

# Functional decoupling between flowers and leaves in the *Ameroglossum pernambucense* complex can facilitate local adaptation across a pollinator and climatic heterogeneous landscape

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## Abstract

Decoupling between floral and leaf traits is expected in plants with specialized pollination systems to assure a precise flower–pollinator fit, irrespective of leaf variation associated with environmental heterogeneity (functional modularity). Nonetheless, developmental interactions among floral traits also decouple flowers from leaves regardless of selection pressures (developmental modularity). We tested functional modularity in the hummingbird-pollinated flowers of the *Ameroglossum pernambucense* complex while controlling for developmental modularity. Using two functional traits responsible for flower–pollinator fit [floral tube length (TL) and anther–nectary distance (AN)], one floral trait not linked to pollination [sepal length (SL), control for developmental modularity] and one leaf trait [leaf length (LL)], we found evidence of flower functional modularity. Covariation between TL and AN was ca. two-fold higher than the covariation of either of these traits with sepal and leaf lengths, and variations in TL and AN, important for a precise flower–pollinator fit, were smaller than SL and LL variations. Furthermore, we show that previously reported among-population variation of flowers associated with local pollinator phenotypes was independent from SL and LL variations. These results suggest that TL and AN are functionally linked to fit pollinators and sufficiently decoupled from developmentally related floral traits (SL) and vegetative traits (LL). These results support previous evidences of population differentiation due to local adaptation in the *A. pernambucense* complex and shed light on the role of flower–leaf decoupling for local adaptation in species distributed across biotic and abiotic heterogeneous landscapes.

## Introduction

The study of phenotypic variation within and among the body parts (e.g. organs and limbs) of organisms is an important topic in evolutionary biology because it

directly affects species adaptability. Because traits within the same body part have higher covariation in size than traits among different parts, these parts can be recognized as modules. Modules can arise by developmental processes (developmental modules) and/or selection on a set of traits to perform a particular function (functional modules). A developmental module is a product of the interactions among the developmental pathways of its traits caused by genetic and/or environmental factors. A functional module, in turn, although not necessarily independent from developmental

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interactions, is mostly a product of selection pressures leading to a tightly coordinated covariation among traits to achieve a particular function. In other words, modularity of morphological structures can be due to developmental relatedness and/or selection pressures, both leading to higher trait covariation (or integration) within body parts and decoupled trait variation among these parts (Olson & Miller, 1958; Wagner & Altenberg, 1996; Klingenberg, 2008; Armbruster *et al.*, 2014).

In plants, functional decoupling of flowers from vegetative trait variation is important to local adaptation of populations along heterogeneous landscapes because it facilitates flower adaptation to local pollinators without hampering the adaptation of leaves (or other vegetative organs) to local abiotic environments (e.g. Chalcoff *et al.*, 2008; Cosacov *et al.*, 2014). Berg (1959, 1960) formerly supposed that functional modularity of traits under selection for pollination accuracy would be particularly crucial in plants whose flowers are exclusively pollinated by one or few groups of morphologically similar pollinators (specialized flowers), such as hummingbird-pollinated flowers. For an accurate pollination, specialized flowers are expected to exhibit high integration and low phenotypic variation to tightly fit pollinator's morphology, assuring a precise pollen deposition and pollen collection on a specific part of the pollinator's body. These predictions have been supported by several studies (Berg, 1959, 1960; Conner & Via, 1993; Armbruster *et al.*, 1999; Chalcoff *et al.*, 2008; Pélabon *et al.*, 2011; Cosacov *et al.*, 2014; Pérez-Barrales *et al.*, 2014), suggesting that specialized flowers tend to be functional modules decoupled from vegetative traits. On the other hand, selection for integration and low phenotypic variation in flowers pollinated by several groups of morphologically variable pollinators (generalist flowers) or by wind are not expected to be as strong as in specialized flowers because pollination in the former does not rely on precise flower–pollinator fit (Berg, 1959, 1960). Nonetheless, floral integration and decoupling from vegetative traits have also been observed in generalist and wind-pollinated flowers (e.g. Armbruster *et al.*, 1999; Pérez-Barrales *et al.*, 2007). For such plants, decoupling between flower and vegetative traits might reflect more developmental than functional modularity. Therefore, an appropriate test for functional decoupling between flowers and leaves requires controlling for flower developmental modularity. This is possible to achieve by comparing patterns of covariation and variance of floral traits directly related to pollination, among floral traits related and not related to pollination and among both types of floral traits and vegetative traits (e.g. Conner & Via, 1993; Baranzelli *et al.*, 2014; Cosacov *et al.*, 2014).

Functional decoupling between flowers and leaves is important to be tested at the within-population level because low covariation among traits from different modules observed in individuals occurring in similar environmental conditions is indicative of lack of pleio-

tropism, genetic linkage and/or developmental interactions underlying trait variation between modules. Testing functional decoupling uniquely at the among-population level may be misleading because decoupled traits at the within-population level can covary among populations due to independent selection for similar changes in trait size (Armbruster, 1991; Herrera *et al.*, 2002; Chalcoff *et al.*, 2008). Conversely, once functional decoupling is observed at the within-population level, testing it among populations allows examining whether flowers and leaves are indeed varying in a decoupled trend over, for example, a species distribution range (Chalcoff *et al.*, 2008; Cosacov *et al.*, 2014). Nonetheless, decoupled variation between functional modules among populations in modular organisms might arise by either neutral divergence (genetic drift/demographic history) or adaptive divergence (local adaptation; Armbruster, 1991; Chalcoff *et al.*, 2008). Therefore, examining functional decoupling among populations in the absence of the knowledge of current local selection agents prevents a better understanding of the role of functional decoupling in local adaptation and therefore in the early stages of speciation (Armbruster, 1991; Chalcoff *et al.*, 2008; Cosacov *et al.*, 2014).

