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The roles of geography and environment in divergence within and between two closely related plant species inhabiting an island-like habitat

Artur Maia Wanderley[1] | Isabel Cristina Sobreira Machado[1,2] | Erton Mendonça de Almeida[3] | Leonardo Pessoa Felix[4] | Leonardo Galetto[5] | Ana Maria Benko-Iseppon[1,6] | Victoria L. Sork[7,8]

[1]Programa de Pós-Graduação em Biologia Vegetal, Universidade Federal de Pernambuco, Recife, PE, Brazil

[2]Departamento de Botânica, Universidade Federal de Pernambuco, Recife, PE, Brazil

[3]Instituto Nacional do Semiárido (INSA), Campina Grande, PB, Brazil

[4]Departamento de Ciências Biológicas, Centro de Ciências Agrárias, Universidade Federal da Paraíba, Areia, Paraíba, Brazil

[5]Instituto Multidisciplinario de Biología Vegetal (UNC-CONICET), Universidad Nacional de Córdoba, Córdoba, Argentina

[6]Departamento de Genética, Universidade Federal de Pernambuco, Recife, PE, Brazil

[7]Department of Ecology and Evolutionary Biology, University of California, Los Angeles, CA, USA

[8]Institute of the Environment and Sustainability, University of California, Los Angeles, CA, USA

Correspondence
Artur M. Wanderley, Programa de Pós-Graduação em Biologia Vegetal, Universidade Federal de Pernambuco, Recife, PE, Brazil.
Email wanderley.artur@gmail.com

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Abstract

Aim: In island-like habitats, geographic isolation facilitates population and species divergence by constraining gene flow, while environmental isolation can enhance divergence. We tested the relative contribution of geographic and environmental isolation in genetic and phenotypic divergence within and between two species of the figwort *Ameroglossum* (Scrophulariaceae) inhabiting spatially isolated habitats, known as inselbergs.

Location: Borborema Plateau, north-eastern Brazil.

Methods: Multivariate models of redundancy (RDAs) and partial redundancy analyses (pRDAs) were used to partition the geographic and climate components of genetic variation in 48 microsatellite alleles, and phenotypic variation in 11 leaf and flower traits. We also used linear mixed-effect models (LMEs) to test independent associations of floral tube length variation among inselbergs with local pollinator phenotypes, climate and geography. In each approach, we analysed the data for each species separately and in pooled models for both species.

Results: RDAs revealed that genetic variation within and between the species of *Ameroglossum* was associated similarly with geography and climate. Phenotypic variation within *A. manoel-felixii* and between species was also associated similarly with geography and climate but, within *A. pernambucense*, phenotype was more strongly associated with climate. Linear mixed-effect models revealed that flower divergence in *A. manoel-felixii* was associated only with the bill length of local hummingbirds, whereas floral variation in *A. pernambucense* was associated with geography, bill length and climate. Only climate was associated with flower divergence between species.

Main conclusions: Genetic and phenotypic variation in *Ameroglossum* are associated with geographic and environmental isolation. These findings indicate a significant role of ecological factors shaping plant divergence among inselbergs, irrespective of their spatial distances.

KEYWORDS
Ameroglossum manoel-felixii, *Ameroglossum pernambucense*, genetic divergence, genetic drift, inselbergs, isolation by distance, isolation by environment, local adaptation, phenotypic divergence

1 | INTRODUCTION

Restricted gene flow fosters evolutionary diversification because it facilitates genetic and phenotypic divergence that may culminate in speciation (Schluter, 2001; Slatkin, 1987). Both geographic and environmental isolation can influence genetic divergence within and among closely related plant species through isolation by distance (IBD) and isolation by environment (IBE), respectively (Wang & Bradburd, 2014). Whereas IBD is the accumulation of genetic differentiation by genetic drift as geographic isolation increases (Wright, 1943), IBE arises by environmental regulation of gene flow or selection against immigrants (Andrew, Ostevik, Ebert, & Rieseberg, 2012; Wang & Bradburd, 2014). For instance, IBE is expected among plant populations distributed along environmental gradients because they can exhibit flowering displacement and/or dissimilar local pollinator assemblies that might result in non-random gene flow, or because immigrant plants are maladapted to local conditions (Andrew et al., 2012; Ellis, Weis, & Gaut, 2006; Nattero, Sérsic, & Cocucci, 2011). Phenotypic divergence associated with geographic isolation can be caused by genetic drift linked to neutral demographic events such as colonisation history and IBD.

When phenotypes are correlated with environmental gradients, they might reflect species responses to the local environment. These responses often arise by selection (local adaptation), phenotypic plasticity, or both, and can influence patterns of gene flow, distribution and evolution (Endler, 1986; Pigliucci, 2001; Riordan et al., 2016). For instance, neutral geographic variation in leaves has been linked to common ancestry (Keller, Sowell, Neiman, Wolfe, & Taylor, 2009), whereas leaf size reduction associated with elevation has been linked to cooler temperatures (Milla & Reich, 2011). Likewise, floral variation among populations and closely related species can arise through selection by local pollinator species that vary along environmental gradients, or by abiotic environmental factors, or through neutral geographic variation (Nattero et al., 2011; Whittall & Hodges, 2007).

In island-like habitats, geographic isolation is a major factor constraining gene flow, creating opportunity for both genetic and phenotypic divergence among populations through genetic drift (Boucher, Zimmermann, & Conti, 2016; Mayr, 1970). Environmental heterogeneity among islands can further enhance population and species divergence beyond expectations based on geographic isolation alone (Schluter, 2001; Wang & Bradburd, 2014). The roles of geographic and environmental isolation, or their combination, in divergence of plants from island-like habitats has often been recognized (e.g. Barbará, Martinelli, Fay, Mayo, & Lexer, 2007; Britton, Hedderson, & Anthony Verboom, 2014; Byrne & Hopper, 2008; Gao, Ai, Kong, Kang, & Huang, 2015; Price & Wagner, 2004) but few studies have explicitly attempted to disentangle their relative roles. Moreover, although some studies have shown that the significance of geographic and environmental isolation varies among types of islands (e.g. Boucher et al., 2016; Ellis et al., 2006; McGlaughlin & Friar, 2011), their relative contributions have not been quantified. Therefore, the roles of these types of isolation in population and species divergence in terrestrial-island systems remains poorly understood.

