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CONEXÃO E EVOLUÇÃO ENTRE PEQUENAS POPULAÇÕES INSULARES DE PLANTAS RUPÍCOLAS DO NORDESTE BRASILEIRO

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Tese apresentada ao Programa de Pós-Graduação em Biologia Vegetal do Departamento de Botânica da Universidade Federal de Pernambuco (UFPE) como parte dos requisitos necessários à obtenção do título de Doutor em Biologia Vegetal (área de concentração: Ecologia e Conservação; linha de pesquisa: Ecologia de Populações e Comunidades).

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Os pedaços de lajeiro que afloravam então, apresentavam-se cobertos de coroas-de-frade e macambiras, rubras, amarelas ou roxas, às vezes com maravilhosas flores escarlates luzindo entre as folhas espinhosas, mas sempre selvagens, incendiadas pelo sol, como se fossem enormes tochas, ou lampadários... (Trecho de *A Pedra do Reino*, de Ariano Suassuna)

.

RESUMO

Várias espécies de plantas ocorrem em habitats discretos (i.e. descontínuos) como ilhas oceânicas, topos úmidos de montanhas e afloramentos rochosos. Quando populações dessas plantas ocorrem em paisagens ambientalmente heterogêneas, o isolamento espacial pode facilitar sua adaptação local. Isso acontece devido à redução do fluxo gênico com outras populações adaptadas a condições distintas, o que poderia diluir a adaptação local. Por outro lado, populações de habitats discretos tendem a ser pequenas e, portanto, mais suscetíveis à perda de variabilidade genética por endogamia (cruzamento entre parentes) e deriva genética (fixação aleatória de alelos), o que reduz seu potencial adaptativo. Entretanto, quando esses habitats discretos ocorrem na forma de arquipélagos compostos por conjuntos isolados de pequenas populações adjacentes e potencialmente conectáveis, há maior chance de redução dos níveis de endogamia e deriva, e consequentemente, adaptação local. Além disso, populações expostas a diferentes assembleias de polinizadores e condições abióticas distintas (ex. diferenças na temperatura e precipitação) devem ser favorecidas quando suas flores podem ajustar-se morfologicamente aos polinizadores locais independentemente dos ajustes impostos às folhas por fatores abióticos (desacoplamento). Utilizando principalmente como modelo arquipélagos de populações discretas (exclusivas de afloramentos rochosos) do complexo *Ameroglossum pernambucense* (Scrophulariaceae) expostos a diferentes condições ambientais, esta pesquisa teve como objetivo principal investigar processos ecológicos (interação planta-polinizador), evolutivos (seleção natural *versus* deriva genética) e morfofuncionais (desacoplamento entre caracteres reprodutivos e vegetativos) que podem facilitar ou constranger a adaptação local de pequenas populações discretas. O potencial de cruzamento, inferido a partir da dispersão de partículas fluorescentes, entre plantas dentro e entre populações de um mesmo arquipélago revelou que a atividade dos polinizadores deve reduzir potencialmente os níveis de endogamia e deriva dentro dos arquipélagos, podendo favorecer adaptação local. Analisando a variação geográfica e ambiental da morfologia (folhas e flores) e composição genética (marcadores microssatélites) das populações do complexo *A. pernambucense*, foi observado que essas variações são melhor explicadas por modelos de diferenciação por seleção natural, e não por modelos de deriva genética, indicando a presença de adaptação local. A observação de um sistema de polinização funcionalmente desacoplado das folhas, indica que as

populações do complexo *A. pernambucense* podem se adaptar às assembleias locais de polinizadores sem serem constrangidas pelas pressões seletivas impostas às folhas.

Palavras-chave: *Ameroglossum*, especiação, inselbergs

ABSTRACT

Several species of plants are distributed in discrete habitats such as oceanic islands, humid mountain tops and rock outcrops. When the populations of these plants occur across environmental heterogeneous landscapes, spatial isolation might favor local adaptation. This is due to reduced gene flow with populations adapted to different environmental conditions, what could dilute local adaptation. On the other hand, populations in discrete habitats tend to be small, and thus more prone to losses of genetic variation by endogamy (mating between relatives) and genetic drift (random allele fixation), both affecting population adaptability. However, when discrete habitats form archipelagos of small adjacent populations with potential connectivity the levels of endogamy and drift are more likely to reduce and increase the adaptive potential of archipelago populations. Furthermore, populations exposed to different pollinator assemblies and different climatic conditions (e.g. differences of temperature and precipitation) might be favored when flowers are able to adapt to local pollinators independently from leaf adaptation to local climates (flower-leaf decoupling). Using mainly the *Ameroglossum pernambucense* complex (Scrophulariaceae) as a model of archipelagos of small disjunct populations (exclusive from rock outcrops) exposed to a highly heterogeneous landscape, the main goal of this research was to investigate ecological (plant-pollinator interactions), evolutionary (natural selection *versus* genetic drift) and morpho-functional (flower-leaf decoupling) aspects that could favor or constrain local adaptation of small isolated discrete populations. The mating potential of the studied plants within and among archipelago populations revealed that pollinator activity has potential to reduce endogamy and genetic drift, and might favor local adaptation. Examining the geographical and environmental variation of the phenotypes (flowers and leaves) and the genetic composition of the *A. pernambucense* complex populations, the results suggested that natural selection models better explained these variations than genetic drift models, providing evidence of local adaptation. The evidences also supported a pollination system functionally decoupled from leaves, suggesting the *A. pernambucense* complex population are able to adapt to local pollinator assemblies without constraining the adaptation of leaves to local climates.

Key-words: *Ameroglossum*, inselbergs, speciation

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Duas forças evolutivas são responsáveis pela maior parte da diversidade biológica, deriva genética e seleção natural. Pequeno tamanho populacional e isolamento geográfico são dois fatores que favorecem a deriva genética (i.e. fixação aleatória de alelos), a qual, por sua vez, leva à diferenciação entre populações e pode reduzir seu potencial adaptativo. Por outro lado, quando populações estão distribuídas ao longo de uma paisagem ambientalmente heterogênea, o isolamento geográfico pode favorecer a adaptação dessas populações às condições ambientais locais (adaptação local) porque dificulta a diluição da adaptação local devido a fluxo gênico com populações adaptadas a ambientes distintos. Portanto, conjuntos de pequenas populações adjacentes e potencialmente conectáveis (arquipélagos), mas geograficamente isolados de outros conjuntos populacionais, ao longo de uma paisagem heterogênea, podem se diferenciar por deriva genética ou adaptação local. Além disso, propriedades morfofuncionais, como desacoplamento de diferentes estruturas morfológicas, podem diminuir a interferência entre a adaptação local dessas estruturas aos seus diferentes agentes de seleção (ex. flores aos polinizadores, folhas ao clima). Entretanto, desacoplamento não garante adaptação.

Essa pesquisa teve como objetivo principal investigar (1) o papel dos polinizadores para a manutenção da adaptabilidade de plantas em arquipélagos de pequenas populações disjuntas; (2) estimar a contribuição relativa da deriva genética e seleção natural na diferenciação dessas populações; e (3) investigar se propriedades morfofuncionais dessas plantas estão associadas à adaptação local. O principal modelo utilizado nessa investigação foi o complexo *Ameroglossum pernambucense* (Scrophulariaceae), embora *Encholirium spectabile* (Bromeliaceae) também tenha sido estudado.