The *Ameroglossum pernambucense* complex is a group of shrubs exclusively pollinated by hummingbirds and restricted to the granitic inselbergs – terrestrial island-like environments inhabited by a wide range of exclusive taxa (Porembski, 2007) – from the Borborema Plateau and adjacent areas in North-eastern Brazil (Wanderley *et al.*, 2014a,b). The plants of this species complex are self-compatible, although hummingbirds are necessary to increase fruit and seed formation (Wanderley *et al.*, 2014a). The *A. pernambucense* flowers are tubular and show bilateral symmetry with the reproductive organs (anthers and stigma) positioned in the upper portion of the flower, just before the entrance of the floral tube (Wanderley *et al.*, 2014a). This taxon is considered a species complex because its populations can vary in plant size, phyllotaxy, and leaf and flower shapes (Wanderley *et al.*, 2014a,b). The landscape within the distribution range of this species complex is a mosaic of highly heterogeneous environments, where the lower areas (200–500 m.a.s.l.) of this region are dominated by Caatinga tropical dry forests and the higher lands (> 500 m.a.s.l.) are mainly dominated by Atlantic rainforest enclaves (Andrade-Lima, 1960).

(A.M. Wanderley, I.C.S. Machado, E.M. Almeida, L.P. Felix, L. Galetto, A.M. Benko-Iseppon & V.L.S. Sork, unpublished) in a recent study that examined the role of allopatry and ecology on genetic and phenotypic variation in the *A. pernambucense* complex observed that whereas floral variation among populations was associated with the bill length of the main local pollinators, leaf phenotypic variation was associated with local climates, suggesting that morphological variation in this species complex is likely a product of local adaptation of

populations that generated at least three different ecotypes. These findings also suggest that functional decoupling between flowers and leaves in this species complex might have an important contribution in local adaptation. To assure an accurate pollination, the floral tube length of the *A. pernambucense* complex flowers needs to be integrated with the position of the reproductive organs (anthers and stigma). This is because whereas a hummingbird is seeking for nectar, the floral tube determines the direction to which the hummingbird will access the flower (always unidirectional) and positions the hummingbird's forehead for a standardized contact with the reproductive organs. If floral tube length or the position of the reproductive organs is not integrated, pollination will be inaccurate. Furthermore, if the bill length of the pollinator varies among populations, then a coordinated change in the floral tube length and the position of the reproductive organs will be necessary to maintain flower–pollinator fit (Wanderley *et al.*, 2014a).

The central goal of this study was to test whether the flowers of the *A. pernambucense* complex are functionally decoupled from leaves, allowing flower adaptation to local pollinators without constraining leaf adaptation to local abiotic environments, while controlling for developmental decoupling between these organs. Functional decoupling between flowers and leaves in the *A. pernambucense* complex was tested within and among populations by comparing the strength of integration between two floral traits responsible for pollination accuracy (floral tube length and anther–nectary distance), between each of these floral traits and a floral trait not related to pollination [sepal length] and between all three floral traits and a leaf trait [leaf length]. Additionally, the role of flower–leaf functional decoupling in local adaptation was tested by examining whether the association between flower and main pollinator phenotypes among populations observed by (A.M. Wanderley, I.C.S. Machado, E.M. Almeida, L.P. Felix, L. Galetto, A.M. Benko-Iseppon & V.L.S. Sork, unpublished) was decoupled from sepal and leaf phenotypic variation. Within- and among-population analyses used in this study supported the central hypothesis that the hummingbird-specialist flowers of the *A. pernambucense* complex are sufficiently decoupled from leaves to favour local adaptation.

## Materials and methods

### Study plant and population sampling

The *A. pernambucense* complex has only one described species, *A. pernambucense* Eb. Fisch., S. Vogel & A.V. Lopes, which is included in the IUCN Red List as an Endangered plant (Wanderley *et al.*, 2014b). The flowers of this complex exhibit an orange or red (except for the yellow lower lip) bilabiate and tubular corolla. Flowers have four didynamous exerted stamens with filaments

adnate to the corolla at the base. The gynoecium has a conical ovary and a filiform exerted style. The 5-lobed calyx has the largest upper lobe and is located at the base of the corolla. Although the calyx has apparently the function of protecting the floral buds and possibly the capsules after fruit formation, no function can be attributed to this structure during pollination (Fischer *et al.*, 1999; pers. obs.). These flowers can offer copious amounts of nectar (up to 107.9  $\mu$ L and 24.8 mg of sugar per flower) and are exclusively pollinated by hummingbirds. Although spontaneous self-pollination may occur, when exposed to pollinators, flowers produced significantly higher fruit and seed set. Therefore, responses of floral traits to pollinator selection are expected (Wanderley *et al.*, 2014a; (A.M. Wanderley, I.C.S. Machado, E.M. Almeida, L.P. Felix, L. Galetto, A.M. Benko-Iseppon & V.L.S. Sork, unpublished)). The leaves of this complex are lanceolate, cauline, opposite decussate or three to six verticillate (pers. obs.).

(A.M. Wanderley, I.C.S. Machado, E.M. Almeida, L.P. Felix, L. Galetto, A.M. Benko-Iseppon & V.L.S. Sork, unpublished) observed that the *A. pernambucense* complex is composed of at least three distinct ecotypes with varying sizes of flowers and leaves apparently adapted to local pollinators and environmental conditions, respectively (Fig. 1; A.M. Wanderley, I.C.S. Machado, E.M. Almeida, L.P. Felix, L. Galetto, A.M. Benko-Iseppon & V.L.S. Sork, unpublished). The first ecotype (*pernambucense* ecotype) is the species described as *A. pernambucense*, which has the shortest floral tube (~2.8 cm) and the smallest leaves (~5.7 cm long), and occurs on highland inselbergs (1000–1200 m.a.s.l.) exposed to intense wind conditions and low temperatures at night (~4 °C), with average annual rainfall ~1000 mm. The main pollinators observed in two populations of this ecotype are the short-billed hummingbirds *Chlorostilbon lucidus* (bill length ~1.9 cm) and *Amazilia fimbriata* (bill length ~1.97 cm). The second ecotype (*dry-season* ecotype) has flowers with intermediate floral tube length (~3.8 cm) and leaf size (~9.5 cm long). Their populations occur in low–mid-elevation inselbergs (150–800 m.a.s.l.), exposed to high solar irradiation, severe drought periods (~6 months) and annual rainfall of ~600 mm. The main pollinator observed in *dry-season* populations is the medium-billed (bill length ~2.4 cm) hummingbird *Eupetomena macroura*. The third ecotype (*forest-immersed* ecotype) has the longest floral tubes (~4.5 cm) and the largest leaves (~14.35 cm long). Their populations occur in mid-elevation (500–600 m.a.s.l.) forest-immersed inselbergs partially covered by the Atlantic forest canopy with lower levels of solar irradiation, but with an average annual rainfall similar to that faced by the *pernambucense* ecotype. The main pollinator observed in two populations of this ecotype is the long-billed (bill length ~3.5 cm) hummingbird *Phaethornis pretrei* (A.M. Wanderley, I.C.S. Machado, E.M. Almeida, L.P. Felix, L.



