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EXPLORING PHENOTYPIC FLORAL INTEGRATION IN *IRIS PUMILA* L.: A COMMON-GARDEN EXPERIMENT

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Abstract - The angiosperm flower is a complex integrated phenotype, but within this structure there are partly independent units or modules. The interconnections among floral organ traits are hypothesized to be mostly generated by pollinator-mediated selection. In this study, we explore whether floral dry mass per area (DMA) in an insect-pollinated herb, *Iris pumila,* exhibits a modular correlation pattern as has been reported for some size-related traits. We found that the overall pattern of floral organ integration with regard to DMA was uneven in the offspring of *Iris pumila* derived from a sun-exposed and a shaded natural population. Since principal component analysis (PCA) showed that most of the eigenvalue variance was explained by the first two principal components (PCs), these PCs were considered as two floral modules. The greatest factor loadings on the first PC axis was that of the perianth and style arm DMA (PSDMA) and perianth tube DMA (PTDMA),while on the second PC axis, the greatest factor loading was that of stamen DMA (STDMA). The results indicate that the function of the first module would be to attract a pollinating vector, while the second one would reflect male functions. Selection analyses revealed that the targets of phenotypic selection were both intra-floral integration and individual floral traits. Both PSDMA and PC1DMA were under strong linear selection, while PTDMA experienced direct stabilization selection. The level of integration in floral organ DMA expressed in the term of relative eigenvalue variance appeared to be rather low, as was documented for other angiosperm taxa.

Key words: CPCA, dry mass per area (DMA), floral integration, integration index, *Iris pumila*, modularity, phenotypic selection analysis

INTRODUCTION

Phenotypic integration refers to a complex pattern of interconnections among a suite of traits of an organism, which results from the concurrent action of genetic, functional, developmental and evolutionary sources of variation (Pigliucci and Preston, 2004). Within natural populations, phenotypic integration occurs due to genetic and functional relationships between traits, whereas among populations and/or species, correlations can arise because of similar responses of functionally related traits to a spatially and/or temporally unstable selection agent, or to multiple selection pressures that vary jointly (Armbruster and Schwaegerle, 1996).

By comparing the correlations among morphological measurements of specimens from a few or many distinct natural populations, two paleontologists, Everett Olson and Robert Miller observed that within the set of measured variables there are subsets called "ρ-sets", which show relatively high mutual phenotypic correlations, but a rather low number of correlations among variables of different ρ-sets (Olson and Miller, 1958). Measurements within each of these ρ-sets are assumed to be functionally, develop-

mentally, or evolutionarily interrelated. They coined the term *morphological integration* to describe "…the summation of the totality of characters which, in their interdependency of form, produce an organism" (Olson and Miller, 1958). A similar idea was proposed by Terentjev (1931), who claimed that traits are commonly grouped according to their correlations into "correlation pleiades" where correlations among elements of different pleiades tended to be minimal.

Currently, there is a wide agreement among biologists about the concepts of phenotypic integration and modular variation of organismal form, as well as about modularity, which is presumed to be the prerequisite for the evolution of complex adaptations (Mitteroecker and Bookstein, 2007). Moreover, these two concepts were viewed as equivalent one to the other, since both influence the variance/covariance pattern among individual traits (Armbruster et al., 2004; Klingerberg, 2008). However, a careful consideration of the issues revealed that "*modularity* typically denotes the (experimental) dissociability of developmental, functional, or evolutionary processes", while *phenotypic integration* is "about the decomposition of morphological traits according to their (phenotypic) covariance structure compared across adults and/or subadults of a single taxon or across several taxa" (Mitteroecker and Bookstein, 2007). According to this definition, a biological module would be an entity (a structure, a process, or a pathway) that is strongly integrated internally, but relatively independent from other such entities (Bolker, 2000; Magwene, 2006; Klingenberg, 2008).

The angiosperm flower is a highly complex modular structure that interacts with different pollen vectors to promote an efficient pollen transfer. The interconnections among floral organ traits are hypothesized to be mostly generated by pollinator-mediated selection. Berg (1960) was among the first to carry out comprehensive observations of plants to find out under which combination of ecological factors a particular pattern of floral phenotypic variation/covariation will be produced. She revealed that the magnitude of floral phenotypic integration notably differs between plants with specialized and those with generalized pollination. The author concluded that interconnection among floral organs will be much stronger in species with specialized relationships with animal pollen vectors than to those with more generalized relationships. Since flowers with specialized pollination have to be well matched with their pollinators, natural selection is expected to jointly favor phenotypic integration among floral traits as well as the decoupling of these traits from variation in vegetative ones. The outcome of such selection effects is the production of reproductive and vegetative modules, or, in Berg's (1960) terminology, separate correlation pleiades. The Berg hypotheses are corroborated in a large number of studies, which have detected the strong phenotypic and genetic correlations among investigated floral organs (Conner and Sterling, 1996; Fenster and Galloway, 1997; Armbruster et al., 1999; Armbruster et al., 2004).

Recently, Ordano et al. (2008) reported that the flowers of higher plants exhibit a lower level of floral integration than can be expected according to a randomly generated distribution. In addition, by applying a hierarchical selection analysis using their own data, these researchers revealed that only individual floral traits and sets of highly interconnected floral traits are the targets of selection, while the entire flower is likely to be selectively neutral. The low strength of flower phenotypic integration is presumed to result from a small adaptive value of reproductive plant modules, or as the consequence of natural selection favoring instability of reproductive plant organs (Ordano et al., 2008).