2 FUNDAMENTAÇÃO TEÓRICA

2.1 PLANTAS DE HABITATS DESCONTÍNUOS E O POTENCIAL ADAPTATIVO DE PEQUENAS POPULAÇÕES GEOGRAFICAMENTE ISOLADAS

Muitas espécies de plantas são exclusivas de habitats descontínuos, tais como ilhas oceânicas, topos de montanhas e afloramentos rochosos (Ellstrand & Elam, 1993; Prance, 1996; Emerson, 2002; Porembski, 2007). Devido a descontinuidade dos habitats em que ocorrem, frequentemente essas populações, além de geograficamente isoladas, são também de pequeno tamanho (Ellstrand & Elam, 1993). Populações com pequeno tamanho populacional e espacialmente isoladas são mais susceptíveis à depressão endogâmica e deriva genética devido ao baixo número de parceiros sexuais e, portanto, devem ter menor potencial adaptativo do que grandes populações contínuas (Wright, 1931; Slatkin, 1987; Ellstrand and Elam, 1993; Oakley & Winn, 2012). Embora populações pequenas e geograficamente isoladas por dezenas ou centenas de quilômetros tenham chances muito baixas de fluxo gênico suficiente para prevenir a deriva genética (Barbará *et al.*, 2009; Boisselier-Duabayle *et al.*, 2010; Pinheiro *et al.*, 2014), espécies exclusivas de habitats discretos estão comumente distribuídas em arquipélagos formados por pequenas populações (ou subpopulações) adjacentes que são potencialmente conectáveis (Russel-Smith, 1991; Buzan *et al.*, 2013; Boisselier-Duabayle *et al.*, 2010; Millar *et al.*, 2014). Apesar de poder haver extremo isolamento entre arquipélagos, o fluxo gênico dentro e entre as populações de cada arquipélago pode reduzir os níveis de deriva e endogamia, mantendo o potencial de adaptação local dessas populações (Boisselier-Duabayle *et al.*, 2010; Buzan *et al.*, 2013; Millar *et al.*, 2014). Uma vez que o fluxo polínico é geralmente a principal forma de movimento de genes dentro e entre populações de angiospermas (Ennos, 1994; Petit *et al*., 2005), os polinizadores podem potencialmente reduzir os níveis de endogamia e deriva se aumentarem o número de possíveis cruzamentos dentro e entre as populações desses arquipélagos, criando assim potencial para adaptação. Embora tenha sido observado que pequenas populações são menos atrativas para polinizadores do que grandes populações (Fritz & Nilsson, 1994; Mustajärvi *et al.*, 2001; Justino *et al*., 2012), pouco se sabe sobre os padrões de fluxo polínico em arquipélagos de pequenas populações disjuntas (Millar *et al*., 2014; Sampson *et al*., 2014).

2.2 ISOLAMENTO GEOGRÁFICO E ADAPTAÇÃO LOCAL

O surgimento de diferenças genéticas e morfológicas entre populações devido à variação ambiental (seleção divergente) é uma das principais formas de especiação e o primeiro estágio desse processo é a adaptação local. Entretanto, a presença de fluxo gênico entre populações expostas a diferentes pressões ambientais é uma importante força contrária à adaptação local devido ao seu efeito homogeneizador. Dessa forma, barreiras geográficas ao fluxo gênico entre populações de ambientes contrastantes devem facilitar a adaptação local (Mayr, 1942; 1947; Slatkin, 1987; Schluter, 2001; Levin, 2005; Lenormand, 2012). Por outro lado, o isolamento populacional também pode favorecer a diferenciação entre populações por deriva genética devido a diferentes fatores, tais como isolamento por distância, efeito fundador, gargalo genético e pequeno tamanho populacional (Wright, 1943; Slatkin, 1987; Ellstrand and Elam, 1993; Levin, 2005; Keller *et al.*, 2009; Wang *et al.*, 2013). Portanto, pequenas populações geograficamente isoladas podem se diferenciar por seleção divergente, deriva genética ou por esses dois mecanismos conjuntamente. Assim, estudar o potencial adaptativo dessas populações envolve dissociar os efeitos da seleção natural e da deriva genética na sua diferenciação (Keller *et al.*, 2009).

2.3 DESACOPLAMENTO FUNCIONAL ENTRE FLORES E FOLHAS E SUA IMPORTÂNCIA ADAPTATIVA

Organismos complexos são formados por diferentes estruturas (ex. membros e órgãos) que desempenham diferentes funções. Essas estruturas são geralmente consideradas módulos porque os traços que as compõem estão mais associados entre si do que com traços de outros módulos. Os mecanismos que originam esses módulos podem se originar por processos ontogenéticos (módulos ontogenéticos) e/ou por seleção natural em direção a uma íntima associação de conjuntos de traços para o desempenho de uma função específica (módulos funcionais). Módulos ontogenéticos são o produto de interações que ocorrem durante o desenvolvimento dos traços que compõem esses módulos. Já os módulos funcionais, embora também possam estar sujeitos aos processos ontogenéticos, são principalmente resultado de seleção natural em direção ao uma íntima associação entre diferentes traços (não necessariamente relacionados ontogeneticamente) cuja finalidade é desempenhar uma função específica (Wagner & Altenberg, 1996; Klingenberg, 2008).

Flores morfologicamente adaptadas a grupos específicos de polinizadores (ex. abelhas de médio porte, beija-flores ou morcegos) tendem a apresentar uma estreita associação entre os verticilos florais diretamente relacionados ao contato com polinizador para assegurar uma precisa transferência de pólen. A associação entre esses traços deve ser maior que com traços de outras estruturas da planta (ex. folhas), até mesmo com outras partes florais envolvidas em outras funções (ex. sinalização). Além disso, como os polinizadores de flores especializadas são semelhantes em tamanho e forma, para que a parte do corpo do polinizador onde o pólen foi depositado por uma primeira flor seja contatada pelo estigma de outra flor, os traços florais diretamente envolvidos nesse processo devem ser estáveis (i.e. pouco variáveis). Portanto, espera-se que flores especializadas se comportem como módulos funcionais, apresentando maior covariação entres os traços diretamente relacionados à polinização do que com outros traços florais ou vegetativos não envolvidos nesse processo. Além disso, espera-se que os traços envolvidos na polinização tenham menor variância que os demais traços (Berg, 1959, 1960). Embora essas predições tenham sido suportadas por vários estudos (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), desacoplamento entre flores e partes vegetativas também foi reportado para várias espécies com sistemas de polinização não especializado (ex. flores polinizadas por ampla gama de animais ou pelo vento; Armbruster *et al.*, 1999; Pérez-Barrales *et al*., 2007). Uma vez que a intensidade do desacoplamento entre flores não especializadas e traços vegetativos é geralmente menos intenso do que o desacoplamento em plantas com flores especializadas, isso sugere que a modularidade do primeiro grupo de flores se dá principalmente pela maior interação entre os traços florais durante sua ontogênese do que entre esses traços e traços vegetativos (módulos ontogenéticos). Já o segundo grupo de flores se caracteriza como um módulo funcional, já que além dos efeitos ontogenéticos, há também a seleção para um ajuste fino entre traços florais envolvidos na polinização (Armbruster *et al.*, 1999; Herrera *et al.*, 2002; Ordano *et al.*, 2008).

Estudar desacoplamento entre estruturas funcionalmente distintas é importante para entender os mecanismos relacionados à adaptação local dessas estruturas, principalemente quando as populações de uma espécie estão distribuídas em paisagens extremamente heterogêneas. O desacoplamento funcional é uma característica importante nesses casos porque a interferência da adaptação local de uma determinada função sofre pouca ou nenhuma interferência da adaptação de outra função às condições locais (Wagner & Altenberg, 1996). No caso de plantas, desacoplamento

entre flores e folhas em populações expostas a diferentes pressões seletivas em cada uma dessas estruturas – ex. diferentes polinizadores (flores) e diferentes climas (folhas) - permite uma maior variedade de respostas adaptativas da planta (Chalcoff *et al.*, 2008). Portanto, nesses casos a adaptação das flores às assembleias locais de polinizadores não será constrangida pela adaptação das folhas ao clima local. O desacoplamento flor-folha é particularmente crítico em plantas com flores especializadas e cujas variações climáticas entre populações podem levar a drásticos rearranjos fenotípicos de traços vegetativos. Se não houver desacoplamento, a variação dos traços vegetativos levará à instabilidade de traços florais, prejudicando o fino ajuste entre flores e polinizadores necessário para uma eficiente polinização (ex. Cosacov *et al.*, 2014).

Entretanto, ainda que plantas distribuídas em paisagens heterogêneas tenham flores e folhas desacopladas, isso não garante que as populações se adaptarão às condições bióticas e abióticas locais. Processos neutros relacionados a histórias demográficas e deriva genética podem levar a flutuações randômicas entre flores e folhas de diferentes populações, ainda que de uma forma desacoplada (Armbruster, 1991). Pequenas populações isoladas têm maior probabilidade de apresentar esse padrão (e.g. Herrera *et al.*, 2002).