**Fig. 1** Morphological variation of three ecotypes of the *Ameroglossum pernambucense* complex, ecotype *pernambucense* (a), ecotype *dry-season* (b) and ecotype *forest-immersed* (c). Flowers and leaves on the left are from stem cuttings of wild plants grown in common garden.

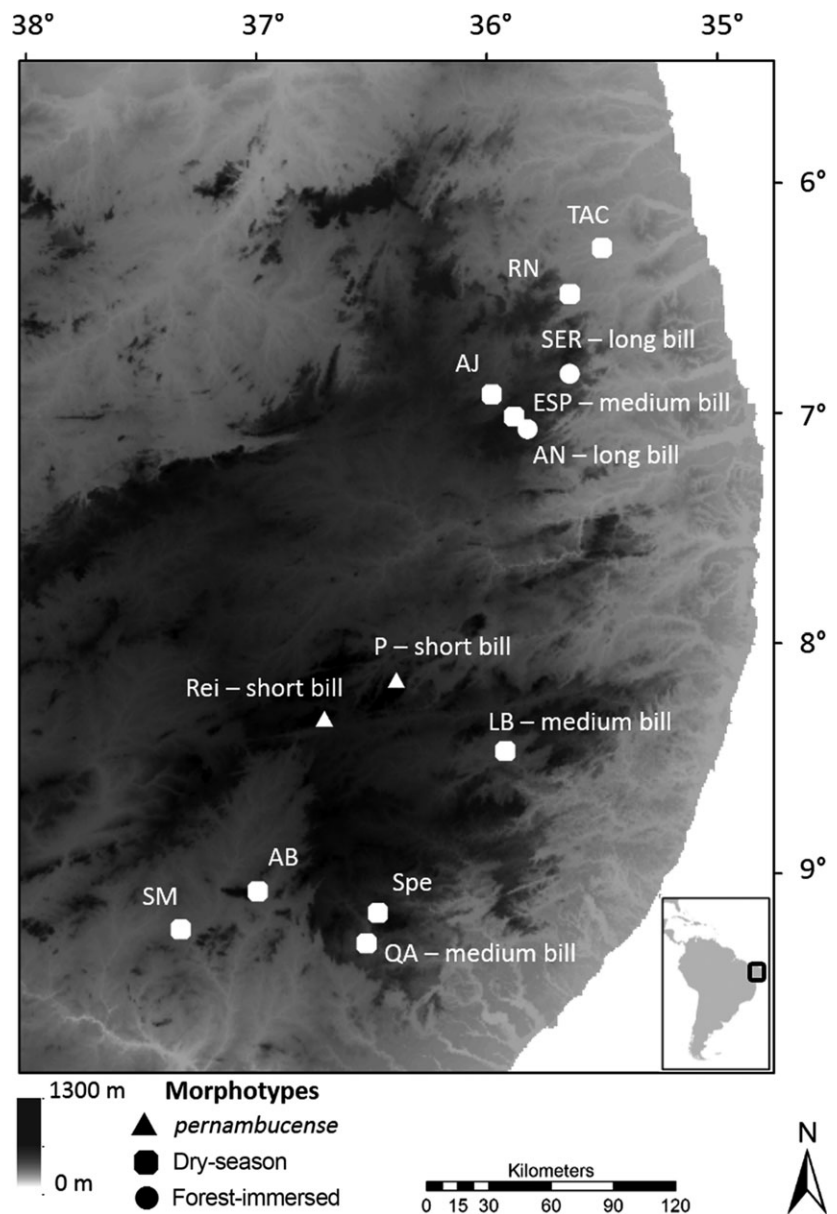
Galetto, A.M. Benko-Iseppon & V.L.S. Sork, unpublished).

Flowers and leaves were sampled in 13 populations, including seven populations where pollinator information is available (A.M. Wanderley, I.C.S. Machado, E.M. Almeida, L.P. Felix, L. Galetto, A.M. Benko-Iseppon & V.L.S. Sork, unpublished), of the three ecotypes along the entire geographical range known for the *A. pernambucense* complex (Fig. 2). The number of sampled individuals per population depended on population size and varied from eight to 25 (total = 184 individuals). For each plant, 1–3 fully developed flowers and leaves were collected per individual. To reduce subindividual leaf variation along the stems (Herrera, 2009), leaves were always collected at the medial portion of fully developed stems. The overall number of leaves and flowers collected was, respectively, 549 and 307. Because of the rarity of the first and third ecotypes, only two populations of each of these two ecotypes were sampled, against nine populations sampled for the second ecotype (Table 1). The samples from the 13 populations used in this study are a subset of a larger population sampling across 17 populations of the *A. pernambucense* complex used in another study focused on understanding the relative roles of geographical and ecological barriers to gene flow and whether these barriers create opportunity for local adaptation in this complex (A.M. Wanderley, I.C.S.

Machado, E.M. Almeida, L.P. Felix, L. Galetto, A.M. Benko-Iseppon & V.L.S. Sork, unpublished).