Up to now, investigations of phenotypic integration in plants have been mostly centered on morphological leaf traits. However, in the present study, we pay attention to the phenotypic variation in the strength and the pattern of correlations between *functional plant traits* that influence fitness indirectly, through direct effects on fitness components (performance traits), such as survival, growth, and reproduction (Violle et al., 2007). Specifically, we focused on a key functional trait, dry mass per area (DMA), a morphological attribute which is not related to size. DMA has been frequently used as an index of leaf

structure (see Poorter at al., 2009), and in resource-use strategies of plants (Wright et al., 2004).

To explore whether non-size-related morphological traits exhibit a modular correlation pattern as has been reported for size-related ones, we quantified the amount of DMA in the floral organs of an insect-pollinated perennial herb, *Iris pumila*. The experimental plants used in this study originate from two natural *Iris* populations that experienced contrasting light conditions in the Deliblato Sands. One population occupied a sun-exposed site on the top and slope of a small dune, while the other population inhabited the understory of a *Pinus nigra* stand. The offspring of these populations were raised, from seedling stage to adults, under similar ambient conditions in an experimental garden.

The aim of this experiment was to elucidate whether: (1) *I. pumila* plants originating from an exposed and a shaded population express the comparable mean value of floral organ DMA under common-garden conditions; (2) the magnitude and/or the pattern of phenotypic integration of floral organ DMA vary between the two populations; and (3) phenotypic selection targets individual floral traits, sets of tightly correlated traits (i.e., within flower integration), or both of the two.

MATERIAL AND METHODS

Studied species and experimental design

Iris pumila L. (Iridaceae) is a rhizomatous perennial herb native to the protected natural reserve at the Deliblato Sands (44º 48' 39" N/ 21º 20' 00" E to 45º 13' 10" N/ 28º 26' 08" E) in northern Serbia. Natural populations of the species consist of circle-shaped clones differing in flower color. Because flower color polymorphism in *I. pumila* originates from the segregation at several gene loci, each of the flower color variants in a population can be regarded as a unique clonal genotype (Tucić et al., 1988).

Flowers of *I. pumila* are radially symmetrical. They are composed of six, slightly scented lobes, which are not clearly discernible between sepals and petals. The three petal-like falls (sepals) hang down, spreading out from their narrow base into a broader limb. The upper surface of the falls is outfitted with a beard consisting of fuzz and commonly decorated with bright lines leading to the flower's mouth. The three standards (petals) stand upright, somewhat behind the fall bases. The falls and the standards that form a perianth are fused at their base into a perianth tube that lies down to the ovary composed of three carpels. The styles are split toward the apex into petaloid arms, with a stigmatic lip at the top. The style arms are arched over the stamens. While the falls serve as a "landing platform" for pollinators, which hold to the beard as they enter the iris flower in search of nectar, the bright lines above the fall serve as nectar guides. However, the colorful and fleshy standards provide pollinator-attracting power to the iris flower.

The floral organs of *I. pumila* are positioned in four concentric whorls: the falls in whorl 1; the standards in whorl 2; the stamens in whorl 3, and the styles, with stigmatic lip on the top of style arms, in whorl 4 – the center of the flower (Bowman, 1997).

To investigate how parental growth conditions influenced the variation in the degree and the pattern of correlations between functional floral traits in *I. pumila*, we harvested one reproductive ramet from each of the flowering clones grown under common ambient conditions in an experimental garden. Since the outermost ramets of each *Iris* clone represent the 14^{th} vegetative generation, we assume that environmental maternal effects which might influence the variation of a quantitative floral trait are minimized.

For this experiment, two natural populations of *I. pumila* living under contrasting light conditions were selected in nature. In April 1996, during the blooming phase of *I. pumila*, a number of clonal genotypes were randomly chosen according to flower color (genotypic marker) in both populations. Subsequently, the pairs of synchronously flowering clones within each population were hand-pollinated. The offspring of each mating pair consist of full-sibs, and as such

represent a full-sib family. Seedlings obtained from these crosses were grown singly in plastic pots under the same environment-controlled growth-room conditions (light intensity: 110 μmol $m^{-2}s^{-1}$; photoperiod: 16 h; ambient temperature: 21/16 ºC day/night). Six-month-old seedlings were repotted into 20 cm-diameter clay pots, and then placed at random positions into an experimental garden near the Siniša Stanković Institute for Biological Research, in Belgrade, where they still grew as adult clones under common-garden conditions.

In April 2011, during the blooming phase of *I. pumila*, 60 clonal plants were randomly chosen for analysis: 32 plants originated from the exposed population, and 28 plants were from the shaded population. From each of these clones we harvested one flowering ramet between 10 and 12 a.m. during two successive days. To circumvent variation due to floral age, the reproductive ramets were harvested at the early male stage; that is, just after flower opening. Each ramet was placed individually in a plastic bag, and the whole material brought back to the laboratory, where it was replaced in a refrigerator cabinet (at 2ºC) before dissection (~ 20-30 min after collection). To get individual floral organs, including falls, standards, style arms, and stamens, flowers were cut at the base of the perianth. The perianth tubes were obtained by trimming the corresponding flower at the top of the ovary. Fresh floral organs were weighed in two ways: once individually and once as the whole flower. Upon weighing, the ovary, floral tube and stamens of the same flower were mounted on the surface of a glass plate and their digital images taken using a Canon CanoScan 8800F scanner at a 200 dpi resolution. After scanning, these floral organs were placed in separate paper bags, and dried in an oven at 60ºC for 72 h. The other floral organs of a blossom, including falls, standards, style arms, and spates were placed in a 0.5 L bottle filled with 70% ETOH, and stored at room temperature until dissection. Following dissection, parts of each floral whorl were separated into components and then flattened (including a spathe) over a glass plate covered with glycerol, to keep their original size. We took digital images of these floral organs in a similar way as was done for the ovary, floral tube and anthers, and thereafter replaced them in a paper bag for oven drying (72 h at 60ºC). Measurements of floral organ area were done using image-analysis software, the UTHSCSA Image Tools, version 3.0 (San Antonio, TX, USA). The surface area of the ovary and floral tube was estimated as its individual digital image-size multiplied by a factor of 3. We recorded dry mass per area, DMA [(dry biomass)/area; in g cm^2], for each floral organ of 60 harvested reproductive ramets.