2.4 INSELBERGS

Inselbergs (do alemão: insel = ilha; berg = montanha) são afloramentos rochosos distribuídos isoladamente ao longo de paisagens tropicais e temperadas, e habitados por inúmeras espécies endêmicas, onde ca. de 3.500 espécies de angiospermas são exclusivas dessas ilhas terrestres. Devido à descontinuidade desse habitat, populações de espécies exclusivamente rupícolas são geralmente pequenas e sempre isoladas, formando comumente arquipélagos altamente isolados geograficamente de outros arquipélagos (Barthlott & Porembski, 2000; Porembski, 2007; Boisselier-Duabayle *et al.*, 2010; Millar *et al.*, 2014). Além disso, populações de diferentes arquipélagos podem sofrer seleção divergente, já que estes estão frequentemente imersos em diferentes matrizes ambientais, tais como áreas mais xéricas de baixa altitude ou áreas mésicas mais elevadas (Barbará *et al.*, 2009; Pinheiro *et al.*, 2014). Portanto, plantas exclusivas de inselbergs são ideais para estudar fluxo gênico polínico em arquipélagos de pequenas populações disjuntas, bem como para examinar o papel do isolamento geográfico na adaptação local dessas populações.

2.5. *Encholirium spectabile* E *Ameroglossum pernambucense*

Encholirium spectabile (Bromeliaceae) e *A. pernambucense* (Scrophulariaceae) são plantas exclusivas dos afloramentos rochosos do nordeste do Brasil, cujas populações são pequenas e isoladas geograficamente, ocorrendo comumente em arquipélagos de inselbergs graníticos. Embora tenham sistema reprodutivo autocompatível, ambas as espécies dependem de polinizadores para aumentar seu sucesso reprodutivo (Queiroz, 2005; Queiroz, 2014; Wanderley *et al.*, 2014a, b). Os polinizadores de *A. pernambucense* são exclusivamente beija-flores, enquanto *E. spectabile* é principalemente polinizado por morcegos e beija-flores, apesar de vários outros grupos de visitantes (beija-flores, abelhas e vários outros insetos generalistas) também visitem suas flores, podendo polinizá-las (Queiroz, 2014; Wanderley *et al.*, 2014a). Portanto, essas duas espécies são ideais para estudar os padrões de fluxo polínico em arquipélagos de pequenas populações disjuntas.

Embora o gênero *Ameroglossum* esteja descrito como monotípico (Fischer *et al.*, 1999), as populações desse arbusto podem apresentar notáveis diferenças morfológicas, tantos nas estruturas reprodutivas quanto vegetativas, sugerindo que este táxon se trata de um complexo composto por diferentes subespécies ou espécies proximamente relacionadas. As diferenças morfológicas mencionadas podem ser: variações na filotaxia, que pode ser verticilada em *A. pernambucense* ou oposta cruzada nos demais morfotipos, caule cilíndrico em *A. pernambucense* ou quadrangular nos outros morfotipos, assim como diferenças no tamanho e forma de folhas e flores, as quais são menores em *A. pernambucense* (Wanderley *et al.*, 2014a, b; obs. pess.). As diferenças morfológicas observadas no complexo *A. pernambucense*, principalmente nas folhas e flores, parecem ser acompanhadas por variações ambientais, já que este táxon ocorre em um acentuado gradiente ambiental, podendo suas populações ocorrer em arquipélagos de inselbergs localizados desde áreas baixas de Caatinga (ca. 180 m), até elevadas áreas de brejo altitude acima dos 1000 m (Wanderley *et al.*, 2014a, b; obs. pess.). Portanto, o complexo *A. pernambucense* é ideal para examinar o papel da seleção divergente e da deriva genética na diferenciação de pequenas populações geograficamente isoladas.

3 WITHIN AND AMONG POPULATION MATING PATTERNS INFERRED FROM FLUORESCENT DYE PARTICLES IN ARCHIPELAGOS OF SMALL ISOLATED PLANT POPULATIONS IN NORTHEAST BRAZIL

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ABSTRACT

Background and aims Several plant species are naturally small and isolated because they are confined to discrete patches of suitable habitat. Both small population size and isolation increase inbreeding and genetic drift, which might reduce population adaptability because of loss of genetic diversity, inbreeding depression and genetic structuring of populations. High mating potential within (panmixia) and among (at least one among population mating per generation) small adjacent disjunct populations (archipelagos) by pollinator activity are expected to reduce the levels of both inbreeding and genetic structuring caused by drift, leading to higher adaptive potential of these populations. However, because pollinator activity is negatively related to population size, it remains unclear to which extent small isolated population can attract pollinators to increase their mating range. This research investigated whether the potential mating patterns within and among populations of the *Ameroglossum pernambucense* complex (Scrophulariaceae) and *Encholirium spectabile* (Bromeliaceae), both occurring as discrete adjacent populations in inselberg archipelagos, could minimize inbreeding and genetic drift. Specifically, within population panmixia in the *A. pernambucense* complex, and sufficient among population mating potential to prevent drift in both species were tested.

Methods Both within and among population mating potential promoted by pollinator activity were inferred using fluorescent dye particles as pollen analogues.

Key results Evidence of panmixia was not obtained in the examined populations of the *A. pernambucense* complex. However, the observed within population mating radius was expressive and extended over 34 and 47% of each population in a panmictic-like trend. The among population mating potential was eight and 20-folds higher, respectively for the *A. pernambucense* complex and *E. spectabile*, than the minimum expected to prevent population genetic structuring by drift.

Conclusions These data show that the adaptability of the studied populations will unlikely be constrained because of lack of pollinator activity.

Key-words: pollination, pollen flow, mating radius, adaptation, population isolation, inselbergs, ornithophily, chiropterophily, *Ameroglossum pernambucense* complex, *Encholirium spectabile*

INTRODUCTION

Several plants species are confined to discrete patches of suitable habitat, such as oceanic islands, humid mountaintops and rock outcrops. Because these habitats are discontinuous, plant populations from such areas are usually small and always isolated from one another (Ellstrand & Elam, 1993; Prance, 1996; Emerson, 2002; Porembski, 2007). Small population size and isolation usually increases inbreeding and genetic drift. Inbreeding can reduce population fitness (adaptability) and occurs when there is breeding between relatives, leading to an increase of homozygotes and reduction of genetic variation. Inbreeding has also an additional potential to reduce population fitness when the cumulative frequency of homozygous for recessive deleterious alleles leads to inbreeding depression. Genetic drift is the random fluctuation of allele frequencies over generations, an evolutionary force capable to reduce genetic variation and population fitness. Moreover, in the absence of gene flow, genetic drift is also expected to cause genetic structuring, leading to non-adaptive population differentiation and to an uneven distribution of genetic diversity. In small isolated populations in which gene flow is weak (i.e. less than one among population mating per generation; Slatkin, 1987) or absent, genetic drift can easily lead to loss of adaptive alleles, and therefore, affect population adaptability. In summary, small and isolated populations are expected to have lower adaptive potential and higher extinction risks than large continuous populations because of reduced fitness due to genetic impoverishment, inbreeding depression and genetic structuring. Therefore, as much as a small isolated population approaches its mating pattern to a panmictic unit (a set of individuals with similar chances of mating), and the greater is the number of among population mating, the higher are the chances of adaptation (Wright, 1931; Slatkin, 1987; Ellstrand & Elam, 1993; Oakley & Winn, 2012).

Inselbergs (from German: *insel* = island, *berg* = mountain) are isolated rock outcrops spread worldwide and inhabited by several groups of exclusive organisms. It is estimated that ~3500 angiosperm species are exclusive to this environment. Because inselbergs are discrete habitats (i.e. are discontinuous patches of habitat across a landscape), populations of inselberg specialist organisms are usually small and always isolated (Porembski, 2007). Therefore, inselberg populations are ideal systems to study the mating patterns within and among small discrete populations (Gevaert *et al.*, 2013; Millar *et al.*, 2013, 2014). Studies on the genetics of plant populations reported low genetic variability and high genetic structure (F_{ST} ~ 0.3), respectively,

within and among small isolated inselberg populations apart ca. 30-50 km, suggesting strong inbreeding and genetic drift operating in such populations (Barbará *et al.*, 2009; Boisselier-Duabayle *et al.*, 2010; Pinheiro *et al.*, 2014). Conversely, when these small discrete populations occur in archipelagos of adjacent inselbergs, levels of genetic structure are lower ($F_{ST} \sim 0.015$) and genetic diversity is higher, suggesting low inbreeding and gene flow within archipelagos (Boisselier-Duabayle *et al.*, 2010; Gevaert *et al.*, 2013; Millar *et al*., 2013). Nevertheless, it remains unclear if current patterns of genetic variability and gene flow in archipelago populations are due to contemporary gene movement (i.e. mating patterns) or recent events of dispersion or vicariance (but see Millar *et al*., 2014).