### Trait measures and data analyses

Two pollination accuracy-related traits, floral tube length and anther–nectary distance, one floral trait not related to pollination, the length of the upper sepal (sepal length), and one leaf trait, leaf length, were used to test functional decoupling between flowers and leaves in the *A. pernambucense* complex populations. floral tube length and anther–nectary distance variation are expected to be highly coordinated because the former is responsible to place the hummingbird’s forehead against the anthers and the stigma while the hummingbird is seeking for nectar within the flower. Low covariation between these two traits, as well as high phenotypic variation of one or both of these traits, might prevent an efficient pollen transfer. On the other hand, from a functional viewpoint, high covariations of floral tube length and anther–nectary distance with sepal length and leaf length are not necessary for an accurate pollination. Therefore, if the flowers of the *A. pernambucense* complex are functional modules, high covariation is expected between floral tube length and anther–nectary distance, whereas low covariation is expected between each of these two traits and sepal



**Fig. 2** Sampled populations of three ecotypes of the *Ameroglossum pernambucense* complex. Bill length categories of the main local pollinators of seven populations were extracted from (A.M. Wanderley, I.C.S. Machado, E.M. Almeida, L.P. Felix, L. Galetto, A.M. Benko-Iseppon & V.L.S. Sork, unpublished) and are presented as 'short bill', 'medium bill' and 'long bill'.

length and leaf length. The distance between stigma and nectary was not selected instead of anther-nectary distance, because the style of the flowers of the *A. pernambucense* complex is a flexible structure that slips back and forth allowing a better accommodation of the stigma to the hummingbird's forehead, irrespective of the strength of this contact. The filaments, on the other hand, are more sessile and less flexible structures. Therefore, because of the style flexibility, the strength of natural selection for a tight covariation between pistil length and floral tube length, as well as for low variance of the stigma-nectary distance, might be weaker than the strength of natural selection for a tight covari-

ation between floral tube length and anther-nectary distance, and low phenotypic variation of the latter. Indeed, these predictions were supported during the preliminary analyses of this study (results not reported).

The aim of the first analysis of this study is to explore the variation of the four measured traits across the sampled populations. Therefore, one-way analysis of variance (ANOVA) of each trait was performed to test for significant differences of these traits among the study populations. Then, *post hoc* multiple comparisons among each population pair were performed using the Tukey's test. To satisfy the assumption of homoscedasticity, data

**Table 1** Sampled populations of the *Ameroglossum pernambucense* complex. Populations highlighted in bold are those where phenotypic correspondence between flowers and main local pollinator was observed (A.M. Wanderley, I.C.S. Machado, E.M. Almeida, L.P. Felix, L. Galetto, A.M. Benko-Iseppon & V.L.S. Sork, unpublished).

Population	Elevation (m)	Ecotype	N (sampled plants)	Bill length of the main pollinator (cm)
<b>P</b>	<b>1215</b>	<b><i>pernambucense</i></b>	<b>15</b>	<b>1.9</b>
<b>Rei</b>	<b>1086</b>	<b><i>pernambucense</i></b>	<b>16</b>	<b>1.97</b>
<b>QA</b>	<b>514</b>	<b><i>dry-season</i></b>	<b>25</b>	<b>2.4</b>
Spe	808	<i>dry-season</i>	20	-
SM	796	<i>dry-season</i>	9	-
AB	860	<i>dry-season</i>	12	-
<b>LB</b>	<b>443</b>	<b><i>dry-season</i></b>	<b>11</b>	<b>2.4</b>
<b>ESP</b>	<b>640</b>	<b><i>dry-season</i></b>	<b>16</b>	<b>2.4</b>
AJ	650	<i>dry-season</i>	8	-
TAC	162	<i>dry-season</i>	10	-
RN	150	<i>dry-season</i>	13	-
<b>AN</b>	<b>629</b>	<b><i>forest-immersed</i></b>	<b>12</b>	<b>3.5</b>
<b>SER</b>	<b>549</b>	<b><i>forest-immersed</i></b>	<b>17</b>	<b>3.5</b>
		Total	184	

were square-root-transformed. After transformation, only anther-nectary distance did not pass the Levene test of homoscedasticity, showing a marginal *P*-value (0.047). Nonetheless, because natural log and power of two transformations failed to increase homoscedasticity, the square root transformation of anther-nectary distance was used in the ANOVA.

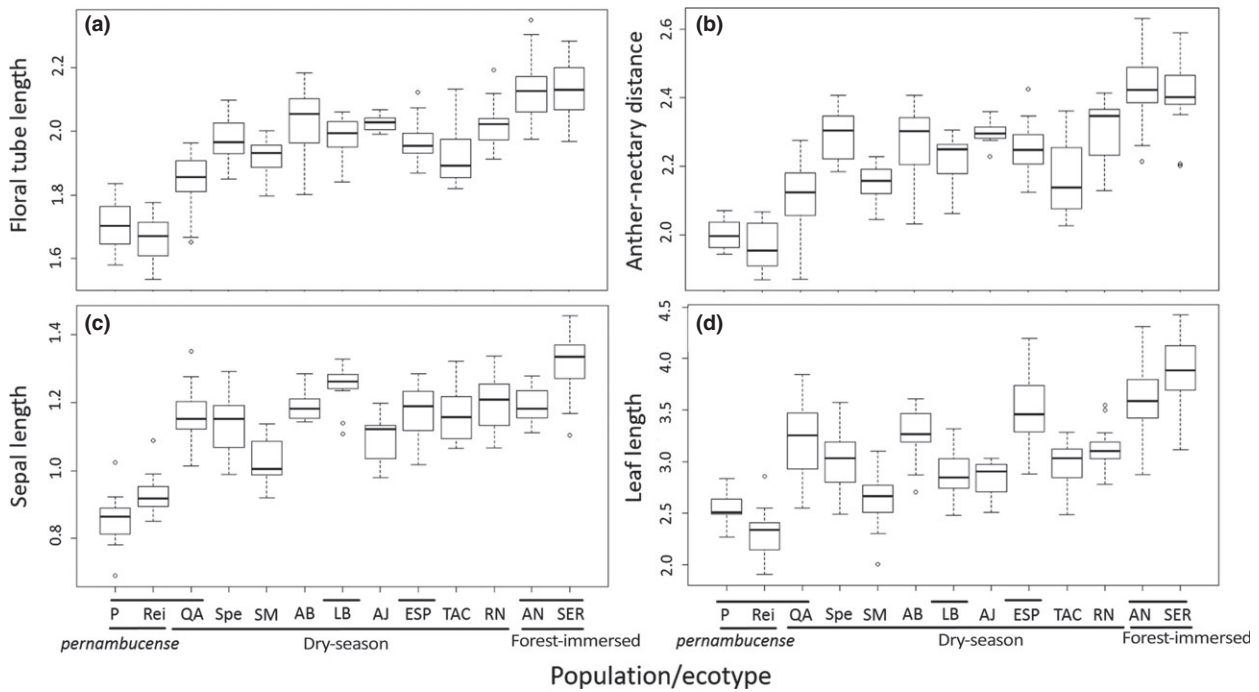
#### *Functional decoupling at the within-population level*