Statistical analyses

To determine the effect of population-of-origin on the mean value of floral organ DMA we computed a series of univariate ANOVAs using the generalized linear model (GLM) in SAS (version 9.1). In these ANOVAs, DMA was the response variable, while the population-of-origin (exposed or shaded) was the explaining variable.

For each population, a Pearson product-moment phenotypic correlation matrix was computed including all traits analyzed (PROC CORR option in SAS). Generally, each correlation matrix has two attributes: the amount and the pattern of correlations. The *amount* of correlations refers to the overall strength of correlations among respective traits, i.e., the degree of integration of a multidimensional phenotype in a particular environment (Cheverud, 1982; Haber, 2011), while the *pattern* of phenotypic correlations denotes the overall arrangement of phenotypic correlations in a correlation matrix. The structures of floral phenotypic correlation matrices between the exposed and the shaded populations were compared using a common principal component analysis (CPCA; Philips and Arnold, 1999). The CPC model effectively tests for a hierarchical progression of relatedness between two covariance matrices whose patterns are compared to four possible similarity classes. The hierarchy of comparisons starts at unrelated matrix structure, and moves to partial common principal components (individual PCs differ significantly between matrices), then to proportionality (eigenvalues in one matrix are proportional to those of another by a constant), and ends with matrix equal-

ity; that is, the compared matrices do not differ either in eigenvectors (PCs) or eigenvalues. Here we used the step-up approach to interpret the results. This approach starts with no relation between the matrices, and moves to a higher hierarchical level (CPCs; proportionality; and equality). The likelihood that a particular model is correct is tested against the previous lower model, which is used as the current null hypothesis (Philips and Arnold, 1999).

The overall level of inter-correlations among floral organ traits was measured using the variance of eigenvalues (Pavlicev et al., 2009) estimated in a principal component analysis (PCA). PCA transforms a correlation matrix composed of several dependent quantitative variables in a set of new orthogonal variables called principal components (Abdi and Williams, 2010). Principal components (eigenvectors) are linear combinations of the original variables, whose importance is determined by the amount of variance (eigenvalue) "explained" by a given component or by the proportion of the total variance explained by that component. The first component explains the most variation, then the second component and so on. High eigenvalue variance is the property of highly integrated phenotypes, whereas low eigenvalue variance indicates that this phenotypic unit exhibits a low level of integration. The amount of floral integration in each of the two *I. pumila* populations was estimated as the relative variance of the eigenvalues – r*VE*, according to the formula (Pavlicev et al., 2009):

$$\mathrm{r}VE = VE / maxVE = VE / N\,(N\text{-}1),$$

where *VE* is the eigenvalue variance

$$(VE = [\sum_{i=1}^{N} (E_i - 1)^2] / N),$$

maxVE is the maximal eigenvalue variance (*maxVE* = *N* – 1), *E* is eigenvalue, and *N* is the number of traits or matrix size. The 95% confidence interval of r*VE* is estimated by a bootstrap analysis.

To elucidate whether the targets of natural selection are individual floral traits or intra-floral modules, we first ran a principal components analysis on the PCA scores of the pooled population data (Ordano et al., 2008). Since principal components are orthogonal (unrelated) to other PCs, we consider each PC with an eigenvalue greater than or close to 1 as a distinct floral module. The linear and quadratic components of phenotypic selection on floral organs were estimated by a regression analysis of relative fitness on individual floral traits, as well as on PC1 and PC2 (Lande and Arnold, 1983). The linear (*S*) and quadratic (*C*) selection differentials estimate the total selection on the trait (using a univariate regression), but confound the direct effects of selection on a trait with the indirect effects due to selection on phenotypically correlated traits. The linear (β) and quadratic (γ) selection differentials estimate selection acting directly on the trait, accounting (using partial regression coefficients from a multiple regression) for indirect selection due to phenotypic correlations with other measured traits. While the linear coefficients quantify directional selection on the trait, the quadratic coefficients measure the stabilizing (variance) selection. To allow comparisons of traits with different scales, all data were standardized to a mean of zero and a variance of 1 (*z*-transformation; Sokal and Rohlf, 1981). We used the dry aboveground biomass of reproductive ramets as a proxy of fitness. The aboveground biomass is the most integrative measure of individual performance of clonal herbs because it encompasses both survivorship and fecundity. The absolute fitness was transformed to relative fitness by dividing the dry biomass of each ramet with the average biomass of all ramets belonging to the corresponding population. All analyses were done using PROC GLM in SAS.