Because pollination usually promotes higher gene movement within and among angiosperm populations than dispersion, pollinators are key agents for the maintenance of genetic diversity in angiosperm species (Ennos, 1994; Petit *et al*., 2005). Therefore, pollinators can reduce inbreeding and drift in archipelagos of small discrete populations if their activity increases the number of possible mates within and among these populations. Nonetheless, pollinator activity is expected to be positively related to population size. Several studies reported lower pollinator activity (measured by stigma pollen loads, number of pollinator visits and/or pollinator-dependent reproductive success) in small isolated than large/continuous populations (Jennersten, 1988; Aizen & Feinsinger, 1994; Ågren, 1996; Molano-Flores *et al*., 1999). Therefore, small isolated plant populations within archipelagos might not necessarily attract pollinators. Despite the importance of pollinator activity as potential reducer of inbreeding and drift in archipelagos of small discrete populations, few is known about the extent of pollen flow in such populations. Recent studies reported evidence of contemporary pollen gene flow among populations within inselberg archipelagos distant up to ca. 2 km in North America and Western Australia (Gevaert *et al.*, 2013; Millar *et al.*, 2014). These findings suggest that despite their small size, inselberg archipelago populations can attract pollinators and reduce inbreeding and genetic drift.

The impact of pollinator activity on pollen movement, and thus on the potential mating patterns, within and among small isolated populations can be inferred using fluorescent dye particles as pollen analogues. Fluorescent dyes were reported by several studies as a reliable method to infer pollinator mediated plant mating because the dispersion of these particles were highly correlated to pollen dispersion (Waser and Price, 1982; Fenster *et al.*, 1996; Adler and Irwin, 2006). Pollen flow estimates using these particles are also the least expensive method to investigate

pollen movement (Fenster *et al.*, 1996). Besides of their low cost, fluorescent dyes may render more precise estimates of pollen movement than paternity analysis using molecular markers because paternity assignments in natural plant populations are often ambiguous (Fenster *et al.*, 1996).

The aim of this study was to investigate mating potential in archipelagos of small discrete populations of two Neotropical inselberg specialist plants, *Ameroglossum pernambucense* Eb. Fisch., S. Vogel & A.V. Lopes (Scrophulariaceae) and *Encholirium spectabile* Mart. ex Schult. f. (Bromeliaceae) using fluorescent dye particles as pollen analogues. Both species are restricted to inselbergs from northeastern Brazil, whose populations commonly occur in archipelagos of adjacent (~ 3 km) inselbergs (pers. obs.). *Ameroglossum pernambucense* is an ornithophilous shrub exclusively pollinated by hummingbirds (Wanderley *et al*., 2014a), whereas *E. spectabile* is a bromeliad mainly pollinated by bats and hummingbirds, but also by moths, generalist insects and the opossum *Didelphis albiventris* (Lund, 1840) (Queiroz, 2014).

Using these two plant models, two questions were addressed regarding the pollinator mediated plant mating patterns in archipelagos of small discrete population. First, do the *A. pernambucense* populations resemble to panmictic units, i.e. is the frequency of mating between individuals from the same inselberg similar regardless to their distance? Secondly, are the pollinators of *A. pernambucense* and *E. spectabile* able to promote at least one mating per generation between plants from different inselbergs, so that levels of gene flow among adjacent populations would suffice the minimum threshold predicted by population genetics theory to prevent structuring by drift? We predicted positive answers for both questions.

MATERIALS AND METHODS

STUDY SITES AND SPECIES

This research was conducted in two inselberg archipelagos, *Serra do Ponto* (*P*) and *Esperança* (*ESP*), both located in Northeastern Brazil (Fig. 1). The inselbergs from archipelago *P* are immersed in mountain Atlantic rainforest (~1200 m.a.s.l.; 08º 9' 36.3", s 36º 23' 31.5" w), whereas *ESP* archipelago is located in the semi-arid *Caatinga*, a Brazilian savanna-like ecosystem (~600 m.a.s.l.; 07º 0' 37.8" s, 35º 53' 58" w). Annual precipitation in *P* and *ESP* are, respectively, ~1000 mm and ~600 mm (Prado *et al.*, 2003).

Ameroglossum pernambucense complex is an ornithophilous endangered shrub included in the IUCN red list with distribution range restricted to northeastern Brazil inselbergs (Wanderley *et al.*, 2014b). Although *Ameroglossum* is monotypic, plants from different populations – including populations from *P* and *ESP* (Fig. 2 A and B) - exhibit morphological variation, suggesting a species complex composed by at least three different species or subspecies (Wanderley *et al.*, 2014a,b; pers. observation). Whereas populations from *P* exhibit 5-6 verticilate phyllotaxy with lanceolate leaves ~5.5 cm long and floral tube length ~2.8 cm, in *ESP*, plants show opposite decusate phyllotaxy and significantly longer leaves (~12.4 cm) and floral tubes (~3.8 cm). However, plants from both archipelagos have red, tubular and zygomorphic flowers exclusively pollinated by hummingbirds (unpublished data). Because of the taxonomic uncertainties caused by morphological variation in this species complex, from now on this taxon will be referred as the *A. pernambucense* complex. Although self-spontaneous, plants of this complex rely on hummingbirds to increase reproductive success (number of fruits and seeds). Plants of this complex can offer copious amounts of nectar (up to 107.9 µl and 24.8 mg of sugar per flower) and produce up to 283 flowers at the same time, what is sufficient to provide 1/2-1/3 of the daily energy demanded by a hummingbird (Wanderley *et al.*, 2014a). The flowering period of the *A. pernambucense* complex lasts from April to October with flowering peak between July and August (Wanderley *et al.*, 2014a; unpublished data).

Encholirium spectabile is an endemic bromeliad from inselbergs of Northeastern Brazil, occurring mostly in the Caatinga domain (Fig 2 C; Forzza, 2005). The flowers of this plant are bright yellow to greenish with an open actinomorphic corolla 1.5-2.5 cm wide and 1-2 cm deep

(pers. observation). The main pollinators of *E. spectabile* are bats and hummingbirds. Similarly to the *A. pernambucense* complex, this bromeliad is self-compatible, but pollinators are required to increase reproductive success. *Encholirium spectabile* flowers also produce copious amount of nectar (190.34 ± 98.52 µl and 30.55 ± 14.0 mg of sugar per flower; mean ± SD) and inflorescences exhibit 28.55 ± 6.6 flowers per day. Flowering of *E. spectabile* occurs from June to October (Queiroz, 2014).

WITHIN POPULATION MATING ESTIMATES

To test whether small isolated populations can be panmictic units, experiments with fluorescent dye particles as within population mating estimates (i.e. potential mating frequency) were performed in two *A. pernambucense* complex populations, one from *ESP* and the other from *P*. Specifically, it was tested whether the potential mating frequency among individuals of the same population was distance-dependent (see Data analysis). In a panmictic situation, an individual is expected to have similar mating frequencies with all fertile plants inhabiting the inselberg, regardless to their spatial distance.

The within population mating frequency was estimated during the flowering peak (August 2011) by choosing one cluster in each population of 4-6 plants (donor plants) and applying fluorescent dye particles (Shanon Luminous Materials, Inc.) at the anthers of all their flowers using a small thin paintbrush. Thirty-four and 49 flowers were marked with dye particles, respectively, in *ESP* and *P* populations. Flower marking occurred in the morning, when hummingbird activity starts. After 24 hours, stigmas from all observed flowers in each population were collected and their distance from donor plants was recorded using a GPS (*Global Positioning System*). In *ESP* population, 75 stigmas distant 10-150 m from the donor were collected. In *P* population, the number of collected stigmas was 407 and their distance to the donors varied from 10 to 498 m. The difference in number and distance range of stigmas collected between these two populations was because in *ESP* the studied *inselberg* had a smaller size – and consequently a smaller population size than the inselberg used for this study in *P*.

AMONG POPULATION MATING ESTIMATES

To test the prediction that pollinators can promote at least one mating between small isolated populations per generation, experiments of dye particles movement among three sympatric populations (*ESP* 1, 2 and 3) of the *A. pernambucense* complex and *E. spectabile* were performed in *ESP* archipelago. Distance among inselbergs where these populations occur vary from 850 to 2400 m. Among population mating estimates for the *A. pernambucense* complex were performed during the same flowering peak of the within population experiments mentioned above. Because one of *ESP* populations (*ESP 2*) was used for both within and among population estimates of mating, colors of fluorescent dye particles (yellow, blue, red and white) did not overlap in each type of estimate. Among population mating estimates for *E. spectabile* occurred in October 2011, during its flowering peak.