Several statistical analyses were performed to test functional decoupling between flowers and leaves. At the within-population level, analyses were as follows. First, four groups of within-population Pearson correlation tests between each pair of measured traits were performed: floral tube length vs. anther-nectary distance (TL vs. AN), floral tube length and anther-nectary distance vs. sepal length (TL/AN vs. SL), floral tube length and anther-nectary distance vs. leaf length (TL/AN vs. LL) and sepal length vs. leaf length (SL vs. LL). Prior to the correlation tests, the distribution normality of each trait for each population was tested using the Shapiro-Wilk normality test. Both significant and nonsignificant coefficients were used in the further analysis. The first test of functional decoupling at the within-population level was conducted by comparing by one-way ANOVA the means of the absolute values (i.e. the modulus) of the correlation coefficients obtained for each group of correlation tests mentioned above (TL vs. AN, TL/AN vs. SL, TL/AN vs. LL and SL vs. LL). Because the correlation coefficients obtained were not fully independent, the significance of differences between these means was tested after 20 000 data permutations. In the case

of functional decoupling, TL vs. AN should have the highest correlation coefficient mean (Berg, 1959, 1960). Conversely, similar correlation coefficient means of TL vs. AN and TL/AN vs. SL, higher than the means of the correlation coefficients of TL/AN vs. LL and SL vs. LL would be an evidence that these flowers are developmental modules. Functional decoupling at the within-population level was further tested by obtaining the coefficient of variation (CV = standard deviation/mean\*100) of each measured trait for each population and comparing the CV means by one-way ANOVA. Significant differences among means were also tested after 20 000 permutations of the data set. In the case of functional modularity, floral tube length and anthers-nectary should have the lowest CV means, that is the highest stability, whereas in the case of developmental modularity, floral tube length, anthers-nectary distance and sepal length would have similar CV means, all higher than the leaf length CV mean (Berg, 1959, 1960). *Post hoc* multiple comparisons to test significant differences among the levels of the factors used in these ANOVAS (correlation categories and trait CVs) were corrected using the Bonferroni method (i.e.  $\alpha = 0.05/\text{number of comparisons}$ ).

#### *Functional decoupling at the among-population level*

Functional decoupling at the among-population level was tested as follows. First, four analyses of covariance (ANCOVAS) were performed to test whether the observed among-population significant differences of floral tube length and anther-nectary distance (see Results) remained significant when controlling for sepal length and leaf length covariation independently. These ANCOVAS used the same data set used in the first ANOVA to explore the variation of the four measured traits across the sampled populations. If among-population differences in floral tube length and anther-nectary distance are adaptations to local pollinator assemblies, and flowers and leaves are functionally decoupled, then these differences should remain significant after controlling for possible covariation with sepal length and leaf length. Seven of the 13 examined populations have pollinator information available (Table 1), and among-population variation in floral tube length in these populations is associated with the bill length of the most frequently observed local pollinators (A.M. Wanderley, I.C.S. Machado, E.M. Almeida, L.P. Felix, L. Galetto, A.M. Benko-Iseppon & V.L.S. Sork, unpublished). Therefore, ANCOVAS in this subset of populations allowed us to investigate the importance of functional decoupling for independent adaptation of flowers and leaves to local pollinators and local environmental conditions, respectively, testing whether floral tube length and anther-nectary distance means of the populations pollinated by hummingbirds with different bill sizes were significantly different after removing sepal length and leaf length covariance. ANCOVAS were performed with



**Fig. 3** Among-population variation of three floral traits (floral tube length, anther–nectary distance and sepal length) and one leaf trait (leaf length) of the *Ameroglossum pernambucense* complex. Bars below population names divide the populations belonging to three different ecotypes. Bars above population names indicate the populations where phenotypic correspondence between flowers and main local pollinators was observed (A.M. Wanderley, I.C.S. Machado, E.M. Almeida, L.P. Felix, L. Galetto, A.M. Benko-Iseppon & V.L.S. Sork, unpublished). The data of the four traits presented were square-root-transformed. P = Serra do Ponto, Rei = Pedra do Rei, QA = Quebrangulo, Spe = Serra do Pedro, SM = Serra de Maravilha, AB = Águas Belas, LB = Lajeiro do Boi, AJ = Algodão de Jandaíra, ESP = Esperança, TAC = Tacima, RN = Rio Grande do Norte, AN = Alagoa Nova, SER = Serraria.

post hoc Tukey's test for pairwise comparisons among populations. Finally, the importance of functional decoupling for local adaptation was also tested using the same subset of the seven populations mentioned above by comparing four simple linear regression models. These models tested whether among-population variations of the bill length of the main local pollinators of the subset of populations better predicted among-population variation of floral tube length and anther–nectary distance than among-population variation of sepal length and leaf length. Under a functional decoupling scenario, higher regression coefficients are expected for the models floral tube length or anther–nectary distance =  $a + b \cdot \text{bill length} + \text{error}$ , than the models sepal length or leaf length =  $a + b \cdot \text{bill length} + \text{error}$ . To achieve homoscedasticity and distribution normality of the error, all four measured traits were square-root-transformed.