RESULTS

Phenotypic variation in DMA of individual floral traits

Under similar ambient conditions prevailing in an experimental garden, the variation of DMA in the floral organs of *I. pumila* appeared to be both population-of-origin- as well as organ-specific (Table 1). The mean value of DMA for each of the analyzed

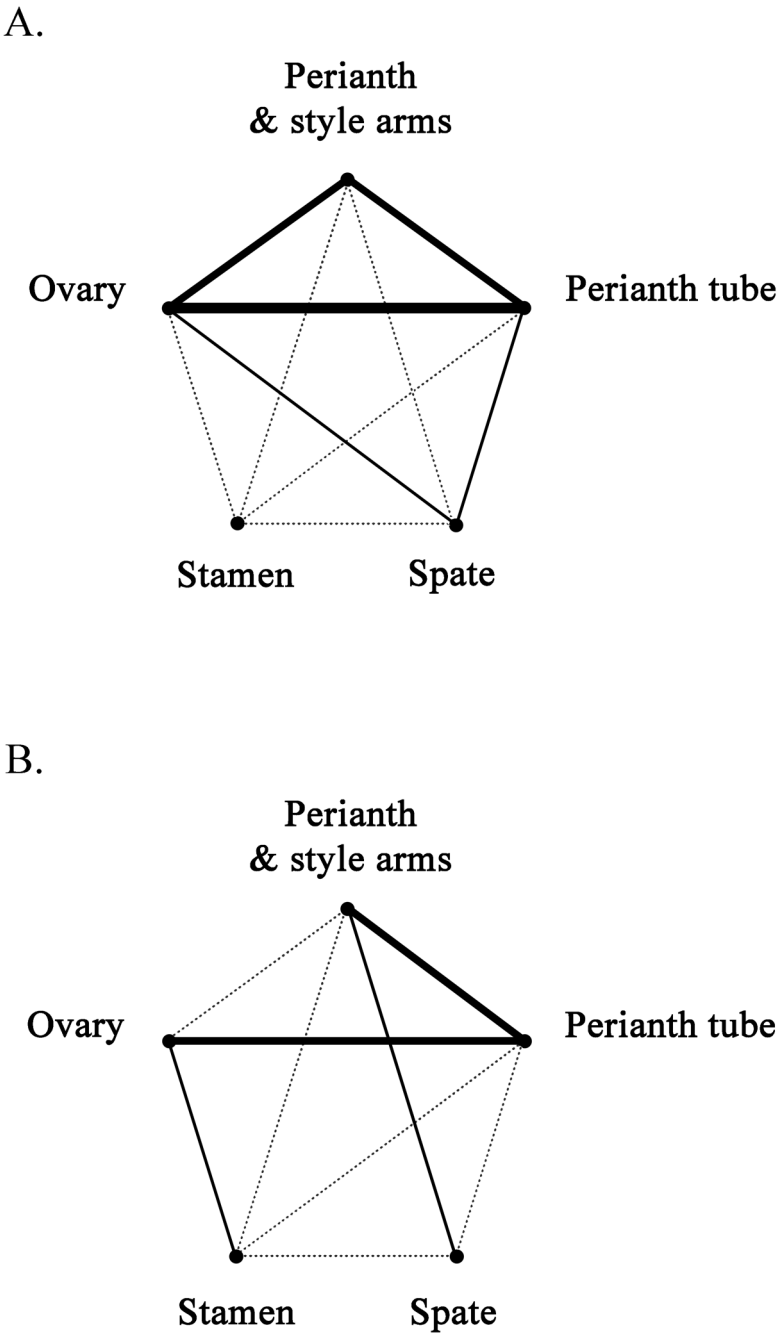

Fig. 1. Phenotypic correlations between floral organ dry mass per area (DMA) in *Iris pumila* plants originating from A. an exposed and B. a shaded population, as expressed after 14 years of growth under common-garden conditions.
Magnitude of significant correlation coefficient, r: = r > 0.80; = 0.80 > r < 0.60; = r < 0.60; = low and/or no significant.

floral organs (PSDMA, PTDMA, SDMA, ODMA, and STDMA) tended to be greater in clones from the exposed population compared to those from the shaded one. Even so, the differences between the two

Table 1. Comparison of the dry mass per area (mean ± 1 SE) in samples (N = sample size) of *Iris pumila* clones, stemming from hand-pollinated seeds in a sun-exposed (Dune) and a shaded (Woods) natural population, as expressed after 14 years of growth under similar environmental conditions in a common-garden. The coefficient of variation (CV, in %) for each floral organ, mean CV, and *F*-values of population effects obtained using an ANOVA are presented for each trait as well. NS, non-significant; bold type, statistical significance (*, $P < 0.05$).

Dry mass per area (DMA, in g cm^{-2})	Population of origin							*F* for comparison of trait means
	Dune (N = 32)			CV	Woods (N = 28)			CV	
Perianth + style arm(PSDMA)	0.0008	±	0.0000	12	0.0007	±	0.0000	14	2.41^{NS}
Perianth-tube (PTDMA)	0.0040	±	0.0001	12	0.0037	±	0.0001	11	**4.97***
Spathe (SDMA)	0.0009	±	0.0000	14	0.0008	±	0.0000	25	0.09^{NS}
Anther (ADMA)	0.0108	±	0.0003	14	0.0097	±	0.0003	11	**5.23***
Ovary (ODMA)	0.0049	±	0.0001	16	0.0047	±	0.0001	19	1.37^{NS}
Mean CV				14				16	

Table 2. Factor loadings of floral organ DMA on three principal components (PC1, PC2, and PC3) in *I. pumila* plants derived from an exposed and a shaded population, and grown under similar environmental conditions in a common-garden. Factor values greater than |0.400| are given in bold type. Relative coefficients of integration, r*VE* (Pavlicev et al., 2009), corrected for sample size, are presented for each of the two populations, as well.

