed significant correlational selection with female fitness. Inspection of the thin-plate spline fit showed that individuals with long corpuscles and caudicles attained the highest pollinia receipt (Fig. 4b).

For the receipt model, directional selection gradients were significant through both male and female fitness for floral display and the inner guide rail length (Table 2). Selection favoured individuals with large coronas (positive gradient) and shorter inner guide rails (negative gradient; Appendix S2). Correlational selection was found acting only through female fitness (Table 2). The combination of inner guide rail length and inner guide rail upper edge width was favoured. The thin-plate spline fit indicated that individuals with low values of these traits received more pollinia (Fig. 4c).

Discussion

Variation and covariation in floral traits

Flower integration analysis showed that the flowers of *M. brachystephana* had a significant but very low integration value (4.04%) and much lower than expected from a distribution of randomly generated and disintegrated hypothetical organisms (21.5%; Ordano *et al.*, 2008). The integration value is even lower than those found for plants with generalized pollination systems (Ordano *et al.*, 2008; Rosas-Guerrero *et al.*, 2011). This low integration index does not agree with Berg's (1960) proposal that specialized flowers are expected to be more highly integrated. Our findings support the more recent idea (Ordano *et al.*, 2008) that whole flower integration values should not be expected to be high when a flower has to perform many functions in which different trait sets are involved. Indeed, our results suggest the existence of intrafloral modules and that flowers seem to be organized in more than just one floral correlation pleiad.

As phenotypic integration is expected to be the combined result of historical, physiological, developmental and adaptive processes (Armbruster & Schwaegerle, 1996; Armbruster *et al.*, 2004; Pérez *et al.*, 2007), competing hypotheses accounting for some of these processes were tested. However, in the present study, functional integration of flower traits appeared to be more important than integration of developmental components, as revealed by the matrix association test, which showed significant correlation of the empirical matrix with a functional hypothesis and not with developmental or purely morphological hypotheses. In the same line, the conditional analysis revealed three major modules linking traits that interact during the pollen export and receipt functions, ignoring their developmental origin. In the same analysis, floral display traits were also included in the pollen receipt module, suggesting that attractiveness is more critically linked to female rather than to male flower function.

The low integration index could have certain adaptive value if we consider that flowers of the Asclepiadoideae are subjected to multiple selective pressures such as the mechanical coordination of sexual functions (pollen export and receipt) and the attraction of pollinators. Thus, it would be unlikely that all flower traits covary optimally for all flower functions; consequently, under selective pressures affecting traits in different directions, the global flower integration is diluted in favour of intrafloral suits of covarying floral trait constellations (Pérez-Barrales *et al.*, 2007; Ordano *et al.*, 2008). This is consistent with phenotypic selection analyses, which demonstrated that selection exerted pressures on subsets of traits in addition to individual traits, but not on the whole floral phenotype (see Maad, 2000; Benítez-Vieyra *et al.*, 2006). The covariation

Table 2 Multivariate phenotypic selection through male and female functions in *Morrenia brachystephana* on floral trait sets for export and receipt models.

						Traits j		
Selection model	Sexual function	Reproductive success	Traits i	β_i (SE)	γ_{ii} (SE)	Corpusculum length γ_{ij} (SE)	Caudicle length γ_{ij} (SE)	OGR length γ_{ij} (SE)
Pollen export model	Male	Pollinarium export/flowers	Corona width	0.050 (0.022)*	0.049 (0.041)	0.015 (0.029)	−0.047 (0.029)	−0.010 (0,028)
			Corpusculum length	0.068 (0.023)**	0.038 (0.032)	–	0.044 (0.025)†	0.063 (0.020)*
			Caudicle Length	0.013 (0.023)	0.088 (0.039)	–	–	0.055 (0.029)
			OGR length	0.068 (0.024)**	0.057 (0.031)	–	–	–
	Female	Pollinarium receipt/flowers	Corona width	0.030 (0.013)**	−0.024 (0.023)	−0.015 (0.017)	0.004 (0.016)	0.022 (0.016)
			Corpusculum length	0.041 (0.013)***	0.008 (0.018)	–	0.032 (0.015)*	0.018 (0.012)
			Caudicle Length	0.023 (0.014)	0.002 (0.022)	–	–	0.018 (0.017)
			OGR length	0.044 (0.013)***	0.043 (0.018)	–	–	–