To assess pollinator mediated dye particles movement among *ESP 1*, *2* and *3* for both species, anthers of all observed flowers that could be accessed in each population (some *E. spectabile* flowers were too high to be reached) were marked with fluorescent dye particles. Each population was marked with particles of different colors, so that it was possible to track their movement. Because *A. pernambucense* complex and *E. spectabile* experiments were performed with a two months interval, using the same colors of particles in both species would unlikely produce misleading results. The number of marked *A. pernambucense* complex flowers was 99, 49 and 39, respectively, in *ESP 1*, *2* and 3 (total = 187). For *E. spectabile*, 130, 31, and 39 flowers (total = 200) were marked in the same sequence of *ESP* inselbergs mentioned above. *Ameroglossum pernambucense* complex flowers were marked during the morning, while *E. spectabile* flowers were marked when flower anthesis begin, before sunset (~17:00). After 24 hours of flower marking, all stigmas of both species that could be reached in *ESP 1*, *2* and 3 were collected and labelled according to their origin (i.e. *ESP 1*, *2* or 3). Forty, 75 and 92 (total = 207) *A. pernambucense* complex stigmas were collected, respectively, in *ESP 1*, *2* and 3. For *E. spectabile*, the number of stigmas collected were, respectively, 251, 239 and 233 (total = 723). The Discrepancy in the number of marked flowers and stigmas collected in *E. spectabile* (200 and 723) was because flower anthesis remained after anthers marking. Therefore, there were more open flowers during stigma collection, than during anthers marking.

DATA ANALYSIS

The presence of fluorescent particles on collected stigmas was verified using stereomicroscope with a UV lamp. When stigma was positive, i.e. with dye particles deposited, it was assumed as an evidence of potential mating. Therefore, the presence of within population panimixy and among population mating potential were considered based on frequency of positive stigmas. To test panmixy, the collected stigmas were grouped into distance classes from donor plants, and the frequency of positive stigmas (potential mating frequency) among these classes was compared using Chi-Square tests. To avoid type I error because of multiple comparisons among several distance classes, the minimum possible number of comparisons were performed by means of stepwise comparisons using pairs of classes with the highest frequencies of pollen transfer. If the highest frequency of dye particle transfer was significantly different from the second highest frequency, then all frequencies lower than the second one were assumed as significantly different from the former. After this, the second and third highest frequencies of dye particle transfers were compared, and so forth. Because in *P* population all frequencies of transfer different from zero, but one, were very similar, only the first and the second highest frequencies were compared (see Fig. 3).

To test whether the number of among population mating potential per generation was higher than one (second prediction), the frequency of among population dye particle transfer was determined. If the number of potential mating among populations in only 24 hours (interval between flower marking and stigma collection) was higher than one, then it would be reasonable to assume that in one generation the among population mating potential is sufficient, at least theoretically (Slatkin, 1987), to prevent differentiation by drift. Additionally, a Chi-Square test was used to test whether the frequency of among population mating potential between *A. pernambucense* complex and *E. spectabile* was different. Significance of all Chi-Square tests used in both within and among population experiments was determined after 1000 permutations.

RESULTS

WITHIN POPULATION MATING ESTIMATES

No evidence of panmixia was observed within both examined *A. pernambucense* complex populations because the frequency of positive stigmas was zero at the farthest distance classes of these populations (Fig 3). In *ESP* population, the more distant positive collected stigma was 61-70 m distant from donor plants, although stigmas were collected up to 150 m apart from donors. In *P* population, stigma collection extended up to 500 m from plant donors. However, the farthest recorded dye particle dispersion was 131-170 m distant from donors. Therefore, the estimated extent of pollen flow (mating radius) in *ESP* and *P* populations comprised, respectively, 47 and 34% of population extensions (farthest recorded dye particle dispersion/farthest stigma collected from donor plants ratio).

Nonetheless, within the radius of observed dye particles movement in both populations (i.e. 70 m in *ESP*, and 170 m in *P*), mating frequency tended to be homogeneous, resembling a panmictic unit. In *ESP* population, the frequency of positive stigmas did not differ significantly (χ^2 = 2.06, df = 1, p = 0.13) among distance classes within the mating radius – except between 21 and 40 m, where positive stigmas were significantly less frequent (χ^2 = 2.06, df = 1, p = 0.13), and between 31 and 50 m, where no positive stigmas were observed (Fig. 3A). In *P* population, the frequency of positive stigmas was constant within the estimated mating radius, except between 61 and 80 m, where the highest frequency of positive stigmas was recorded (Fig. 3 B; χ^2 = 7.01, df = 1, p < 0.0001).

AMONG POPULATION MATING ESTIMATES

In both species among population flow of dye particles was observed among all examined *E. spectabile* populations and between two *A. pernambucense* complex populations (*ESP 1* and *3*; Fig. 4). Whereas *E. spectabile* pollen flow estimation was unidirectional from *ESP 1* (850 m distant) to *ESP 3*, then to *ESP 2* (2400 m distant), it occurred in both directions between the populations *ESP 1* and *2* of the *A. pernambucense complex* (Fig. 4). Although among population dye particles dispersion was more extensive in *E. spectabile* than in *A. pernambucense* complex

occurring, respectively, among three and two populations, the estimated between population pollen flow was more intense (higher frequency of positive stigmas) in *A. pernambucense* complex (χ^2 = 11.97; df = 1; p = 0.001). Allochthonous dye particles were observed in eight of the 207 (8.69%) *A. pernambucense* complex stigmas collected during the experiment of among population mating estimation, whereas only 20 out of 723 collected (2.77%) *E. spectabile* stigmas had allochthonous dye particles deposited. Nonetheless, because in both species evidence of eight and 20 among population mating potential was observed, the prediction of at least one among population mating potential per generation was supported.

DISCUSSION

The first prediction set in this study, i.e. the small isolated *A. pernambucense* complex populations are panmictic units, was not supported. However, the potential mating frequency within the mating radius of these populations tended to be homogeneous, resembling to panmictic units. Although such results do not exclude the possibility of subpopulation structuring, the panmictic-like mating radius extended over 34 and 47% of the *A. pernambucense* complex populations, respectively, for *ESP* and *P* archipelagos. Thus, the observed within population pollinator activity does not seem negligible. Further, the observed *A. pernambucense* complex and *E. spectabile* among population mating potential during a period of only 24 hours was, respectively, eight and 20 folds higher (frequency of positive stigmas with allochthonous dye particles) than the minimum threshold theoretically predicted to prevent population genetic structure by drift (Slatkin, 1987). Therefore, despite the expectation of low pollinator activity in small discrete populations (Jennersten, 1988; Aizen & Feinsinger, 1994; Ågren, 1996; Molano-Flores *et al*., 1999), these findings show that small isolated plant populations from archipelagos of a discrete habitat can be sufficiently attractive to pollinators. In turn, pollinator activity might reduce inbreeding and genetic drift, rendering higher adaptability.

This is in agreement with population genetic studies in this field (Boisselier-Duabayle *et al.*, 2010; Gevaert *et al.*, 2013; Millar *et al*., 2013). For instance, within inselberg archipelago populations (distant < 5 km) of *Pitcairnia geyskesii* L.B. Sm., *Helianthus porteri* (A. Gray) Prusky (Asteraceae) and *Acacia woodmaniorum* Maslin & Buscumb (Fabaceae), inbreeding (F_{IS}) and genetic structure (F_{ST}) levels were different from those expected for populations experiencing high inbreeding and genetic drift. The observed levels of genetic diversity of within archipelago populations of these species were consistent with the evidences for their long-term persistence, which requires adaptability. The same was not true for the remote populations of these same archipelagos isolated from others by ~10 km (or even more) (Boisselier-Duabayle *et al.*, 2010, Gevaert *et al.*, 2013, Millar *et al*. 2013). Although seed dispersal within and among populations within archipelagos, to our knowledge, were not estimated, evidences suggest that gene movement via seed dispersal is weak or ineffective in preventing inbreeding and drift in these populations (Wyatt, 1997; Byrne and Hopper, 2008; Pinheiro *et al*., 2014). *Ameroglossum pernambucense* complex and *E. spectabile* also present limited seed dispersal primarily promoted by gravity

(Wanderley *et al.*, 2014a; pers. observation). Conversely, similar to the results here shown, in *H. porteri* and *A. woodmaniorum* (both mostly pollinated by several groups of insects) pollen flow estimates revealed long distance pollen gene flow (*H. porteri*, ~1500 m; *A. woodmaniorum*, 1870 m). Hence, it seems reasonable to expect pollinators as the primary drivers creating/or maintaining genetic diversity and gene flow within archipelagos of small disjunct populations as reported in previous studies (Boisselier-Duabayle *et al.*, 2010, Gevaert *et al.*, 2013, Millar *et al*. 2013, 2014).