To investigate the impact of geography (here referring only to the spatial location of populations) vs environment on island-like populations, we studied the plant genus *Ameroglossum* (Scrophulariaceae), which is endemic to north-eastern Brazil. This genus comprises two allopatric hummingbird-pollinated shrubs, the southern and endangered *A. pernambucense* Eb. Fisch., S. Vogel & A.V. Lopes, and the northern and narrow endemic *A. manoel-felixii* L.P. Felix & E.M. Almeida (Almeida et al., 2016; Wanderley, Almeida, & Felix, 2014; Wanderley, Lopes & Machado, et al., 2014) (Figure 1). Both species are exclusive to geographically isolated granitic rock outcrops scattered across the landscape, which are "terrestrial habitat islands" known as inselbergs (Porembski, 2007). *Ameroglossum pernambucense* occurs in inselbergs from highland (1000–1200 m) forest enclaves of Atlantic rain forest that rise above the tropical dry forest of Caatinga, and *A. manoel-felixii* occurs in mid-elevation forest enclaves (500–600 m) and in surrounding areas of Caatinga. Leaf and flower size are among the main phenotypic differences between these species (Almeida et al., 2016) but some populations of *Ameroglossum* are taxonomically ambiguous because they exhibit leaf and flower sizes outside the strict taxonomic delimitation of *A. pernambucense* and *A. manoel-felixii* (Wanderley, Lopes & Machado, et al., 2014), which might be due to environmental isolation among inselbergs.

The overall goal of this study is to test the hypothesis that environment creates genetic and phenotypic divergence within and between the species of *Ameroglossum* that is independent from neutral variation associated with the geographic isolation of inselbergs. In doing this, we first confirmed the species identity of our samples using Bayesian inference of genetic clustering implemented in the STRUCTURE program (Pritchard, Stephens, & Donnelly, 2000), based on six neutral nuclear microsatellite loci. Then, we addressed three specific objectives. First, we examined the extent to which genetic structure within and between the species of *Ameroglossum* was associated with IBD or IBE independently. Using multivariate redundancy analysis (RDA) and partial redundancy analysis (pRDA) (Legendre & Legendre, 2012), we tested the strength of association of genetic

FIGURE 1 The two species of the inselberg specialist genus *Ameroglossum*, *A. pernambucense* (a) and *A. manoel-felixii* (b) [Colour figure can be viewed at wileyonlinelibrary.com]

variation from six microsatellite loci with spatial and climate data in separate models for each species, and a pooled model comprising both species. This multivariate approach is similar to a regression model that can separate the effects of multiple factors (Legendre & Legendre, 2012), and has been used elsewhere to distinguish between geographic and climatic effects on population differences (e.g. Gugger, Ikegami, & Sork, 2013; Riordan et al., 2016). Second, also using RDAs and pRDAs, we built separate models for each species of *Ameroglossum*, and a pooled model comprising both species, to estimate the amount of phenotypic variation associated with geography and climate using 11 leaf and flower traits as response variables. Third, we used linear mixed-effect models (LMEs) to test independent associations of floral tube length within and between the two species of *Ameroglossum* with geography, local pollinator phenotypes and climate.

2 | MATERIALS AND METHODS

2.1 | Study species

Ameroglossum pernambucense (Figure 1a) is a pubescent shrub with polygonal cylinder-like stems and 3–5 lanceolate leaves per node. The allopatric species, *A. manoel-felixii* (Figure 1b), was recently described by Almeida et al. (2016) and is predominantly a violet glabrous shrub with quadrangular winged stems and opposite lanceolate leaves. *Ameroglossum pernambucense* has shorter leaves [(5.69 ± 1.12 cm (*M* ± *SD*)] than *A. manoel-felixii* (12.83 ± 3.31 cm) and, although both species present tubular corollas, the floral tubes of *A. pernambucense* are shorter (2.80 ± 0.26 cm) than those of *A. manoel-felixii* (4.28 ± 0.45 cm). Several taxonomically ambiguous plants of *Ameroglossum* are found south of the range of *A. pernambucense* and show affinity with this species because of their cylinder-like pubescent non-winged stems, but they differ from *A. pernambucense* because they share with *A. manoel-felixii* long opposite leaves and long floral tubes. Similarly, plants found at the northernmost portion of the *Ameroglossum* range, which are at the lowest elevations recorded for the genus (100–180 m), fall outside the taxonomic descriptions of either species of *Ameroglossum*. Although they share quadrangular, winged, glabrous stems and opposite leaves with *A. manoel-felixii*, they show bright green vegetative characters instead of the typical violet of *A. manoel-felixii*, and shorter ovate leaves that contrast to the long lanceolate leaves of *A. manoel-felixii* (personal observation).

Plants of *Ameroglossum* are self-compatible and also pollinated by hummingbirds (Wanderley, Lopes & Machado, et al., 2014), which have an ability to fly among adjacent inselbergs (~850 m). Seeds are dispersed by gravity, which makes dispersal to other inselbergs rare (Wanderley, Lopes, & Machado, 2014). Flowers and leaves in *Ameroglossum* are functionally decoupled, allowing the adaptation of flowers to local pollinators without constraining leaf adaptation to local climates (Wanderley, Galetto, & Machado, 2016). Timing of flowering is triggered by photoperiod rather than by local climate in *A. pernambucense*, allowing synchronous flowering among populations in dissimilar climates (Wanderley, Lopes & Machado, et al., 2014). If flowering time overlaps between the two species of *Ameroglossum* due to a shared trigger, IBE within and between species caused by displacement in flowering times is not expected.

2.2 | Study region, and sampling

Ameroglossum occurs predominantly in the Borborema Plateau, north-eastern Brazil (Almeida et al., 2016). This plateau shows a highly heterogeneous landscape with steep gradients in elevation (~250–1200 m) and precipitation (~200–1000 mm/year) (Prado,

2003). We sampled the entire ranges of *A. pernambucense* and *A. manoel-felixii*, and the taxonomically ambiguous plants. We sampled individuals from 17 inselbergs (Figure 2), and considered each inselberg a separate population (Table S1 in Appendix S1). Leaf tissue from 7 to 25 individuals per population was collected for DNA extraction. In 15 of the 17 populations sampled for the genetic analyses, one to three fully developed leaves and flowers per plant were collected for the phenotypic analyses. In each of these populations, we collected leaves and flowers from 4 to 25 plants. Despite our efforts to use the same plants for the genetic and phenotypic analyses, two populations sampled for the genetic analyses (*PC* and *CAT*) could not be sampled for phenotypes because they were inaccessible during the flowering period. In some other populations (e.g. *SJTP*) fewer plants were sampled for phenotypes than for genotypes because few plants were in flower. Therefore, all plants sampled for phenotypes were also sampled for DNA but, in some populations, the genetic sampling was supplemented by additional plants not in flower.

2.3 | Genotyping

Genomic DNA from leaf samples was extracted following methods of Weising, Nybom, Wolff, and Kahl (2005). Genotyping was based on six neutral nuclear microsatellite markers (amg 01, amg 05, amg 06, amg 07, amg 09 and amg 10) developed for *A. pernambucense* with cross-transferability to congeneric taxa (Wanderley, Vasconcelos, Huettel, Machado, & Benko-Iseppon, 2017) using PCR conditions outlined in Appendix S2. The presence of non-amplified (null) alleles was assessed using the program MICRO-CHECKER 2.2.3 (Van Oosterhout, Hutchinson, Wills, & Shipley, 2004).