	Exposed population			Shaded population		
Trait	PC1	PC2	PC3	PC1	PC2	PC3
Perianth & style arm DMA	0.477	0.075	-0.511	0.492	0.075	-0.650
Perianth tube DMA	0.545	0.080	-0.117	0.551	-0.134	0.210
Ovary DMA	0.539	-0.121	-0.102	0.434	-0.593	0.360
Spathe DMA	0.376	-0.510	0.705	0.303	0.730	0.562
Stamen DMA	0.209	0.845	0.467	0.417	-0.896	0.835
Eigenvalue	2.863	1.041	0.578	2.607	1.174	0.719
Proportion variance explained	0.573	0.208	0.116	0.521	0.235	0.144
Cumulative proportion			0.896			0.900
Relative coefficient of integration (95% Confidence interval)						
rVE (PC scores)	0.030 (0.008 - 0.069)			0.036 (0.011 – 0.077)		

Table 3. Factor loadings of floral organs DMA on three principal components (PC1, PC2, and PC3) in a pooled sample of *I. pumila* plants derived from an exposed and a shaded population, and grown under similar environmental conditions in a common-garden. Factor values greater than |0.400| are given in bold type.

	Pooled datasets		
Trait	PC1	PC2	PC3
Perianth & style arm DMA	0.497	-0.169	0.800
Perianth tube DMA	0.581	-0.193	-0.117
Ovary DMA	0.528	-0.275	-0.102
Stamen DMA	0.369	0.927	0.467
Eigenvalue	2.560	0.758	0.518
Proportion variance explained	0.640	0.184	0.129
Cumulative proportion		0.829	0.959

Table 4. Standardized directional (*S'*) and stabilizing/disruptive (*C'*) selection differentials, and directional (*β'*) and stabilizing/disruptive (*γ'*) selection gradients for floral organ dry mass per area (DMA) in a pooled dataset of *I. pumila* plants derived from an exposed and a shaded population, as expressed after 14 years of growth in a common-garden.

Linear selection coefficients	Pooled dataset			
	S'	P	β'	P
Perianth & style arm DMA (PSDMA)	0.155	<0.0001	0.106	0.0015
Perianth tube DMA (PTDMA)	0.151	<0.0001	0.044	0.3395
Ovary DMA (ODMD)	0.116	<0.0001	0.038	0.5090
Stamen DMA (ADMA)	0.074	0.0158	0.008	0.764
PC1DMA	0.152	<0.0001	0.152	<0.0001
PC2DMA	-0.023	0.4573	-0.023	0.3340
Nonlinear selection coefficients	Pooled dataset			
	C'	P	γ'	P
PDMA x PDMA	0.149	<0.0001	0.338	0.6231
PTDMA x PTDMA	0.132	<0.0001	-2.616	0.0102
ADMA x ADMA	0.071	0.0183	0.204	0.5838
SDMA x SDMA	0.110	0.0001	0.660	0.3334
$PC1_{DMA}$ x $PC1_{DMA}$	-0.061	0.0463	-0.106	0.0272
PC2DMA x PC2DMA	0.0173	0.5755	-0.0058	0.9059

populations were significant only for PTDMA and STDMA (Table 1). The results indicate that a greater DMA would be an adaptation to the high ambient light intensity in open habitats. The mean inter-individual variation of floral organs DMA, as expressed in terms of a coefficient of variation (CV%) appeared to be slightly higher in clonal plants originating from the shaded population relative to those clones from the exposed population (16% vs. 14%, respectively; Table 1), likely due to greater microenvironmental heterogeneity.

Given that the two *Iris* populations were grown under common-garden conditions, the significant differences between the two populations detected for the mean value of PTDMA and STDMA could be the outcome of heterogeneous past selection that had affected their ancestors' clones, naturally growing within contrasting light habitats.

Within flower integration among floral organ DMA

Pearson's product moment correlations among floral organ DMA were positive in sign and similar in number in both populations of *I. pumila* (Fig. 1A and 1B), but their pattern appeared to be population-specific (Fig. 1A and 1B). The CPC analysis used to compare the correlation matrices between the exposed and the shaded population in a hierarchical manner revealed that the structure of the two correlation matrices was unrelated [CPC (1) vs. Unrelated: $\chi^2 = 13.12$; df = 4; $P = 0.011$). Because a covariance matrix of quantitative traits combines two distinct features, the magnitude and the pattern (Olson and Miller, 1958), the obtained results suggest that the two *Iris* populations differed not only in the arrangement of correlation among floral organ DMA, but also in their correlatedness, that is, the overall strength of correlations between the floral organ DMA (Pavlicev et al., 2009).

To assess whether *Iris* flowers consist of one or a few subsets of correlated floral organs with regard to DMA, the magnitude and sign of each PC factor loading was examined for each population separately. As can be seen from Table 2, most of the variation appeared to be related to traits included in flower size and pollinator attraction (PC1: PSDMA, PTDMA, ODMA), as well as to pollen transport/reception (PC2: STDMA). The results suggest that flowers of *I. pumila* are indeed composed of two phenotypic modules or correlation pleiades (*sensu* Berg, 1960). The overall magnitude of phenotypic integration among floral organ DMA, expressed in terms of r*VE*, appeared to be rather low in both populations of *I. pumila* (Table 2), similar to the values recorded for other plant taxa (Ordano et al., 2008).

Selection analysis

Our study revealed that phenotypic selection influenced both individual floral traits and intra-floral integration (PC1 scores) of *I. pumila* plants (Table 3). Both individual floral trait DMA as well as PC1 DMA appeared to be significantly affected by positive total linear selection (Table 4). The greatest values of standardized selection differential were estimated for PSDMA and PC1DMA (S' = 0.155*** and 0.152****, respectively). Apart from a significant negative value computed for PC1DMA, nonlinear selection coefficients (C') for all individual traits were significantly positive in sign (Table 4).