## Results

The first one-way ANOVA revealed significant differences among populations for all examined traits (Fig. 3;

Table 2). Although populations of the ecotypes *pernambucense* and *forest-immersed* were significantly different for all the traits, populations of the *dry-season* ecotype were more morphologically heterogeneous, and some traits did not vary significantly from the other ecotypes. This was due to some overlap in trait size of *dry-season* ecotype with both *pernambucense* and *forest-immersed* ecotypes, and to significant differences among some populations of the former ecotype (Table 2). All populations, but QA, which main hummingbird pollinators differed in bill length also showed significant differences in floral tube length, anther–nectary distance and sepal length means. Although QA, LB and ESP populations are mainly pollinated by the same hummingbird (*E. macroura*; (A.M. Wanderley, I.C.S. Machado, E.M. Almeida, L.P. Felix, L. Galetto, A.M. Benko-Iseppon & V.L.S. Sork, unpublished), anther–nectary distance and floral tube length means of QA were significantly different from LB and ESP means. However, this result is likely due to outliers (Fig. 3). Among-population patterns of leaf length variation were slightly different from the variation patterns of floral tube length, anther–nectary distance and sepal length (Fig. 3; Table 2).

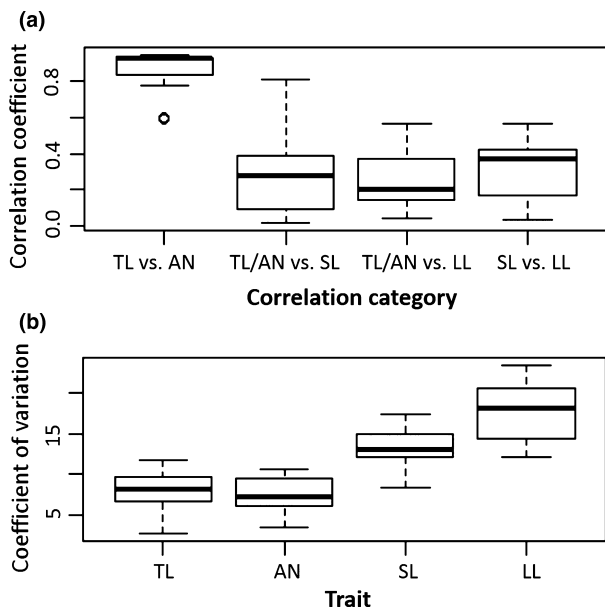
**Table 2** Results of the one-way ANOVA comparing the variation of three floral traits and one leaf trait among 13 populations of the *Amerigoosum pernambucense* complex. Significance of *post hoc* multiple comparisons (Tukey's test) is represented by letters ( $\alpha = 0.05$ ).

Square root of tube length				Square root of anther-nectary distance			
Population	Mean	SE	N	Population	Mean	SE	N
Rei	1.66	0.02	16	Rei	1.97	0.02	16
P	1.7	0.02	15	P	2	0.02	15
QA	1.85	0.02	25	QA	2.11	0.02	25
SM	1.92	0.03	9	SM	2.15	0.03	9
TAC	1.93	0.03	10	TAC	2.17	0.03	10
ESP	1.97	0.02	16	LB	2.22	0.03	11
Spe	1.97	0.02	20	ESP	2.25	0.02	16
LB	1.97	0.02	11	AB	2.27	0.03	12
RN	2.02	0.02	13	Spe	2.29	0.02	20
AJ	2.03	0.03	8	AJ	2.3	0.03	8
AB	2.03	0.02	12	RN	2.31	0.02	13
SER	2.13	0.02	17	SER	2.41	0.02	17
AN	2.13	0.02	12	AN	2.43	0.03	12

Square root of sepal length				Square root of leaf length			
Population	Mean	SE	N	Population	Mean	SE	N
P	0.86	0.02	15	Rei	2.3	0.07	16
Rei	0.93	0.02	16	P	2.52	0.08	15
SM	1.03	0.03	9	SM	2.62	0.1	9
AJ	1.09	0.03	8	AJ	2.84	0.1	8
Spe	1.14	0.02	20	LB	2.9	0.09	11
QA	1.16	0.02	25	TAC	2.98	0.09	10
TAC	1.17	0.02	10	Spe	3.02	0.07	20
ESP	1.17	0.02	16	RN	3.13	0.08	13
AB	1.19	0.02	12	QA	3.23	0.06	25
AN	1.19	0.02	12	AB	3.26	0.08	12
RN	1.21	0.02	13	ESP	3.51	0.07	16
LB	1.24	0.02	11	AN	3.63	0.08	12
SER	1.3	0.02	17	SER	3.87	0.07	17





**Fig. 4** Means of within population Pearson correlation coefficients (A) and coefficients of variation (B) of floral and leaf traits from 13 populations of the *Ameroglossum pernambucense* complex. TL = floral tube length, AN = anther-nectary distance, SL = sepal length and LL = leaf length.

#### Functional decoupling at the within-population level

One-way ANOVA ( $F_{3,48} = 33.49$ ;  $P = 0.0$ ) revealed that the correlation coefficient mean of TL vs. AN ( $0.86 \pm 0.04$  – mean  $\pm$  SE) was significantly greater than the means of TL/AN vs. SL ( $0.29 \pm 0.06$ ), TL/AN vs. LL ( $0.27 \pm 0.05$ ) and SL vs. LL ( $0.30 \pm 0.05$ ; Fig 4a), thus supporting functional decoupling indicated by higher covariation (integration) between floral tube length and anther-nectary distance than between any other pair of measured traits. Although TL vs. AN correlations were significant within all populations, significant TL/AN vs. SL was found in Spe and TAC populations, and significant correlations between anther-nectary distance and leaf length were observed in AB and LB populations. These results indicate that despite the evidence of flower functional modularity, developmental and incomplete functional decoupling between flowers and leaves in the *A. pernambucense* complex is also present. The correlation matrices of the four measured traits within the studied populations are shown in Table S1. Functional decoupling was also supported by the one-way ANOVA comparing the CV means of the investigated traits. Both floral tube length and anther-nectary distance showed lower variation, respectively, represented by the following CV means,  $8.09 \pm 0.65$  and  $7.27 \pm 0.63$ , which were significantly smaller than the CV means of sepal length and leaf length, respectively,  $13.10 \pm 0.73$  and  $17.73 \pm 0.95$

( $F_{3,48} = 41.81$ ;  $P = 0.001$ ). Nonetheless, evidence of developmental decoupling was also observed, as sepal length showed significantly smaller CV mean than leaf length ( $P = 0.001$ ; Fig. 4b). The CVs of all the traits per population are shown in Table S2.