2.4 | Genetic structure among populations and species

To identify the number of genetic clusters (*K*) present in the sampled populations of *A. pernambucense* and *A. manoel-felixii*, and to determine the species assignments of the taxonomically ambiguous

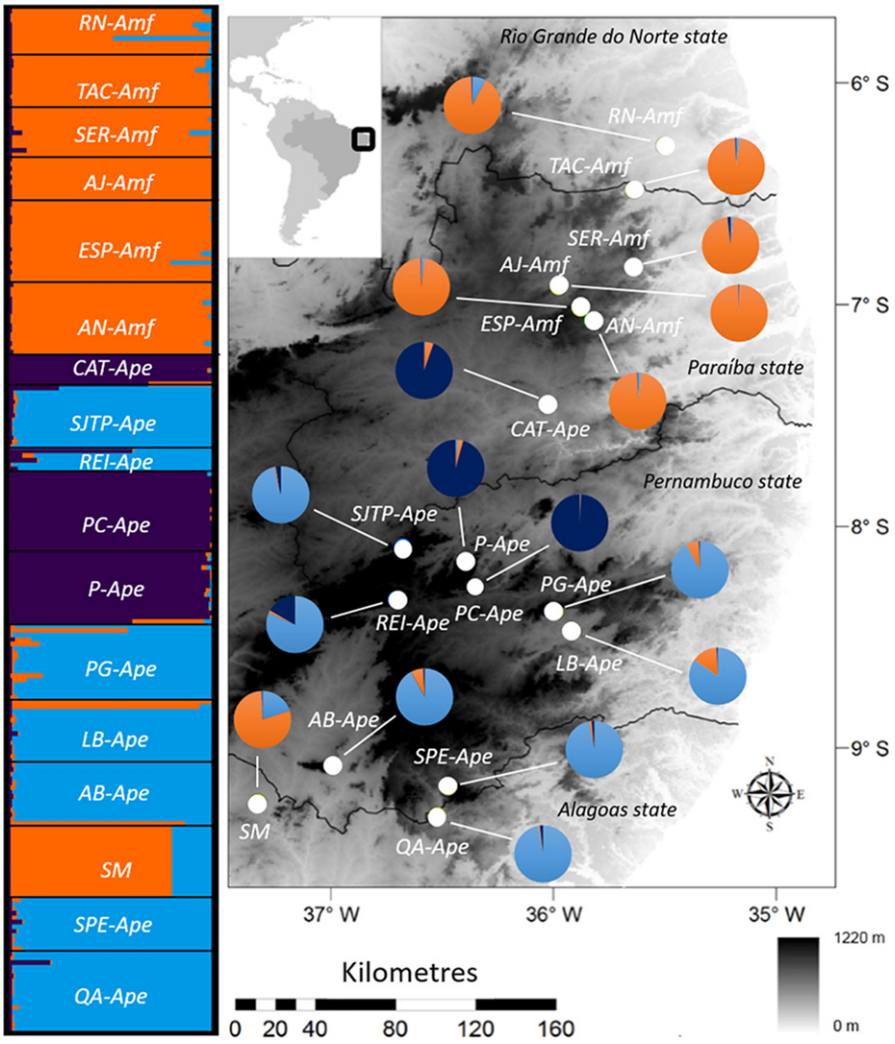

FIGURE 2 Distribution map of the sampled populations of *Ameroglossum pernambucense* (*Ape*) and *A. manoel-felixii* (*Amf*), and one population (*SM*) unassigned because of high interspecific genetic admixture. Barplot and pie charts represent the proportions of individual genetic assignments in three distinct genetic clusters, based on Bayesian inference implemented in the STRUCTURE program. Blue, violet and orange represent *K1*, *K2* and *K3*, respectively [Colour figure can be viewed at wileyonlinelibrary.com]

populations, we used Bayesian inference implemented in STRUCTURE 2.3.4 (Pritchard et al., 2000). We ran STRUCTURE assuming admixture and correlated allele frequencies and conducted 10 independent runs with 500,000 Markov chain Monte Carlo (MCMC) cycles, following 250,000 burn-in steps for each 1–17 *K*. The most likely *K* fitting the microsatellite data was defined by visualisation of the posterior log probability [Pr(*X*|*K*)] plot for each *K* (Pritchard et al., 2000), and the Δ*K* method (Evanno, Regnaut, & Goudet, 2005), using Structure Harvester (Earl & vonHoldt, 2012).

Genetic differentiation among populations and species was estimated by pairwise F_{ST} comparisons among all sampled populations, and hierarchical analysis of molecular variance (AMOVA) (Excoffier, Smouse, & Quattro, 1992). Both analyses were conducted in GENALEX 6.501 (Peakall & Smouse, 2006), and significance was assessed with permutation tests (999 permutations). In addition, to better understand whether there was opportunity for gene flow due to overlap in flowering times of populations of *Ameroglossum*, we performed a 2-year phenological survey of two populations of *A. pernambucense* and four populations of *A. manoel-felixii* (Table S1 in Appendix S1).

2.5 | Association of genetic variation with geography and climate

To test the independent roles of IBD and IBE in genetic divergence within and between the species of *Ameroglossum*, we used RDA and pRDA (Legendre & Legendre, 2012). Redundancy analysis is a constrained version of principal component analysis (PCA), in which it is possible to constrain a few ordination axes that summarise a multidimensional set of response variables to be linear combinations of a set of explanatory variables. We used RDA instead of the Mantel test because the former is more powerful when testing complex associations between spatial genetic data and multiple response variables (Legendre & Fortin, 2010).

To test for IBD and IBE within species, we ran separate RDA and pRDA models for each species. Each model included populations in which ≥80% of the individuals were assigned to *A. pernambucense* or *A. manoel-felixii* genetic clusters detected by STRUCTURE with membership coefficients >90%. To test for IBD and IBE between species, we pooled the entire sample of successfully genotyped *Ameroglossum* individuals, irrespective of their assignments to species. In both separate and pooled models, we first conducted the overall model, which included microsatellite alleles as response variables and geographic and climate variables as predictors, as explained in Appendix S3. Then, we used pRDA to partition the pure geographic and climatic components of genetic variation. We ran these models in the R package 'vegan' 2.3-3 (Oksanen, Blanchet, Kindt, Legendre, & O'Hara, 2016) in R 3.3.1 (R Core Team, 2016), with model significance defined by permutation test (999 permutations).

The genetic data used as response variables in the RDA and pRDA models were microsatellite single locus genotypes converted into allelic variables (as per Smouse & Williams, 1982). The geographic explanatory variables (*PCNMs*) were the eigenvectors corresponding to the positive eigenvalues of principal coordinates of neighbour matrices, which are able to detect the spatial structure in response data at all scales allowed by the sampling design (Borcard & Legendre, 2002). The climate predictors in the models were five climate variables (temperature of coldest quarter [T_{coldq}], temperature seasonality [T_{seas}], precipitation seasonality [P_{seas}], precipitation of driest quarter [P_{dq}], and precipitation of warmest quarter (P_{wq}]), downloaded from WorldClim (www.worldclim.org) (Hijmans, Cameron, Parra, Jones, & Jarvis, 2005), and elevation (*Elev*). Elevation was labelled as a climate variable because it captures microclimatic variation associated with elevation. To avoid bias towards IBD or IBE and model over-parameterisation, equal numbers of selected geographic and climate predictors were used in all models (see Appendix S3 for a detailed description of the variables used in the RDA and pRDA models and the criteria used for selecting the predictor variables of the separate and pooled models).