Multivariate selection analysis also detected a significant positive direct selection on PSDMA (β' = 0.106, P ≤ 0.0015) and PC1DMA (β' = 0.152, P ≤ 0.0001) in the pooled data set of *I. pumila* (Table 4).

The standardized quadratic nonlinear selection gradient, γ', was found to be significantly negative in sign exclusively for PTDMA (γ' = -2.6, $P \leq$ 0.0102) and PC1DMA (γ' = -0.106, $P = 0 \leq 0.0272$). A simultaneous occurrence of insignificant linear and negative quadratic gradients obtained for PTDMA indicated this trait was under stabilizing selection, meaning that plants with an average amount of DMA invested in PTDMA will be selectively favored compared to those having extreme values of this trait. Since the linear selection gradient for PC1DMA was positive, while the non-linear gradient had a negative sign, the results reflect a monotonic relationship between this phenotypic module

and fitness, rather than stabilizing selection (Table 4; Mitchell-Old and Shaw, 1989). However, we failed to find evidence either for total and/or direct selection or for total and/or quadratic selection on PC2DMA.

DISCUSSION

The flower of higher plants is a highly complex modular structure which comprises a suite of functionally, developmentally and genetically interrelated traits, some of which interact with different pollen vectors (e.g., corolla; Smyth, 2005) to promote an efficient pollen transfer (Wagner, 1996; Galen, 1999; Murren, 2002). The mechanisms of the interconnections among floral organs are still controversial. Although there are many experimental studies that found that interconnections among floral traits are shaped by pollinator-mediated selection (Alexandersson and Johnson, 2002; Pérez et al., 2007; Ordano et al., 2008), others appeared as well, pointing out that a tight association among floral organs may originate as a by-product of the genetic and developmental architecture of a flower (e.g., Armbruster et al., 2004).

Mean values of functional floral traits

Our study provides evidence that, even under common-garden conditions, the production of DMA within the same floral organs depended on the habitat type that the *I. pumila* plants were derived from. The mean values of floral-organ DMA tended to be greater in plants stemming from an exposed population relative to those from their shaded conspecific. However, the ANOVA results have shown that only PTDMA and STDME differed significantly between the two habitat types. The observed differences in average values of analyzed floral traits signify an adaptation to the local environmental conditions prevailing in their native habitats, rather than the adaptive phenotypic plasticity to heterogeneous light conditions (Kawecki and Ebert, 2004; Strauss and Whittall, 2006). Given that floral organ DMA reflects the amount of structural tissue invested per area (Poorter et al., 2009), the results indicate that sun-exposed *Iris* taxa suffer a greater carbon cost as compared to shaded ones.

Floral phenotypic integration

In the present study, we focused on both the overall level and the pattern of correlations among floral organ traits in *I. pumila.* Investigations of the variation/covariation patterns among organism traits are especially attractive from an evolutionary perspective, because they may highlight the processes that have created the evolutionary trajectories of the traits under study, and, possibly, the trajectories of their potential evolution (Lande and Arnold, 1983). The evolutionary models of phenotypic integration propose that developmental and functional integration produce genetic integration, which, in turn, lead to evolutionary integration (Lande, 1980; Cheverud, 1982).

There is growing evidence that the magnitude of floral integration varies among different plant taxa (Schlichting, 1986; Armbruster, 2001; Murren et al., 2002; Pigliucci and Preston, 2004). In comparative studies of phenotypic integration that aimed to illuminate the relative role of constraints vs. selection on the variation pattern in magnitudes of phenotypic integration, the occurrence of a significant difference in the magnitude of phenotypic integration between taxa from the same hierarchical level was interpreted as the consequence of past natural selection, while similarity would indicate that genetic and/or developmental constraints have played the key role (Tucić and Avramov, 1996; Murren et al., 2002; Armbruster et al., 2004; Ordano et al., 2008). The quantitative-genetic theory proposes that significant genetic correlations between polygenic traits reflect either an overlap between loci that regulate flower development (i.e., pleiotropy), or linkage disequilibrium of the genes that independently determine floral traits (Falconer and Mackay, 1996). Recently, Juenger et al. (2005) have shown in a mapping experiment with an *A. thaliana* recombinant inbred line that the significant genetic correlations found within the flowers of this species could be attributed to multiple gene effects, rather than to linkage disequilibrium. Howev-

er, the correlations among traits can be generated by developmental processes as well, leading to phenotypic integration and modularity (Olson and Miller, 1958; Wagner, 1996; Wagner and Altenberg, 1996; Klingenberg, 2008).

In *I. pumila*, the number of significant correlations between floral organ DMA appeared to be similar in plants derived from the exposed and the shaded population (6 vs. 5, respectively; Fig. 2). As a curiosity, in *Iris* clones originating from the sun-exposed population, STDMA was unrelated to any DMA value recorded for other floral organs in the study. Because the correlation matrices between the exposed and the shaded populations were found to be unrelated (CPC test) under common-garden conditions, it seems reasonable to conclude that the observed population divergence in the pattern of functional floral integration in *I. pumila* would be the outcome of divergent past selection that occurred within contrasting light environments in the wild.

In a recent review on floral integration, Ordano et al. (2008) documented that the magnitude of floral integration estimated by the Wagner index of integration (Wagner, 1984) in 36 angiosperm species is lower than expected by chance. The estimated integration values ranged from 0.069% to 73.23%, relative to the maximal integration, with the mean magnitude of 21.5% (SD = 15.4) for the observed, and 32.7% (SD = 8.5) for the expected datasets. Numerical simulation based on the hypothesis that the magnitude of integration is independent of either adaptive, developmental, genetic, or physiological constraints indicates that the low level of phenotypic integration in flowering plants could be related to natural selection promoting intra-floral integration rather than whole-flower integration and, consequently a more flexible floral evolution.