The breeding system is also an important factor preventing or contributing to inbreeding and genetic structuring by drift (Ellstrand and Ellam, 1993). *Ameroglossum pernambucense* complex and *E. spectabile* are self-compatible, although pollinators are required to increase their reproductive output (Queiroz, 2014; Wanderley *et al.*, 2014a). Self-compatible plant species are generally expected to be less susceptible to inbreeding depression, because of genetic purging, than self-compatible plants (Charlesworth & Charlesworth, 1987; Barret & Charlesworth, 1991a; Sampson *et al.*, 2014). Nonetheless, different studies reported inbreeding depression in populations of self-compatible plants (Karron, 1989; Barret & Charlesworth, 1991b). Furthermore, small populations may fail to purge deleterious alleles when genetic drift overcome partial or completely natural selection, leading to inbreeding depression in self-compatible populations (Hedrick & Miller, 1992). Finally, self-compatibility strengthens population genetic structuring (Barbará *et al*., 2007). Therefore, pollinators have potential to reduce inbreeding depression in both self-compatible and self-incompatible small isolated populations. Furthermore, because population genetic structuring is stronger in self-compatible populations, a more intense among population pollen gene flow within archipelagos is required in self-compatible species to prevent or at least reduce genetic structuring by drift.

An important factor that might contribute to the observed pollinator activity is the amount of floral nectar offered by the study species. In a previous study, the maximum estimated amount of floral nectar offered by a single *A. pernambucense* complex plant (~3.49 kcal) was sufficient to supply 1/2-1/3 of the daily energy demand of a hummingbird (Wanderley *et al.*, 2014a). Queiroz (2014) also reported high amounts of sugar production by *E. spectabile* flowers (total volume = 190.34 ± 98.52 µl; mean sugar concentration = 16.58 ± 8.86%). Differences in the extent of among population dye particle transfer (*A. pernambucense* complex, 850 m; *E. spectabile*, up to 2400 m), in turn, is likely explained by the foraging amplitude of the pollinator guilds of each species. *Ameroglossum pernambucense* complex is exclusively pollinated by hummingbirds, whereas *E.*

spectabile is mostly pollinated by hummingbirds and bats. Bats seem to forage longer distances than hummingbirds. For instance, bats were commonly observed moving over up to ~10 km during a single foraging period (Medina *et al.*, 2007). On the other hand, the maximum foraging distance observed for the hummingbird *Phaethornis guy* (Lesson, 1833) in a fragmented tropical landscape was ~1500 m (Hadley & Bets, 2009). Conversely, the highest efficiency of among population dye particle transfer observed in *A. pernambucense* complex – 8.69% of positive stigmas with allochthonous particles for *A. pernambucense* complex, against 2.77% for *E. spectabile* – might be a result of pollinator specialization. While *A. pernambucense* complex is pollinated only by hummingbirds (Wanderley *et al.*, 2014a), *E. spectabile* flowers were observed been visited by a wide range of insects (e.g. generalist bees, butterflies and moths) besides their legitimate pollinators. Therefore, higher pollen waste by non-legitimate pollinators might occur in *E. spectabile* than in *A. pernambucense* complex, explaining the lower efficiency of among population transfer of dye particles observed in the former.

The genetic diversity and, thus, the adaptability of plant populations do not depend uniquely on the extension of their mating radius. Demographic factors (e.g. dispersion, vicariance and bottleneck/founder effect), as well as breeding system (self-compatible or incompatible) have also major impacts on populations genetic diversity (Keller *et al.*, 2009; Gevaert *et al*., 2013; Millar *et al*., 2014; Sampson, *et al.*, 2014). Therefore, it is not possible to conclude from the results here presented that the study populations are not experiencing inbreeding depression or genetic drift. Nonetheless, these data show that small populations from archipelagos of discrete habitats will not necessarily lose genetic diversity and face genetic structuring because their small size and isolation prevent pollinator activity. This conclusion is in agreement with recent studies in ancient inselberg archipelago populations (i.e. with more or less stable demographic histories) that found long distance pollen dispersal, and levels of genetic diversity and gene flow within archipelagos different from those expected in high inbreeding and genetic drift conditions (Gevaert *et al.*, 2013; Millar *et al.*, 2013, 2014). Therefore, pollinator mediated gene movement within isolated archipelagos of small discrete populations might have a primary role in maintaining suitable levels of genetic diversity necessary for adaptation in such populations.

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FIGURES

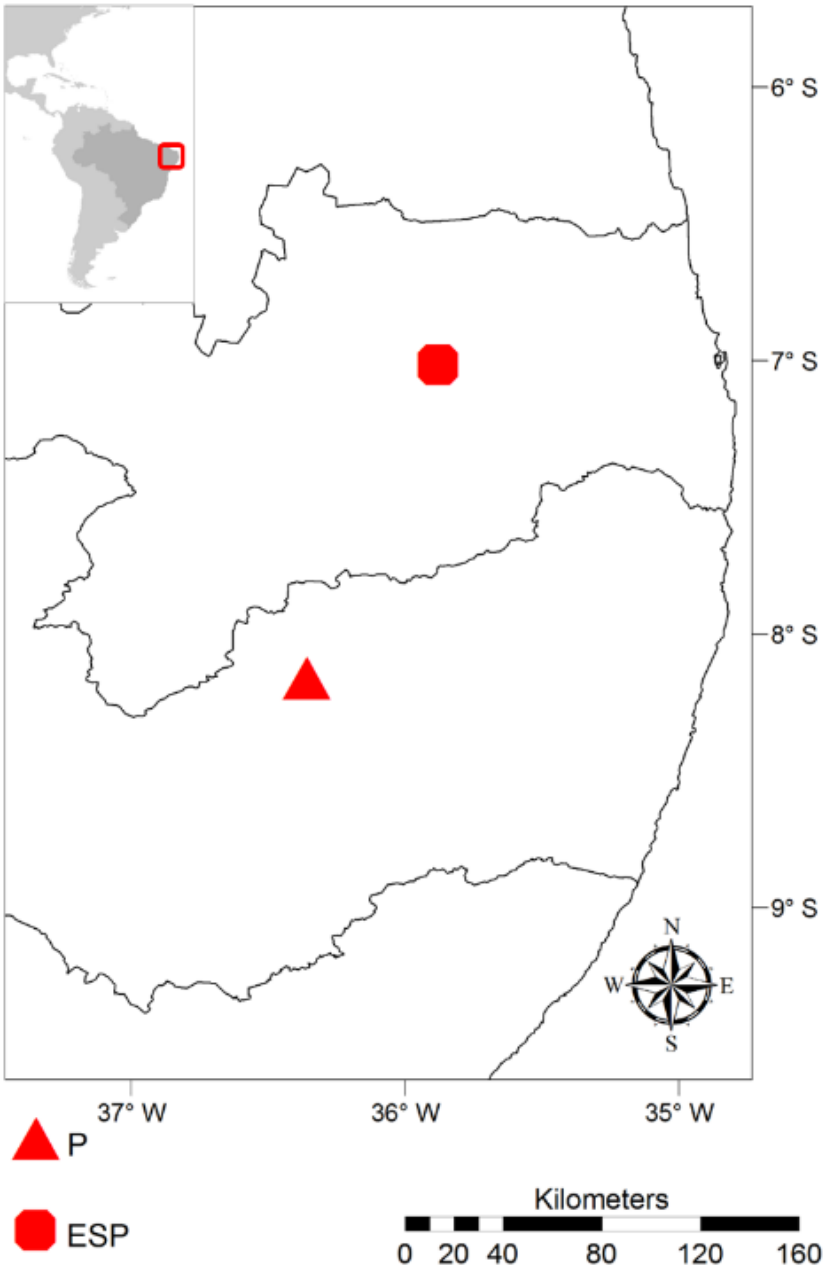

FIGURE 1 - Geographical location of the inselberg archipelagos *Serra do Ponto* (*P*) and *Esperança* (*ESP*)