2.6 | Association of phenotypic variation with geography and climate

To partition the geographic and climate components of phenotypic variation in leaves and flowers of *Ameroglossum*, we also built separate models for each species, and a pooled model including all phenotypically sampled individuals using RDA and pRDA. Leaf traits used as response variables were: length, width, area, perimeter, length–width ratio and perimeter–area ratio; and floral measurements were: corolla tube length, distance from the corolla base to stamens insertion, mean length of the lower and upper stamens pairs, pistil length, and distance from the nectary to the anthers. To facilitate comparisons between the genetic and phenotypic models of each species, and both species pooled, the predictors used in the phenotypic models were the same as those selected for the corresponding separate and pooled genetic models. Both response and explanatory variables were standardized to have zero mean and unit variance.

2.7 | Association of flower variation with geography, pollinators and climate

To test whether differences in flower phenotypes within and between the species of *Ameroglossum* were independently associated with geography, local pollinator phenotype and local climate, we ran separate and pooled LMEs using the R package 'lm4' (Bates, Maechler, Bolker, & Walker, 2014). The response variable used in these models was floral tube length, which is expected to be associated with the bill length of the most frequent local pollinator (e.g. Whittall & Hodges, 2007). The predictors included in the LMEs were bill length of the main local pollinator, and the least correlated geographic and climate variables showing highest loadings on the first two axes of the corresponding separate and pooled phenotypic pRDA models. All explanatory variables were standardized to have zero mean and unit variance. The data used in the LMEs are from five populations of *A. pernambucense* (*QA*, *P*, *REI*, *LB* and *PG*) and

three populations of *A. manoel-felixii* (*AN*, *ESP* and *SER*). Except for populations *LB* and *PG*, for which pollinator information was extracted from Wanderley, Lopes, & Machado, 2014, the most frequent local pollinators in the field were determined by recording the frequency of flower visits in which visitors contacted the anthers and stigma. The mean bill length (*bill length*) of each main local pollinator species was determined by measuring specimens from the study locations deposited in the Ornithology Collection of the *Universidade Federal de Pernambuco*, Brazil.

The full separate and pooled models were fitted with geography, *bill length* and climate as fixed effects, and populations as random effect. The best models predicting floral tube length were identified by backward elimination of non-significant fixed effects, and the *p-values* of the fixed effects were calculated by Satterthwaite's approximation using the R package 'lmerTest' (Kuznetsova, Brockhoff, & Christensen, 2016). Marginal (R^2_m) and conditional (R^2_c) coefficients of determination, which represent the variance explained only by the fixed effects or by both types of effects, respectively, were calculated using the R package 'MuMIn' (Barton, 2014). The assumptions of homoscedasticity and normality of the residuals of each model were visually validated.

3 | RESULTS

3.1 | Genetic structure within and between species

We successfully genotyped 217 individuals (Table S1 in Appendix S1). There were 54 alleles obtained across the six loci, and null alleles were not detected. After converting genotypes into allelic variables, 48 allelic variables were obtained and used to test for IBD and IBE.

STRUCTURE analyses revealed $K = 3$ to be the most likely number of populations, as log likelihoods increased slightly for $K > 3$ and the ΔK method estimated the best value of K as $K = 3$ (Figure S1 in Appendix S1). In the taxonomically unambiguous populations of *A. pernambucense*, 97% of the individuals were assigned to clusters *K1* and *K2* with individual membership coefficients >90%. Similarly, 96% of the individuals from the taxonomically unambiguous populations of *A. manoel-felixii* were assigned to cluster *K3* with individual membership coefficients >90%. Thus, the taxonomically ambiguous populations where considered identified as *A. pernambucense* or *A. manoel-felixii* when >80% of their individuals were assigned to the *A. pernambucense* clusters (*K1* and *K2*) or to the *A. manoel-felixii* cluster (*K3*) with membership coefficients >90% (Figure 2). Only one taxonomically ambiguous population (*SM*) was not assigned to either species, which was also the only population in which all loci were monomorphic. Hereafter, populations assigned to *A. pernambucense* or to *A. manoel-felixii* will be designated by their acronym followed by *Ape* or *Amf* (e.g., *QA-Ape* and *TAC-Amf*).

Overall F_{ST} among the populations of *Ameroglossum* was extremely high (0.506) despite overlap in flowering time across populations of both species (Figure S2 in Appendix S1). Within species, non-significant ($F_{ST} = 0.062$, *REI-Ape* and *SJTP-Ape*) and high (e.g., $F_{ST} = 0.481$, *SJTP-Ape* and *CAT-Ape*) pairwise F_{ST} values were found, and extremely high pairwise F_{ST} were found between populations of each species (0.763 *CAT-Ape* and *AJ-Amf*) (Table S2 in Appendix S1). The greatest genetic structure was found between the unassigned *SM* population and *AJ-Amf* ($F_{ST} = 0.904$). Hierarchical structuring of genetic variation provided by AMOVA revealed that 37% of the genetic variation was found among populations in *A. pernambucense*. In *A. manoel-felixii*, the amount of genetic variation among populations was 38%. For the pooled model, most of the genetic variation was found among populations (31%) rather than between species (19%) (Table S3 in Appendix S1).

3.2 | Association of genetic variation with geography and climate

One hundred and twenty-eight individuals from 10 populations of *A. pernambucense* and 73 individuals from six populations of *A. manoel-felixii* were used in the separate RDA and pRDA models. The pooled model included 217 individuals from all 17 sampled populations. The selected variables used in the *A. pernambucense* models were the first (*PCNM1*), second (*PCNM2*), and fifth (*PCNM5*) eigenvectors of the spatial distance matrix among the sampling sites, T_{coldq}, P_{wq} and *Elev*. In the *A. manoel-felixii* models, the predictors used were *PCNM1*, *PCNM2*, T_{seas} and P_{wq}, whereas the predictors of the pooled RDA and pRDAs were *PCNM1-5*, and all climate variables presented in the methods, but T_{coldq}, which was highly correlated with *Elev* ($r_s = -0.83$).