						Traits j		
Selection model	Sexual function	Reproductive success	Traits i	β_i (SE)	γ_{ii} (SE)	IGR length γ_{ij} (SE)	IGR width γ_{ij} (SE)	Upper edge of IGR width γ_{ij} (SE)
Pollen receipt model	Male	Pollinarium export/flowers	Corona width	0.079 (0.024)**	0.013 (0.052)	−0.001 (0.031)	−0.013 (0.032)	−0.001 (0.034)
			IGR length	−0.097 (0.029)**	> 0.001 (0.038)	–	−0.003 (0.032)	0.011 (0.033)
			IGR width	0.022 (0.027)	0.013 (0.053)	–	–	−0.044 (0.047)
			Upper edge of IGR width	−0.016 (0.028)	> 0.001 (0.059)	–	–	–
	Female	Pollinarium receipt/flowers	Corona width	0.042 (0.014)**	−0.020 (0.030)	−0.011 (0.017)	0.013 (0.019)	−0.029 (0.020)
			IGR length	−0.054 (0.017)**	0.003 (0.022)	–	−0.029 (0.020)	0.040 (0.019)*
			Total guide rail length	0.023 (0.017)	−0.016 (0.030)	–	–	0.003 (0.027)
			Upper edge of IGR width	−0.014 (0.013)	−0.008 (0.034)	–	–	–

$n = 125$ individuals. †$P < 0.1$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Standardized directional selection gradients (β_i). Stabilizing/disruptive selection gradients (γ_{ii}) correlational selection gradients (γ_{ij}) and standard errors (SE) are indicated. Linear and nonlinear selection gradients were estimated in separate regressions.