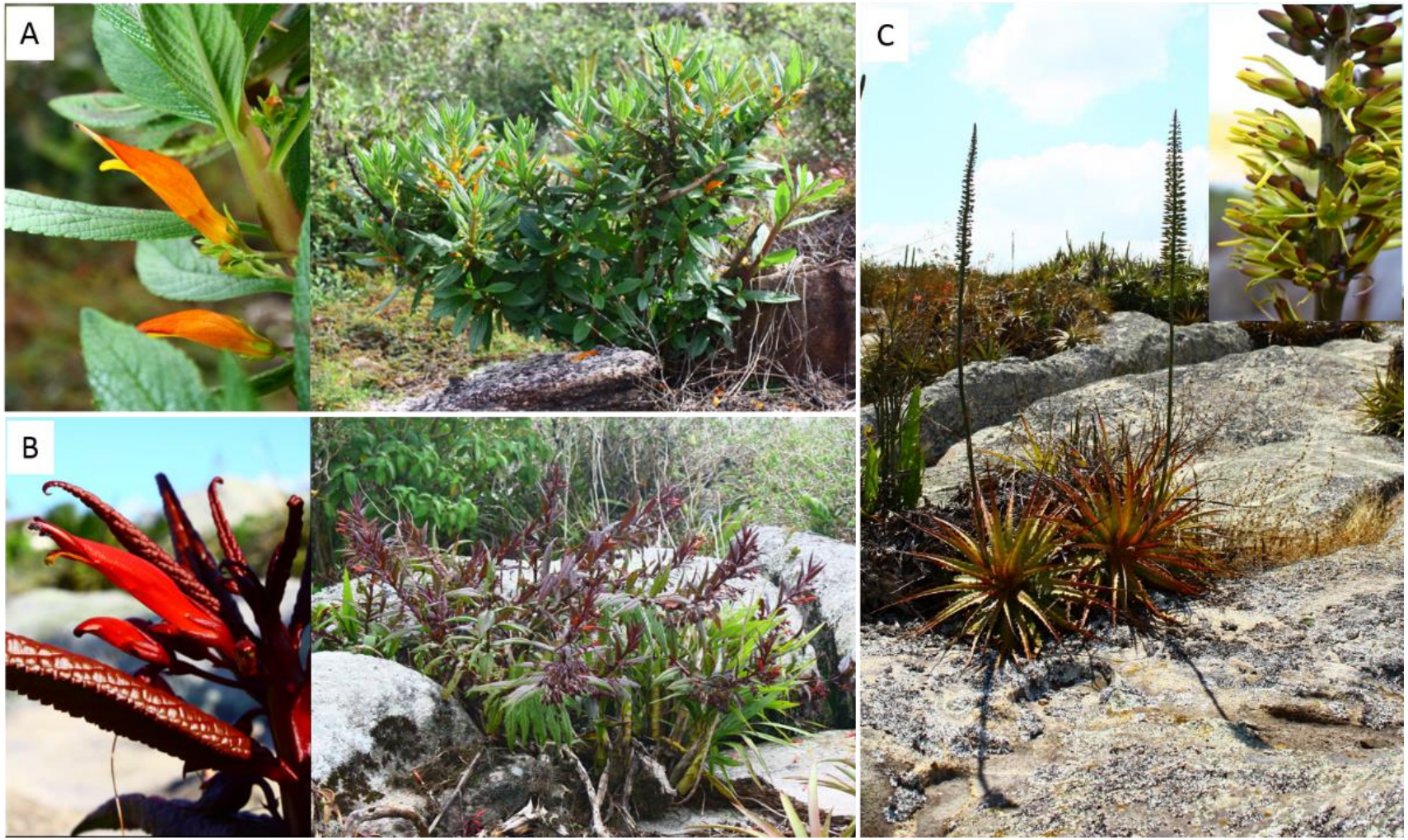

FIGURE 2 - Plants of the *Ameroglossum pernambucense* complex from *P* (A) and *ESP* (B) archipelagos. *Encholirium spectabile* (C).

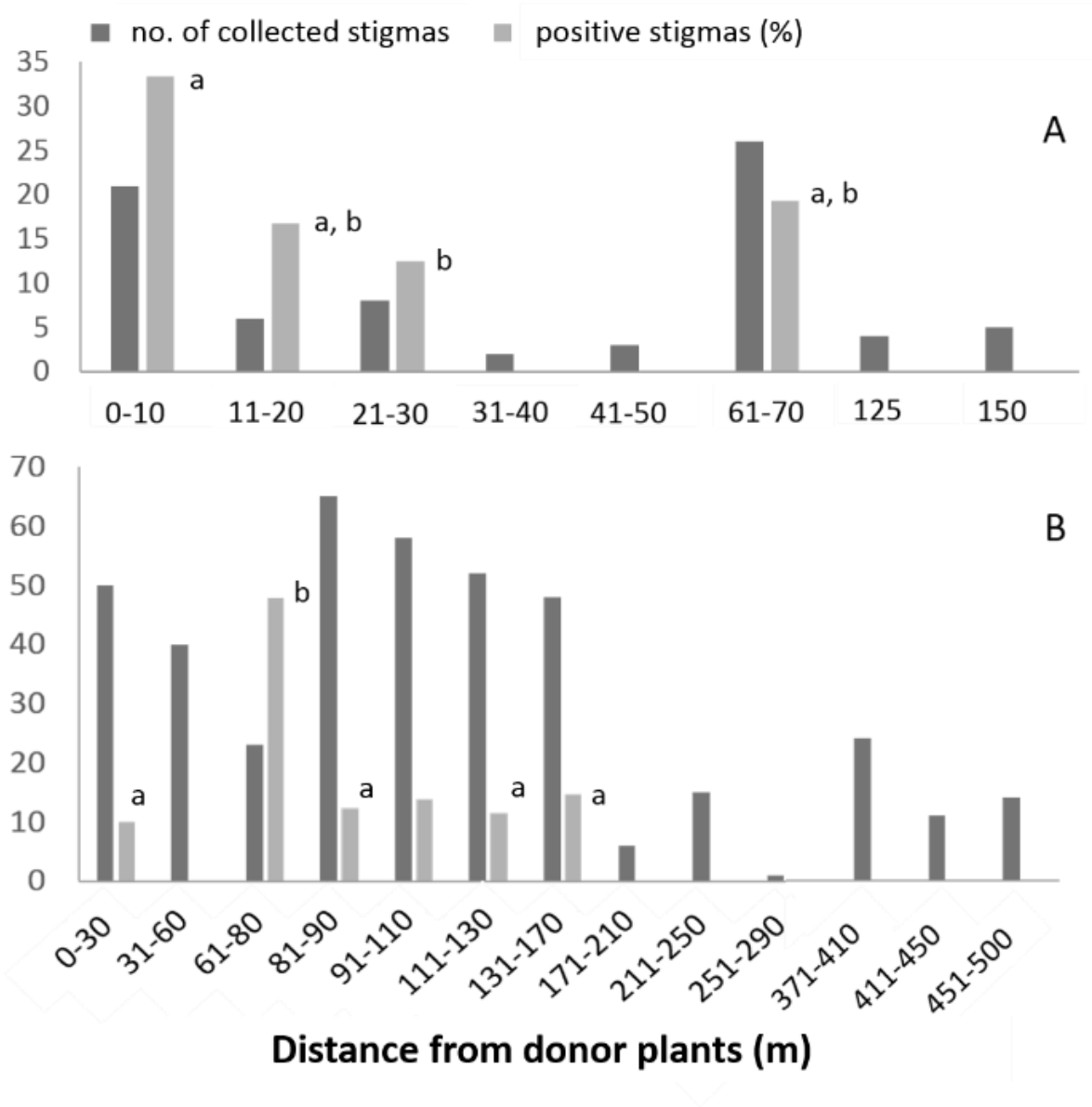

FIGURE 3 - Estimated within population mating potential using fluorescent dye particles as pollen analogues in two small discrete populations of the hummingbird pollinated *Ameroglossum pernambucense* complex belonging to two distinct inselberg archipelagos (*ESP*, A; and *P*, B). Significant differences (letters on column tops) in frequency of dye particle transfers were tested by Chi-Square.