The separate (*A. pernambucense*: $df = 121$; pseudo-$F = 13.041$, $p = .001$; *A. manoel-felixii*: $df = 68$; pseudo-$F = 12.249$, $p = .001$) and pooled ($df = 206$; pseudo-$F = 23.083$, $p = .001$) RDA overall models testing for IBD and IBE were significant. In *A. pernambucense*, 13.3% (pseudo-$F = 8.859$, $p = .001$) and 14.4% (pseudo-$F = 9.591$, $p = .001$) of the genetic variation was purely associated with geography and with climate, respectively. In *A. manoel-felixii*, the percentages of genetic variation associated with geography and climate alone were 12.5% (pseudo-$F = 7.293$, $p = .001$) and 10.6% (pseudo-$F = 6.229$, $p = .001$), respectively. In the pooled model, 23.1% of the genetic variation was associated with geography (pseudo-$F = 20.150$, $p = .001$), and 18.0% was associated with climate (pseudo-$F = 15.764$, $p = .001$). The percentages of genetic variation associated with geography and climate together due to their collinearity were 11.5% (*A. pernambucense*), 18.8% (*A. manoel-felixii*) and 11.7% (both species pooled) (Table S4 in Appendix S1). The percentages of genetic variation found in *A. pernambucense*, *A. manoel-felixii* and both species pooled that were not explained by the RDA models were 60.8%, 58.1% and 47.2%, respectively. The predictors with the highest scores in the first two axes (RDA1 and RDA2) of the RDA and pRDA models were not the same. In the climate pRDAs, the predictors loading highest in at least one of the first two axes were P_{wq} for *A. pernambucense*, T_{seas} for *A. manoel-felixii* and P_{dq} for both species pooled (Figure 3, Table S5 in Appendix S1).

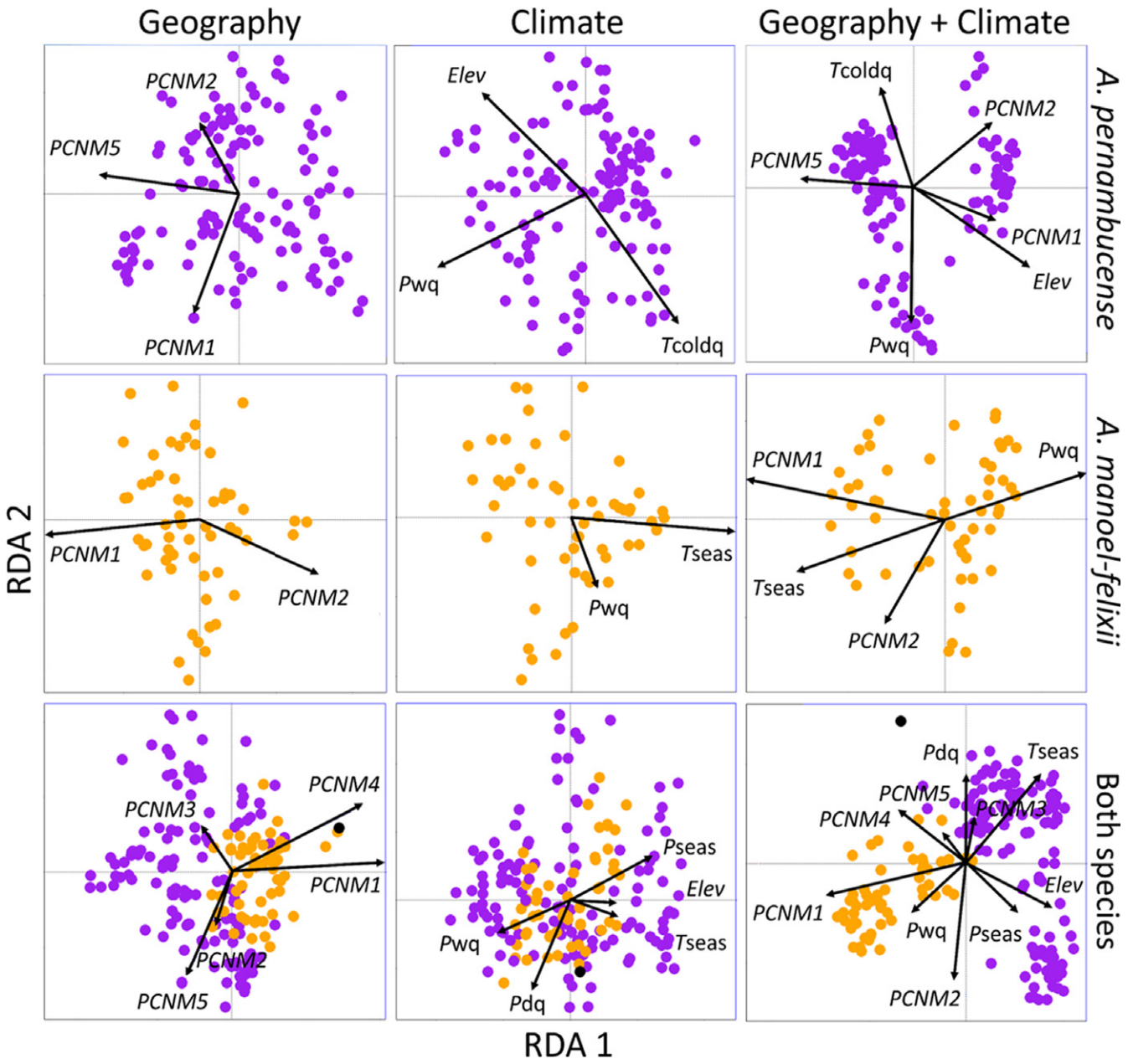

FIGURE 3 First two axes of redundancy analysis (RDA) and partial RDA models testing the association of genetic variation in *Ameroglossum*, based on six nuclear microsatellite loci, with geography and climate. Geographic predictors (*PCNMs*) are eigenvectors of truncated matrices of geographic distances among sampling sites, calculated by principal coordinates of neighbour matrices. Climate predictors used in the models are the following: temperature of coldest quarter (T_{coldq}), temperature seasonality (T_{seas}), precipitation seasonality (P_{seas}), precipitation of driest quarter (P_{dq}), precipitation of warmest quarter (P_{wq}), and elevation (*Elev*). The black circle represents the individuals from a population (*SM*), where all sampled plants ($N = 16$) showed identical genotypes with interspecific genetic admixture, and thus were not assigned to either species [Colour figure can be viewed at wileyonlinelibrary.com]

3.3 | Association of phenotypic variation with geography and climate

The RDA and pRDA models for *A. pernambucense* and *A. manoel-felixii* used to partition the phenotypic variation included 107 individuals from eight populations and 77 individuals from six populations, respectively, whereas the pooled model included 192 individuals from all 15 phenotypically sampled populations.