#### Functional decoupling at the among-population level

Evidence of functional decoupling between flowers and leaves was also obtained at the among-population level. ANCOVAs revealed that most among-population variation in floral tube length and anther-nectary distance observed in the former ANOVA remained significant after removing both sepal length and leaf length covariances (Table 3). However, only sepal length showed significant covariation with floral tube length ( $R^2 = 0.57$ ;  $F_{1,170} = 47.81$ ;  $P = 0.0001$ ) anther-nectary distance ( $R^2 = 0.64$ ;  $F_{1,170} = 39.99$ ;  $P = 0.0001$ ). ANCOVAs also revealed that the significant differences in floral tube length and anther-nectary distance among the subset of populations of the three ecotypes where (A.M. Wanderley, I.C.S. Machado, E.M. Almeida, L.P. Felix, L. Galetto, A.M. Benko-Iseppon & V.L.S. Sork, unpublished) observed floral tube length variation associated with the bill length of the main local pollinators remained significant after controlling for sepal and leaf length covariances (Table 3). This result indicates that functional decoupling between flowers and leaves facilitates local adaptation in the *A. pernambucense* complex, permitting flower adaptation to local pollinators without preventing leaf adaptation to local environments. In contrast, all regression models used to test decoupled adaptation of flowers to local pollinators by comparing the strength of association between all four measured traits and the bill length of the main local pollinators were significant, indicating that decoupling between flowers and leaves is not complete. Nonetheless, the highest regression coefficients were obtained for the models using as response variables floral tube length ( $adj R^2 = 0.70$ ;  $F_{1,111} = 255.00$ ;  $P < 0.0001$ ) and anther-nectary distance ( $adj R^2 = 0.69$ ;  $F_{1,111} = 249.52$ ;  $P < 0.0001$ ), rather than using sepal length ( $adj R^2 = 0.46$ ;  $F_{1,111} = 94.23$ ;  $P < 0.0001$ ) and leaf length ( $adj R^2 = 0.55$ ;  $F_{1,111} = 138.69$ ;  $P < 0.0001$ ) as response variables (Table 4).

#### Discussion

Overall, the results of this study suggest that the flowers of the *A. pernambucense* complex are sufficiently decoupled from leaves to allow flower adaptation to local pollinator faunas without hampering leaf adaptation to environmental selection. At the within-population level, functional decoupling was supported by strong integration between two traits important for an accurate hummingbird pollination, floral tube length

**Table 3** ANCOVAs of among-population variation of two floral traits important for pollination accuracy, floral tube length and anther–nectary distance, of the *Ameroglossum pernambucense* complex, using sepal length and leaf length as covariates.

Square root of floral tube length						Square root of anther–nectary distance					
Covariate: sepal length			Covariate: leaf length			Covariate: sepal length			Covariate: leaf length		
$F = 17.66$ ; d.f. = 12; $MS = 0.11$ ; $P < 0.0001$						$F = 19.12$ ; d.f. = 12; $MS = 0.14$ ; $P < 0.0001$					
Tukey's test: $LSD = 0.1$ , d.f. = 170						Tukey's test: $LSD = 0.11$ , d.f. = 170					
Population	Mean	SE	N			Population	Mean	SE	N		
Rei	1.73	0.03	16	A		Rei	2	0.03	16	A	
P	1.79	0.03	15	A	B	P	2.02	0.03	15	A	A
QA	1.84	0.02	25	B	C	QA	2.11	0.02	25	A	B
TAC	1.92	0.02	10	C	D	SM	2.17	0.03	9	B	C
LB	1.94	0.03	11	C	D	TAC	2.17	0.03	10	B	C
SM	1.95	0.03	9	D	E	LB	2.23	0.03	11	C	D
ESP	1.95	0.02	16	D	E	ESP	2.24	0.02	16	C	D
Spe	1.97	0.02	20	D	E	AB	2.26	0.03	12	C	D
RN	2	0.02	13	D	E	Spe	2.29	0.02	20	D	E
AB	2.01	0.02	12	D	E	AJ	2.31	0.03	8	D	E
AJ	2.04	0.03	8	D	E	RN	2.31	0.02	13	D	E
SER	2.07	0.02	17	D	E	SER	2.38	0.03	17	D	E
AN	2.11	0.02	12	D	E	AN	2.41	0.03	12	D	E

**Table 4** Results of four linear regression models where the same predictor variable 'bill length of the main local pollinator' was regressed against two floral traits directly related to pollination, floral tube length (TL) and anther–nectary distance (AN), and two traits not related to pollination, sepal length (SL) and leaf length (LL). The data set used in this analysis is from seven populations of the *Ameroglossum pernambucense* complex where phenotypic correspondence between flowers and main local pollinators was observed (A.M. Wanderley, I.C.S. Machado, E.M. Almeida, L.P. Felix, L. Galetto, A.M. Benko-Iseppon & V.L.S. Sork, unpublished).

Response variable	<i>N</i>	Adjusted <i>R</i> <sup>2</sup>	d.f.	MS	<i>F</i>	<i>P</i> -value
TL	112	0.7	1	2.75	255.0	< 0.0001
AN	112	0.69	1	2.67	249.52	< 0.0001
SL	112	0.46	1	1.41	94.23	< 0.0001
LL	112	0.55	1	23.41	138.69	< 0.0001

and anther-nectary distance, which were poorly integrated with a floral trait not related to pollination, and to a leaf trait, respectively, sepal length and leaf length. Furthermore, the low phenotypic variation expected for key traits to assure a precise pollination in specialized flowers (Berg, 1959, 1960) was observed for floral tube length and anther-nectary distance, but the same was not observed for sepal and leaf lengths, as expected for functional modules (Klingenberg, 2008; Armbruster *et al.*, 2014). Populations strongly differed in the two traits associated with the accuracy of the pollen transfer, tube length and anther-nectary distance, even when the among-population differences in sepal length and leaf length were accounted for statistically, and this also included a subsample of populations where flowers fit to local pollinator phenotypes were previously observed by (A.M. Wanderley, I.C.S. Machado, E.M. Almeida, L.P. Felix, L. Galetto, A.M. Benko-Iseppon & V.L.S. Sork, unpublished). Nonetheless, developmental decoupling between flowers and leaves was suggested by the statistically smaller variation of sepal length when compared to leaf length variation and by the significant covariation between the bill length of the main local pollinators and sepal length. Also, the significant covariations between anther-nectary distance and leaf length in two populations, and between local pollinators' bill length and leaf length suggested incomplete flower–leaf decoupling in the *A. pernambucense* complex. Interestingly, even in the presence of developmental and incomplete flower–leaf decoupling, among-population variation of the flower traits important for a precise pollination was independent from among-population leaf variation in the *A. pernambucense* complex. This result suggests that independent evolution of flower size and leaf size does not necessarily require complete decoupling between their phenotypic variations.