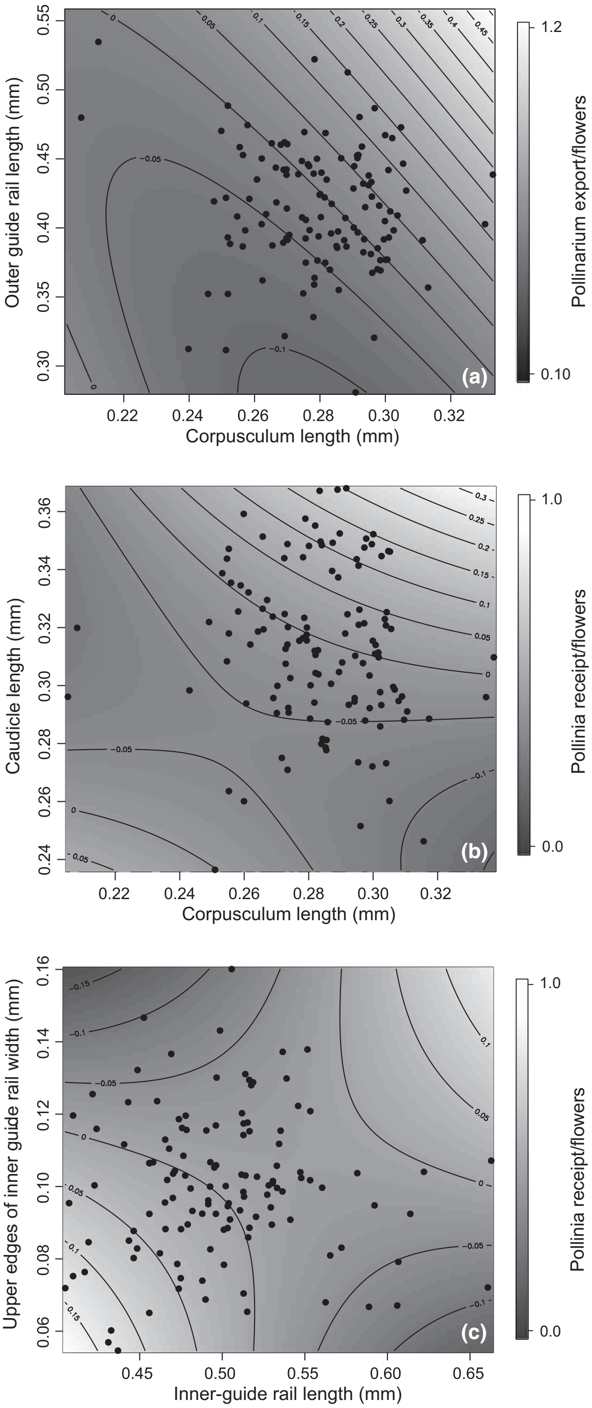

Fig. 4 Selection surfaces between floral traits and fitness in the *Morrenia brachystephana* studied population. (a) Male success (pollinarium export) as a function of corpuscle length and outer guide rail length. (b) Female success (pollinia receipt) as a function of corpuscle length and caudicle length. (c) Female success (pollinia receipt) as a function inner guide rail length and the width of the upper edge of the inner guide rail.

patterns, the INT index and the modularity analyses lend support to the hypothesis that selection may promote the establishment of subsets of functional traits that covary more coordinately among them than with traits involved in the other functions, which results in a low but significant global integration index (Berg, 1960; Herrera *et al.*, 2002).

Natural selection

The study of phenotypic selection on floral traits of Asclepiadoideae has allowed us to explore the incidence of the selective pressures on two different sexual components (Morgan & Schoen, 1997; Maad, 2000). Most previous studies focused only on female fitness, due to the difficulties in evaluating male fitness in most Angiosperms (Armbruster *et al.*, 2005; Nattero *et al.*, 2010; Sletvold & Agren, 2010). We were particularly interested in determining whether these two fitness components had an impact on the selection of flower features in agreement with predictions based on our knowledge of their mechanical functioning in pollen export and receipt. We also expected that, even fine-scaled features, could be targets of selection, as they are engaged in mechanical interaction with small-sized appendages of the insect pollinators.

A positive directional selection was found, strongly acting through male and female fitness on a floral display trait (corona width) in the two selection models. This is consistent with the fact that a large display is more effective at attracting pollinators, critically affecting both functions (Willson & Rathcke, 1974; Broyles & Wyatt, 1995). In other words, the successful attraction of pollinators would favour fitness through both its components.

For a functional module including a set of traits acting in pollen export, both directional and correlational selection gradients were detected. Corpusculum length was subjected to positive directional selection through male and female fitness in favour of larger corpuscula. Selection on this pollinarium trait was as expected for its mechanical role in this model. Previous knowledge in the mechanical role of this pollinarium part in pollen export (Wiemer *et al.*, 2011) suggested that the advantage of a prominent corpusculum lies in that it can more efficiently clasp onto both insect appendages and caudicles of concatenated pollinaria, thus increasing pollen export efficiency. Positive directional selection on the outer guide rail length was also detected through both male and female fitness. Selection on this trait was also expected because of its role in this model: long outer guide rails were favoured suggesting that this state of the trait would allow the guide rail to better capture caudicles or appendages of insects, consequently increasing pollinaria export. It is not obvious why these traits were also impacted through the female fitness component, because we do not see any way in

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which corpusculum or outer guide rail may improve retaining pollinia in the stigmatic chamber, that is, they do not mechanically interact with an incoming pollinarium to capture a pollinium. However, we should expect that fitness is correlated between both sexual functions, that is, individuals that are more successful in exporting pollinaria are also those which are more successful in receiving them. Such a correlation was previously reported for several species of *Asclepias* (Wyatt, 1976; Morse & Fritz, 1985), and we presume that the same is occurring here.

For the pollen export model, we also found three significant correlational selection gradients that agreed with the predictions of concerted selection on (possibly functionally linked) trait constellations. Very few studies have reported correlational selection of floral traits (O'Connell & Johnston, 1998; Benítez-Vieyra *et al.*, 2006, 2009, 2012) and even fewer provided an explicit functional explanation for their expected coordination. The correlational gradient obtained for the combination of corpusculum and outer guide rail is explained by a functional interaction, which would increase pollinarium export. These two traits are involved in the mechanical interaction with the pollinator and with other pollinaria carried by them: the outer guide rail captures either the insect appendages or the caudicles of other pollinaria and guides them to the clasping corpusculum, which consequently makes pollinarium export possible. The correlational selection gradient between corpuscle and caudicle length is marginally significant and significant through male and female fitness, respectively. This result would be expected for male fitness due to reciprocal adjustments required between both traits to promote the concatenation process. Through the above-mentioned correlation of both sexual functions, pollen receipt could be favoured indirectly (Wyatt, 1976; Morse & Fritz, 1985).