Both separate (*A. pernambucense*: $df = 100$; pseudo-$F = 38.262$, $p = .001$; *A. manoel-felixii*: $df = 71$; pseudo-$F = 9.446$, $p = .001$) and pooled ($df = 181$; pseudo-$F = 38.792$, $p = .001$) overall RDA models were significant. The phenotypic variation associated purely with geography was 22.7% in *A. pernambucense* (pseudo-$F = 24.905$, $p = .001$) and 7.8% in *A. manoel-felixii* (pseudo-$F = 4.266$, $p = .002$), whereas the phenotypic variation associated purely with climate was 33.7% (pseudo-$F = 37.023$, $p = .001$) and 6.1% (pseudo-$F = 3.308$,

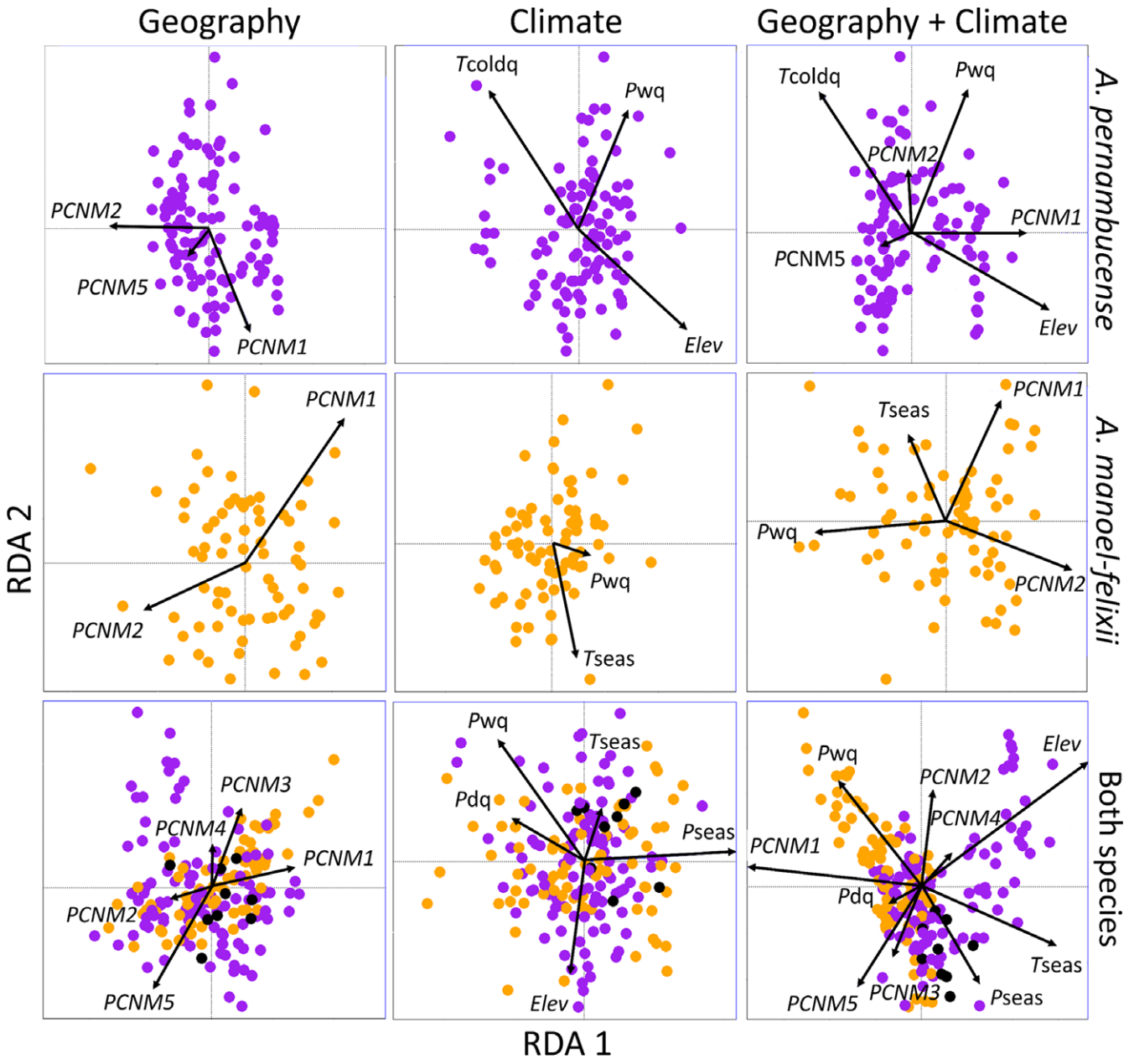

FIGURE 4 First two axes of redundancy analysis (RDA) and partial RDA models testing the association of leaf and flower phenotypic variation in *Ameroglossum* with geography and climate. Geographic predictors (*PCNMs*) are eigenvectors of truncated matrices of geographic distances among sampling sites, calculated by principal coordinates of neighbour matrices. Climate predictors used in the models are the following: temperature of coldest quarter (T_{coldq}), temperature seasonality (T_{seas}), precipitation seasonality (P_{seas}), precipitation of driest quarter (P_{dq}), precipitation of warmest quarter (P_{wq}), and elevation (*Elev*). Leaf traits used as responses variables are: length, width, area, perimeter, length–width ratio and perimeter–area ratio; whereas floral measurements included: corolla tube length, distance from the corolla base to stamens insertion, average length of the lower and upper stamens pairs, pistil length, and distance from the nectary to the anthers. Black circles represent individuals from a population (*SM*) that was not assigned to either species due to high genetic admixture [Colour figure can be viewed at wileyonlinelibrary.com]

p = .014), respectively. In the pooled model, 12.8% of the phenotypic variation was associated with geography (pseudo-F = 14.563, p = .001) and 17.1% was associated with climate (pseudo-F = 19.445, p = .001). The phenotypic variation associated with geography and climate together were 13.3% (*A. pernambucense*), 20.8% (*A. manoel-felixii*) and 38.3% (pooled) (Tables S6, S7 and S8 in Appendix S1). The unexplained phenotypic variation was 30.3% (*A. pernambucense*), 65.3% (*A. manoel-felixii*) and 31.8% (pooled). The predictors with the highest scores in the first two RDA axes varied between the RDA and pRDA models. In the axes RDA1 and RDA2 of the climate pRDAs, the predictors with highest scores were related to temperature in the separate models (*A. pernambucense*: T_{coldq}; *A. manoel felixii*: T_{seas}) and to precipitation (P_{seas}) in the pooled model (Figure 4, Table S7 in Appendix S1).