The level of integration in floral organ DMA of *I. pumila* was rather low in each of the two populations (r*VE* = 3.0% in sun vs. 3.6% in shade population). Although the estimated indices of floral phenotypic integration in *I. pumila* plants were within the range documented for a vast number of flowering plants, their magnitude was rather low, regardless of population origin. Our results are comparable to those reported for Rosaceae species, where the magnitude of floral integration across species was found to be low, while their variance/covariance structure significantly different (Ordano et al., 2008).

The angiosperm flower is a complex integrated phenotype, but within this structure, there are partly independent units or modules. The primary goal of this study was to recognize and compare patterns of integration between different floral parts, as well as to find out whether the floral modules are targets of phenotypic selection. Hence, to identify the overall pattern of floral integration we applied a pooled within-species PCA analysis (on scores of principal components) which minimizes the effects of population means (Mitteroecker and Bookstein, 2008). The overall pattern of floral organ DMA integration was shown to be an uneven pattern, particularly between sterile (perianth) and fertile (stamen) flower parts. Most of the independent variance was concentrated in the first two eigenvalues that resulted in a high eigenvalue variance. Since a high eigenvalue variance is characteristic of highly integrated phenotypic units, and because most of the independent variance was concentrated in PC1 and, to lesser extent in PC2, these two PCs could be referred as distinct floral modules. In the case of *I. pumila* flowers, the greatest factor loading on PC1 have PSDMA (0.50) and PTDMA (0.58), while on PC2 the greatest factor loading has STDMA (0.93). This result indicates that the function of the first module is to attract a pollinating vector, whereas the second one is most likely related to male functions.

Selection analyses revealed that the targets of phenotypic selection were both within flower modules as well as individual floral traits. The intensity of direct selection appeared to be both trait-and module-specific. For example, the linear selection gradient for PSDMA was significant, positive in sign, and with greatest value compared to other traits. The result indicates that plants having a higher than aver-

age PSDMA value are selectively more advantageous than those with lower PSDMA. Conversely, PTDMA is under stabilization selection, which means that plants expressing extreme values for this trait will be less fit than those with average values. As we expected, PC1DMA was affected by a high direct linear selection ($\beta' = 0.152$), which was likely one of the principal causes leading to intra-floral modularity. Our results oppose the statement of Armbruster and colleagues that "rather than reflecting the results of adaptive evolution ... the tight integration of floral parts ... could be by-products of the genetic/developmental architecture of the organism" (Armbruster et al., 2004; p. 24).

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REFERENCES

Alexandersson, R., and *S. D. Johnson* (2002). Pollinator-mediated selection on flower-tube length in a hawkmoth-pollinated *Gladiolus* (Iridaceae). *P. Roy. Soc. B-Biol. Sci.* **269**, 631-636.

Abdi, H., and *L. J. Williams* (2010). Principal component analysis. *Wiley Interdiscip. Rev. Comput. Stat.* **2**, 433-459.

Armbruster, W. S., and *E. K. Schwaegerle* (1996). Causes of covariation of phenotypic traits among populations. *J. Evol. Biol.* **9**, 261-276.

Armbruster, W. S., Di Stilio, V. S., Tuxill, J. D., Flores, T. C., and *J. L. Velasquez Runk* (1999). Covariance and decoupling of floral and vegetative traits in nine neotropical plants: A re-evaluation of Berg's correlation-pleiades concept. *Am. J. Bot.* **86**, 39-55.

Armbruster, W. S. (2001). Evolution of floral form: electrostatic forces, pollination, and adaptive compromise. *New Phytol.* **152**, 181-183.

Armbruster, W. S., Pélabot, C., Hansen, T. F., and *C. Mulder* (2004). Floral integration and modularity: distinguishing complex adaptation from genetic constraints. In: *Phenotypic Integration*, (Eds. M. Pigliucci, and K. A. Preston), 23-42. Oxford University Press, Oxford.

Berg, R. L. (1960). The ecological significance of correlation pleiades. *Evolution* **13**, 171- 180.

Bolker, J. A. (2000). Modularity in development and why it matters in Evo-Devo. *Am. Zool.* **40**, 770-776.

Bowman, J. L. (1997). Evolutionary conservation of angiosperm flower development at the molecular and genetic levels. *J. Biosci.* **22**, 515-527.

Cheverud, J. M. (1982). Phenotypic, genetic, and environmental morphological integration in the cranium. *Evolution* **36**, 499-516.

Conner, J. K., and *A. Sterling* (1996). Selection for independent floral and vegetative traits: evidence from correlation patterns in five species. *Can. J. Bot.* **74**, 642-644.

Falconer, D. S, and *T. F. C. Mackay* (1996). *Introduction to quantitative genetics*. Prentice Hall, New York, New York, USA.

Fenster, C. B., and *L. F. Galloway* (1997). Developmental homeostasis and floral form: evolutionary consequences and genetic basis. *Int. J. Plant Sci.* **158**, S121-S130.

Galen, C. (1999). Why do flowers vary? The functional ecology of variation in flower size and form within natural populations. *BioSci.* **49**, 631-640.

Haber, A. (2011). A comparative analysis of integration indices. *Evol. Biol.* **38**, 476-488.