The significant covariation between the bill length of the main local pollinators and leaf length is one of the results indicating incomplete functional decoupling

between flowers and leaves in the *A. pernambucense* complex. Nonetheless, a pollinator–climate association may be an alternative process explaining bill–leaf covariation. (A.M. Wanderley, I.C.S. Machado, E.M. Almeida, L.P. Felix, L. Galetto, A.M. Benko-Iseppon & V.L.S. Sork, unpublished) observed that the ecotype *pernambucense* presents the shortest floral tube length and anther-nectary distance and is pollinated by the hummingbirds with the shortest bills among all pollinators observed for the *A. pernambucense* complex. This ecotype also has the smallest leaves. *Pernambucense* ecotype populations are located at the highest elevations recorded for the complex (A.M. Wanderley, I.C.S. Machado, E.M. Almeida, L.P. Felix, L. Galetto, A.M. Benko-Iseppon & V.L.S. Sork, unpublished), and leaf size is generally negatively correlated with altitude (Milla & Reich, 2011). Similarly, populations of the ecotype *forest-immersed* have the longest floral tube length, anthers-nectary distance and leaf length, and are pollinated by the hummingbirds with the longest bills when compared to the pollinators of the other ecotypes. However, the populations of the *forest-immersed* ecotypes occur under the most combined humidity and shaded conditions (A.M. Wanderley, I.C.S. Machado, E.M. Almeida, L.P. Felix, L. Galetto, A.M. Benko-Iseppon & V.L.S. Sork, unpublished). Humidity tends to be positively associated with leaf size, whereas solar irradiation is negatively related to it (Pélabon *et al.*, 2011). Finally, the populations of the *dry-season* ecotype exhibit intermediate floral tube length, anthers-nectary distance and leaf length, and are pollinated by hummingbirds with intermediate bill lengths. Nonetheless, these populations are also found under intermediate elevation and humidity conditions, relative to the conditions experienced by the other two ecotypes (A.M. Wanderley, I.C.S. Machado, E.M. Almeida, L.P. Felix, L. Galetto, A.M. Benko-Iseppon & V.L.S. Sork, unpublished). Thus, independent selection pressure leading to similar changes in floral tube length and anther-nectary distance (by pollinators) and leaf length (by environment) is possibly underestimating the strength of functional decoupling in the *A. pernambucense* complex. An additional support for this hypothesis is the covariation between bill length and sepal length, which, although significant, showed the smallest regression coefficient (Table 4). sepal length in this species complex is not expected to respond to neither pollinators nor environmental selection pressures. Therefore, if the observed bill–leaf covariation purely resulted from flower–leaf coupling, then bill length covariation with sepal length should be as strong as it was with floral tube length and anther-nectary distance.

The results here presented, together with flower variation associated with local pollinator phenotypes observed by (A.M. Wanderley, I.C.S. Machado, E.M. Almeida, L.P. Felix, L. Galetto, A.M. Benko-Iseppon &

V.L.S. Sork, unpublished) in the *A. pernambucense* complex, indicate that functional decoupling can not only potentially facilitate local adaptation in this species complex, but also that this is indeed allowing local adaptation of flowers without constraining leaf's local adaptation. Among-population covariation between specialized flower morphology and pollinator morphology is not an obvious expectation. For instance, the specialized flowers of *Helleborus foetidus* and *Viola cazorlensis*, respectively, pollinated by few species of bees and a single hawkmoth species, did not show an among-population variation consistent with the morphology of their local pollinators (Herrera, 1993; Herrera *et al.* 2002). This lack of among-population fit between flowers and pollinators highlights the assertion summarized by Armbruster (1991), who stresses the importance of neutral factors such as demographic history and genetic drift underlying among-population trait variation. Conversely, the results here presented are in accordance with results obtained by Cosacov *et al.* (2014). These authors also reported an among-population covariation between pollinator size (two oil-collecting bees) and the floral traits directly related to pollinator fit in *Calceolaria polyrhiza*, whereas these traits were decoupled from corolla size, a trait not directly related to pollination efficiency in this species. Moreover, the variation of the key pollination traits examined by Cosacov *et al.* (2014) was also decoupled from the variation of vegetative traits associated with local climates, thus facilitating local adaptation in this species.

Testing functional decoupling of flowers from leaves, while controlling for the almost inherent developmental modularity of flowers, likely avoided misleading results due to the confounding effects of functional and developmental factors affecting flower and leaf variation at both within- and among-population levels. Although incomplete functional decoupling between flowers and leaves was observed, it did not prevent independent variation of flower and leaf phenotypes among populations of the *A. pernambucense* complex, increasing chances of local adaptation of these populations across a highly heterogeneous pollinator and climatic landscape.

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### Supporting information

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

**Table S1** Within population correlation matrices between pairs of floral and leaf traits in 13 populations of the *Ameroglossum pernambucense* complex. The values in the lower triangular hemi-matrix are the Pearson correlation coefficients between trait pairs, whereas in the upper triangular hemi-matrix are the *P-values* of these correlation tests. TL = floral tube length; AN = anther-nectary distance; SL = sepal length; LL = leaf length.

**Table S2** Coefficient of variation of three floral traits and one leaf trait in 13 populations of the *Ameroglossum pernambucense* complex. TL = floral tube length; AN = anther-nectary distance; SL = sepal length; LL = leaf length.

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