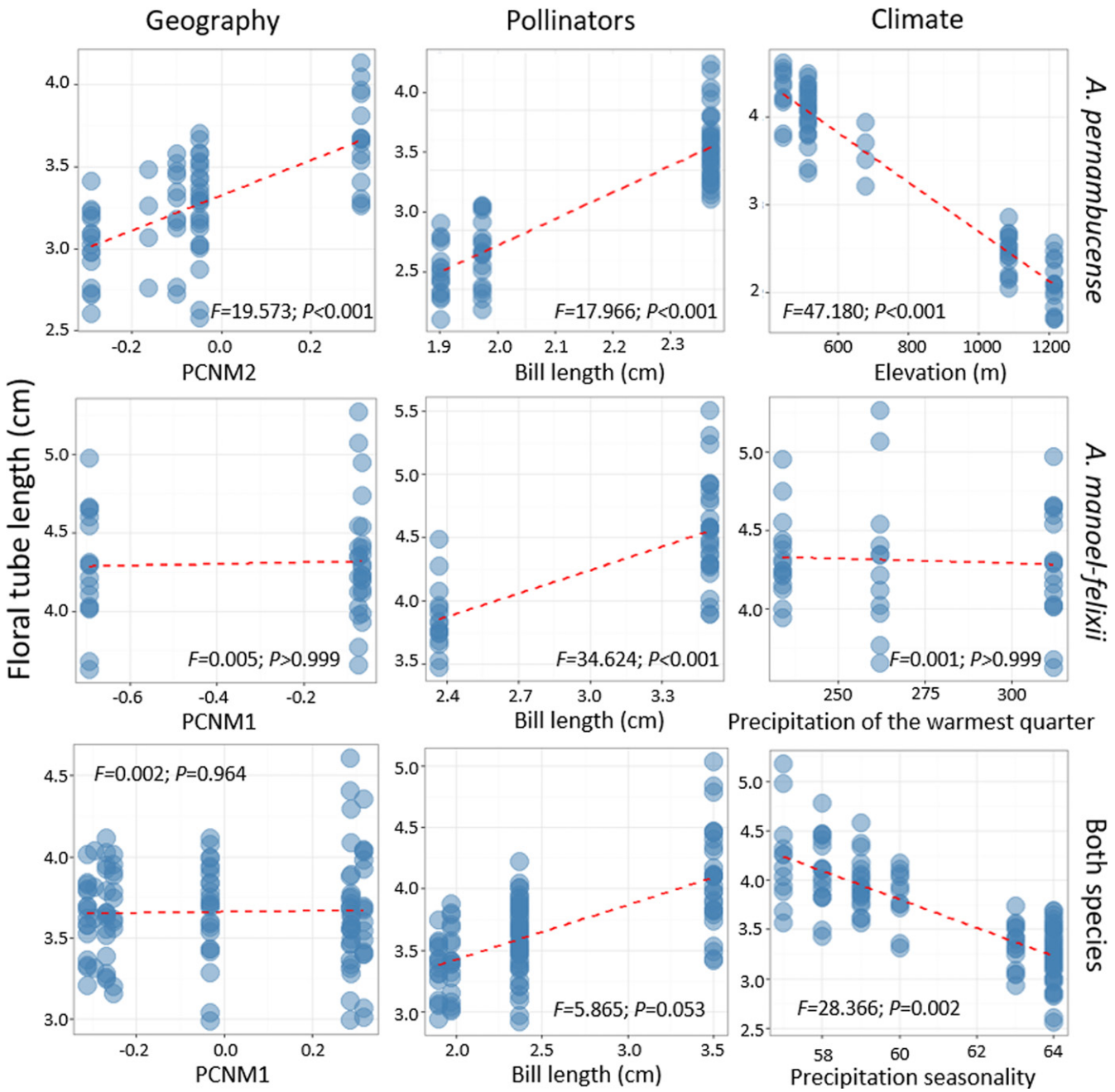

FIGURE 5 Independent associations (partial effects) of floral tube length variation in *Ameroglossum* with geography (*PCNMs*), local pollinator phenotypes (*bill length*) and climate (elevation, precipitation of the warmest quarter and precipitation seasonality), which were tested through linear mixed-effect models (LMEs). *PCNMs*, *bill length* and climate variables were the fixed effects and the sampled *Ameroglossum* populations were the random effects. Partial effects on floral tube length were extracted using the *remef* R package (Hohenstein & Reinhold, 2015), and complete results of the LMEs are presented in Table S10 in Appendix S1. Geographic predictors (*PCNMs*) are eigenvectors of truncated matrices of geographic distances among sampling sites, calculated by principal coordinates of neighbour matrices; *bill length* is the average bill length of the most frequent local pollinator [Colour figure can be viewed at wileyonlinelibrary.com]

3.4 | Association of flower variation with geography, pollinators and climate

Mean floral tube length in *A. pernambucense* was 3.66 ± 0.71 cm (*M* ± *SD*) and the average bill length of the most frequent local pollinators ranged from 1.97 to 2.37 cm, whereas in *A. manoel-felixii* floral tube length mean was 4.17 ± 0.46 cm (*M* ± *SD*) and the average bill length of the most frequent local pollinators ranged from 2.37 to 3.50 cm (Table S9 in Appendix S1).

The number of individuals used in the separate and pooled LMEs was 71 (*A. pernambucense*), 45 (*A. manoel-felixii*), and 116 (pooled). In addition to *bill length*, the following geographic and climate variables were included as predictors in each LME: *PCNM2* and *Elev* for the *A. pernambucense* model, *PCNM1* and P_{wq} for the

A. manoel-felixii model, and *PCNM1* and P_{seas} for the pooled model. Fixed effects showing significant associations with floral tube length kept in the final models were *Elev* ($F = 47.180$; $p < .001$), *PCNM2* ($F = 19.573$; $p < .001$) and *bill length* ($F = 17.966$; $p < .001$) in the LME for *A. pernambucense* ($R^2_m = 0.582$, $R^2_c = 0.756$); only *bill length* ($F = 34.624$; $p < .001$) was kept in the LME for *A. manoel-felixii* ($R^2_m = 0.195$, $R^2_c = 0.746$); and only P_{seas} ($F = 28.366$, $p < .002$) remained in the final pooled LME ($R^2_m = 0.717$, $R^2_c = 0.819$). The random factor *Population* was not significant in the LME for *A. manoel-felixii* ($\chi^2 = 0$; $p > .999$) (Figure 5, Table S10 in Appendix S1).

4 | DISCUSSION

Strong genetic differentiation was found among most populations of *Ameroglossum* (high F_{ST} values), as expected in island-like systems, with evidence for environment (climate and pollinators) playing a significant role in genetic and phenotypic divergence, irrespective of the geographic distances among inselbergs. Partial RDAs showed that geography and climate were, in general, associated similarly with both genetic and phenotypic divergence in the genus.

4.1 | Genetic differences among populations and species

Several studies of island-like habitats have been mainly concerned with the role of IBD in driving intra- and interspecific genetic divergence among island populations without testing for IBE (e.g. Barbará et al., 2007; Britton et al., 2014; Byrne & Hopper, 2008), even when environmental differences (edaphic) among islands are pronounced (e.g. Gao et al., 2015). Our findings complement these earlier studies by providing evidence for a significant role of IBE, in addition to IBD, in potentially driving genetic divergence in plants inhabiting island-like habitats.