Juenger, T., Perez-Perez, J. M., Bernal, S., and *J. L. Micol* (2005). Quantitative trait loci mapping of floral and leaf morphology traits in *Arabidopsis thaliana*: evidence for modular genetic architecture. *Evol. Dev.* **7**, 259-271.

Kawecki, T. J., and *D. Ebert* (2004). Conceptual issues in local adaptations. *Ecol. Lett.* **7**, 1225-1241.

Klingerberg, C. P. (2008). Morphological integration and developmental modularity. *Annu. Rev. Ecol. Evol. S.* **39**, 115-132.

Lande, R. (1980). The genetic covariance between characters maintained by pleiotropic mutations. *Genetics* **94**, 203-215.

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

Magwene, P. M. (2006). Integration and modularity in biological systems: a review. *Acta Zool. Sinica* **52**, (Supplement), 490-493.

Mitchell-Old, T., and *R. Shaw* (1989). Regression analyses of natural selection: statistical inference and biological interpretation. *Evolution* **41**, 1147-1161.

Mitteroecker, P., and *F. L. Bookstein* (2007). The Conceptual and Statistical Relationship between Modularity and Morphological Integration. *Syst. Biol.* **56**, 818–836.

Murren, C. J. (2002). Phenotypic integration in plants. *Plant Spec. Biol.* **17**, 89-99.

Murren, C. J., Pendleton, N., and *M. Pigliucci* (2002). Evolution of phenotypic integration in *Brassica* (Brassicacae*). Am. J. Bot.* **89**, 655-663.

Olson, E. C., and *R. L. Miller* (1951). A mathematical model applied to a study of the evolution of species. *Evolution* **5**, 325-339.

Olson, E. C., and *R. L. Miller* (1958). *Morphological Integration*. University of Chicago Press, Chicago,USA.

Ordano, M., Fornoni, J., Boege, K., and *C. Domínguez* (2008). The adaptive value of phenotypic floral integration. *New Phytol.* **179**, 1183-1192.

Pavlicev, M., Cheverud, J. M., and *G. P. Wagner* (2009). Measuring morphological integration using eigenvalue variance. *Evol. Biol.* **36**, 157-170.

Pérez, F., Arroyo, M. T. K., and *R. Medel* (2007). Phylogenetic analysis of floral integration in Schizanthus (Solanaceae): does pollination truly integrate corolla traits? *J. Evol. Biol.* **20**, 1730-1738.

Philips, P.C., and *S. J. Arnold* (1999). Hierarchical comparison of genetic variance-covariance matrices. I. Using the Flury hierarchy. *Evolution* **53**, 1506-1515.

Pigliucci, M., and *K. Preston* (2004). *Phenotypic Integration: Studying the Ecology and Evolution of Complex Phenotypes.* Oxford University Press, Oxford.

Poorter, H., Niinemets, Ü., Poorter, L., Wright, I. J., and *R. Villar* (2009). Causes and consequences of variation in leaf mass per area (LMA): a meta-analysis. *New Phytol.* **182**, 565-588.

Schlichting, C. D. (1986). Phenotypic plasticity: an evolving plant character. *Biol. J. Linn. Soc.* **29**, 37-47.

Smyth, D. R. (2005). Morphogenesis of flowers – our evolving view. *Plant Cell* **17**, 330-341.

Sokal, M., and *F.J. Rohlf* (1981). *Biometry*. Freeman, New York, USA.

Strauss, Sh. Y., and *J. B.Whittall* (2006). Non-pollinator agents of selection on floral traits. In: *Ecology and Evolution of Flowers*, (Eds. H. D. Lawrence and C.H. Spencer), 120-138. Oxford University Press, New York, USA.

Terentjev, P. V. (1931). Biometrische Untersuchtung über die morphologischen Merkmale von *Rana ridibunda* Pall. (Amphibia, Slientia). *Biometrika* **23**, 23-51.

Tucić, B., and *S. Avramov* (1996). Morphological integration in seedlings of *Iris pumila* L. *Russ. J. Genet.* **32**, 1072-1078.

Tucić, B., Milojković, S., Vujčić, S., and *A. Tarasjev* (1988). Clonal diversity and dispersion in *Iris pumila*. *Acta Oecol.* **9,** 211-219.

Violle, C., Navas, M. L., Vile, D., Kazakou, E., Fortunel, C., Hummel, I., and *E. Garnier* (2007). Let the concept of trait be functional! *Oikos* **116**, 882-892.

Wagner, G P. (1984). On the eigenvalue distribution of genetic and phenotypic dispersion matrices: evidence for a nonrandom organization of quantitative character variation. *J. Math. Biol.* **21,** 77-95.

Wagner, G. P. (1996). Homologues, natural kinds, and the evolutionary modularity. *Am. Zool.* **36**, 36-43.

Wagner, G. P., and *L. Altenberg* (1996). Perspective: Complex adaptations and the evolution of evolvability. *Evolution* **50**, 967-976.

Wright, I. J., Reich, P. B., Westoby, M., Ackerly, D. D., Baruch, Z., Bongers, F., Cavender-Bares, J., Chapin, T., Cornelissen, J. H. C., Diemer, M., Flexas, J., Garnier, E., Lusk, C., Groom, P. K., Gulias, J., Hikosaka, K., Lamont, B. B., Lee, T., Lee, W., Midgley, J. J., Navas, M. L., Niinemets, U., Oleksyn, J., Osada, N., Poorter, H., Poot, P., Prior, L., Pyankov, V. I., Roumet, C., Thomas, S. C., Tjoelker, M. G., Veneklaas, E. J., and *R. Villar* (2004). The worldwide leaf economics spectrum. *Nature* **428**, 821-827.