For the model that included a set of traits acting on pollen receipt, directional and correlational selection gradients were detected. The length of the inner guide rail was subjected to directional selection. The negative sign of this gradient in both fitness functions indicates that short guide rails are favoured. Again there appears to be a correlation between both fitness functions in the selection of this trait. In this case, there is an obvious explanation for this correlation; pollinarium export can be promoted through receipt by means of concatenations, that is, pollinaria captured by the inner-guide rail may serve as an attachment for exported pollinaria. Moreover, the correlational gradients obtained for the combination of length and width of the inner guide rail can also be explained by a functional interaction: shorter and narrower entrances into the inner guide rail should allow flowers to capture pollinia more easily. Once again, this finding is supported by concerted evolution of functionally linked traits.

The correlational gradients detected are consistent with the modularity analyses: three correlational gradients were included in the hypothetical functional matrix, one of them, between corpusculum length and caudicle length was detected in a conditional independence analysis, which is weakly correlated across the population. This would mean that the current selection pattern at least partially explains the realized selection in the population (Herrera *et al.*, 2002). Despite these agreements, correlational selection was found on trait pairs with low or very low correlations, we assume that this low correlation has allowed us to detect correlational selection precisely because there was enough codispersion for selection to act on. Conversely strongly correlated trait pairs, correlational selection should be more difficult to detect.

A recent meta-analysis (Roff & Fairbairn, 2012) found empirical evidence for the hypothesis, which predicts that correlational selection on two traits will cause the main axis of the genetic matrix (G), or the phenotypic matrix (F; Cheverud, 1988) to orient in the same direction as the correlational selection gradient (Lande, 1980; Brodie, 1992).

As , in the present study, linear and correlational selection gradients had the same sign and the fitness curves and the same shape for both fitness functions, we argue that there is no conflict between pollen export and receipt functions. Though this may suggest that both fitness functions would jointly drive selection in one direction (Benítez-Vieyra *et al.*, 2009), we are aware that we are only considering two steps in the process leading to total fitness (number of offspring per individual).

Conclusion

This study evaluates the evolution of floral morphology and of the pollination mechanism in *Asclepiadoideae* involved in fine-scaled interactions with their pollinators. Hypotheses that may explain the covariation patterns in relation to functional, morphological and developmental grouping of traits were addressed. Through *a priori* and *a posteriori* approaches, it was possible to independently detect modules made up by trait sets of different developmental domains, consistent with our hypothesis of functional coordination within trait sets. These findings are still not evidence that functionally linked components represent pleiads of evolutionary change in this species. Though this possibility is suggested by the phenotypic selection analyses, it only supports the adaptive value of the functional correlations between traits.

The use of both sexual components for the phenotypic selection analyses allowed for the identification of non-conflicting and rather mutually congruent selective effects on traits and trait pairs. Selective effects that are specific to each sexual function can, in part, be explained by our knowledge about the interplay between these traits in the functions of pollen export and receipt.

Finally, the finding of correlational selection patterns is partly consistent with the structuring of the flower in functional modules or pleiads of traits suggesting that the modules are the result of realized selection. Though the functional hypothesis has a high explanatory power in relation to the phenotypic correlation structure and the current outcome of selection, there are still unexplained patterns, which are not attributable to mechanical functioning. For example, phenotypic modules might not be as rigid as required by the tested hypotheses, and there may still be underlying and concurring constrains that we were not able to capture in the present approach. The next step of this study should be the experimental manipulation of the flower phenotype, in order to advance from a correlational to a causational approach. The unravelling of the underlying and concurring constrains would require an extension of this study from the population to the phylogenetical level.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Modularity patterns: hypothetical correlation matrices.

Appendix S2 Cubic spline regressions between floral traits and reproductive success in the *Morrenia brachystephana* studied population.

Data deposited at Dryad: doi:10.5061/dryad.kq72j

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