The general expectation of geographic isolation as the only, or main, cause of genetic divergence among spatially isolated populations (IBD) implies that allopatric (neutral) speciation predominates in islands (Boucher et al., 2016; Mayr, 1970). In *Ameroglossum*, the evidence of IBE revealed from analyses of presumed neutral microsatellite markers indicates ecological processes are also likely to be driving genetic divergence in island plants, either because of biased gene flow due to habitat preferences of pollinators or selection against maladapted immigrants (Wang & Bradburd, 2014). Other studies that have tested for IBE in island-inhabiting plants have found conflicting results. Pinheiro et al. (2014), studying orchid populations (*Epidendrum secundum*) sympatric with *Ameroglossum*, found evidence only for IBD when analysing plastid microsatellites but for neither IBD nor IBE when analysing nuclear microsatellites, indicating genetic homogenisation among populations through gene flow by pollen. Unique signs of IBD driving populations and species divergence in island-systems have been found in a member of the Hawaiian silversword alliance (*Dubautia laxa*) and in sister-species of Primulaceae from sky-islands in Europe (Boucher et al., 2016; McGlaughlin & Friar, 2011). Similar to *Ameroglossum*, a combination of IBD and IBE associated with genetic variation within and between species from isolated habitat patches was found for *Argyroderma* in the Cape Floristic Region of South Africa (Ellis et al., 2006). Therefore, although a predominant role of IBD in speciation is more likely in some plants in island-systems, for others the combination of IBD and IBE indicates a combination of allopatric and ecological speciation (Schluter, 2001). Nonetheless, the evidence for IBD and IBE reported in previous studies are qualitative and precludes understanding the extent to which ecological processes are important for genetic divergence in island plants.

Similar to our study, independent IBD and IBE have been quantified in Californian oaks (*Quercus*) continuously distributed over a highly heterogeneous landscape (Gugger et al., 2013; Riordan et al., 2016). A smaller importance of IBE relative to IBD is expected in *Ameroglossum* than in oaks because gene flow in the oaks is not constrained by habitat discontinuity. In *Quercus engelmannii* and *Q. cornelius-mulleri*, the sign of IBE was ca. 1.5-fold greater than the sign of IBD, whereas similar amounts of genetic variation were associated with IBD and IBE within and between the species of *Ameroglossum*. This indicates a greater role of geographic isolation in disrupting gene flow in *Ameroglossum* than in the two oaks (Riordan et al., 2016). In contrast, *Q. lobata* showed similar portions of genetic variation associated with IBE (17.8%) and IBD (18.9%), as occurs in *Ameroglossum*, despite the continuity and heterogeneity of its habitat (Gugger et al., 2013). The evidence for similar roles of IBD and IBE in *Ameroglossum* and in *Q. lobata* indicates that ecological processes can have similar importance for genetic divergence in plants no matter whether they inhabit non-island or island-systems.

The significant evidence of IBD and IBE found within and between the *Ameroglossum* species is reflected in the STRUCTURE results. Overall, STRUCTURE revealed a coarse geographic gradient from *K1* in the southern populations to *K3* in the northern populations (IBD). However, greater genetic similarities among geographically farther than closer *A. pernambucense* populations (e.g. *SJTP-Ape* and *QA-Ape* or *PC-Ape* and *CAT-Ape*), and interspecific genetic admixture, as in *SM*, do not fit a pure IBD model (Figure 2) (Wang & Bradburd, 2014). Given that we found evidence for overlap in flowering times within and between the species of *Ameroglossum*, we know that pollen exchange is possible between populations sharing the same pollinators (Table S9 in Appendix S1). So, the long-distance genetic similarities revealed by the STRUCTURE results may be the outcome of long-distance pollen dispersal by hummingbirds with habitat preferences. Alternatively, selection against immigrants from neighbour populations with dissimilar environments in favour of immigrants from distant populations with similar environments might also explain the long-distance genetic similarities. The interspecific genetic admixture observed in *Ameroglossum* indicates that gene flow between species is possible. Thus, IBE through selection against immigrants might play a role in keeping species apart. Although incomplete sorting, parallel evolution in the microsatellite loci, or

even PCR-amplification errors could also resemble interspecific genetic admixture, we assume these effects are negligible in our data because it is very unlikely they fit a geographic and environmental structure as revealed by the pooled RDA model.

4.2 | Leaf and flower variation

The IBE sign detected in neutral markers within and between species in *Ameroglossum*, together with the associations between phenotypes and climate, indicate that patterns of gene flow are, in part, associated with the suitability of phenotypes to local environments. If so, the distribution of the species of *Ameroglossum* might partially depend on phenotypic responses to local environments, either by local adaptation or phenotypic plasticity, and not only on the constraints to dispersal imposed by geographic isolation. Our results for *Ameroglossum* are similar to those of *Argyroderma* (Ellis et al., 2006) in that independent phenotypic associations with environment were observed within and between species from separate habitat patches. Unless genetic sweeping due to strong gene flow prevents local adaptation and phenotypic plasticity is absent, greater pure phenotype-environment associations are expected for plants in continuous habitats because geographic isolation among islands hampers migration to environmental optima (Wang & Bradburd, 2014). However, this is not supported when comparing *Ameroglossum* to the Californian oaks continuously distributed along steep environmental gradients. In *Q. berberidifolia*, phenotypic variation (in leaves) showed an association 1.2-fold greater with climate than with geography, whereas in *A. pernambucense* phenotypes were 1.5-fold more associated with climate than with geography. More surprisingly, whereas between the species of *Ameroglossum* and within *A. manoel-felixii* phenotypes were similarly associated with geography and climate, in *Q. engelmannii*, the association of phenotypic variation in leaves with geography was ca. 2.7-fold greater than with climate, despite a greater sign of IBE than IBD in this species indicating lack of genetic sweeping (Riordan et al., 2016).

The association of flower phenotype with climate was found only between the species of *Ameroglossum* because populations from dissimilar environments (Atlantic forest and Caatinga) showed greater pollinator sharing between than within species (Table S9 in Appendix S1). Since pollinator-mediated gene flow hampers species divergence through IBE (Wang & Bradburd, 2014), interspecific reproductive barriers, in addition to IBE, might possibly strengthen the flower-climate association between species despite sharing of pollinators.

5 | CONCLUSIONS

Based on correlative evidence, our study suggests the importance of local environments in creating divergence among populations and species inhabiting separate islands beyond the impacts of neutral divergence associated with geographic isolation. By quantifying the portions of genetic and phenotypic divergence associated with geography and climate, we found evidence for these two factors playing similar roles in driving divergence in *Ameroglossum*. Overall, environmental factors are associated with strong population and species divergence among inselbergs, irrespective of how far apart they are.

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ORCID

Artur Maia Wanderley http://orcid.org/0000-0003-1654-9674

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DATA ACCESSIBILITY

Genetic, phenotypic, geographic and environmental data used in this study available from https://doi.org/datadryad.org/doi: 10.5061/dryad.217fn.

BIOSKETCH

Artur Wanderley is a post-doctoral researcher at Federal University of Pernambuco, Brazil. His main research interest is to understand how geography, and abiotic and biotic factors shape genetic and phenotypic variation in plants.

Author contributions: All authors conceived the ideas; A.M., E.A., and L.P. collected the data; A.M., V.L.S., and L.G. analysed the data; and A.M. led writing with substantial contributions from V.L.S., L.G., A.B.-I., and I.M.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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