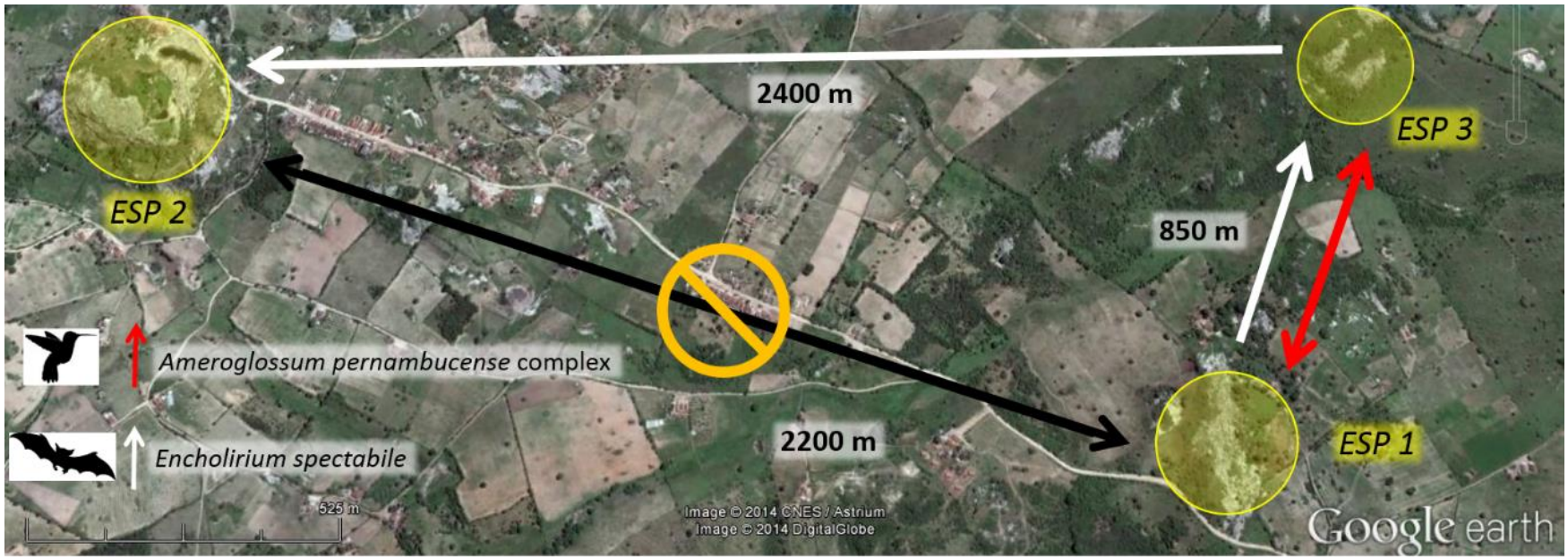

FIGURE 4 - Intraspecific mating potential among three sympatric populations of the *Ameroglossum pernambucense* complex and *Encholirium spectabile*, inferred from fluorescent dye particles used as pollen analogues. Red arrows indicate among population mating potential observed for the *A. pernambucense* complex, mostly pollinated by hummingbirds. White arrows indicate among population mating potential observed for *E. spectabile*, mostly pollinated by bats and hummingbirds. No mating potential was observed among *ESP 1* and *ESP 2* for neither species (black arrow).

4 IMPACT OF SELECTION VERSUS GENETIC DRIFT ON POPULATION DIVERGENCE AMONG HIGHLY ISOLATED PLANT POPULATIONS

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ABSTRACT

Background and aims Geographical isolation usually constrains gene flow, and often enhances the likelihood of genetic drift in small populations. Nonetheless, small isolated populations from patchy habitats are also likely to diverge due to local adaptation that is not diluted by gene flow. Using the naturally distributed patchy populations of the inselberg-specialist shrubs of the genus *Ameroglossum*, we tested whether population differentiation in this taxa showed evidence of local adaptation.

Methods First, we investigated whether geographical barriers were indeed more important than environmental or temporal barriers constraining gene flow and, thus, creating opportunity for population differentiation by genetic drift or selection. Then, based on microsatellite genotypes and leaf and floral phenotypes, we examined the extent to which genetic and morphological interpopulation variations were associated with local environmental factors (evidence of local adaptation) and geographical location (evidence of genetic drift because neutral demographic histories of populations are spatially dependent). Additionally, we searched for evidence of local adaptation by testing for specific associations between leaf and floral traits and abiotic and biotic selection pressures.

Key results Geographical isolation was observed as more important than environmental or temporal barriers limiting gene flow, and the studied populations revealed high levels of genetic structure. We found evidence that divergent selection is an important mechanism driving genetic and morphological differentiation among populations of the *Ameroglossum pernambucense* complex, although genetic drift also contributes. Floral tube length was highly associated with the most frequent local pollinators and leaf morphology was associated to key climatic variables.

Conclusions Small isolated populations can be locally adapted despite their susceptibility to the effects of genetic drift. Increase of population size due to pollinator-mediated gene flow among close patches, genetic purging of unfavorable alleles, and rapid fixation of locally adapted traits, are all potential factors that counteract the impact of genetic drift.

Keywords: divergent selection, local adaptation, genetic drift, genetic and phenotypic divergence, microsatellite, *Ameroglossum*, inselbergs

INTRODUCTION

Two major evolutionary mechanisms create population divergence - selection by local environments and genetic drift (Endler 1986; Slatkin 1987). For geographically isolated small populations, genetic drift can hamper the impact of natural selection (Wright, 1931; Ellstrand and Elam, 1993). At the same time, disruption of gene flow in isolated populations can allow response to local selection pressures, resulting in local adaptation and eventually speciation if the populations are sufficiently isolated (Mayr, 1942; 1947; Slatkin, 1987; Schluter, 2001; Levin, 2005; Lenormand, 2012). Isolation among populations lacking geographical barriers might also arise due to environmental heterogeneity. This is the case when environmental differences among populations lead to non-random gene flow within similar environments, or when there is selection against immigrants adapted to contrasting environments (Andrew *et al.*, 2012; Edellar and Bolnick, 2012). In both cases, gene flow is reduced and local adaptation is favored. Nonetheless, geographical isolation is not required. In plants, temporal barriers among adjacent populations occurring in contrasting environments are common due to interpopulation differences in the timing of flower phenology. Like geographical or environmental isolation, flowering displacement might constrain gene flow and induce divergence among populations (Keller *et al*., 2009; Levin 2009; Ortego *et al.*, 2012). For example, Thomasset *et al*. (2014) reported genetic structure among adjacent populations of *Fraxinus excelsior* L. with different flowering periods. Hence, environmental and temporal isolation can confound geographical isolation and reduce gene exchange. Several plants species present small and highly isolated populations because they are restricted to small patches of suitable habitat (e.g. oceanic islands, humid mountaintops and rock outcrops). Therefore, to study appropriately the impact of geographical isolation on the population differentiation of these plants when they occur in heterogeneous landscapes, it is necessary to disentangle the relative roles of genetic drift and selection, while controlling for environmental and temporal barriers.

Divergence among local climates are expected to create selection pressures on local populations, leading to genetic structuring among populations from different environmental envelopes. When climatic divergence is strong enough, footprints of natural selection are left on neutral genomic regions, and revealed when testing for genetic and environmental associations (Sork *et al.*, 2010; Andrew *et al*., 2012; Ortego *et al*., 2012; Gugger *et al.*, 2013; Wang *et al*., 2013).

The observed associations between neutral molecular markers and adapted phenotypes are a clear example of how natural selection and genetic composition are often linked (e.g. Wang and Summers, 2010). Similarly, morphological variation of leaf and floral traits among populations are expected under divergent selection. Leaf size and leaf area generally correlate negatively with temperature and elevation, and positively correlate with water availability (Milla and Reich, 2011; Chitwood *et al.*, 2012; Ramírez-Valiente *et al*., 2014). Flowers are expected to vary according to pollinator environments (Schemske and Bradshaw, 1999; Boyd, 2002; Anderson and Johnson, 2008). Therefore, divergent selection predicts covariation between neutral markers or morphological traits and the environmental sources of selection. On the other hand, because geographically related populations usually share similar demographic histories, random events promoting population divergence by genetic drift tend to be geographically structured. Thus, drift predicts covariation between molecular or morphological variation with geographical location (Sork *et al.*, 2010; Keller *et al.*, 2009; Andrew *et al*., 2012; Ortego *et al*., 2012; Gugger *et al.*, 2013; Wang *et al*., 2013).

Plants exclusive to inselbergs (i.e. rock outcrops; from German: *insel* = island, *berg* = mountain) are excellent models to study the role of selection and genetic drift contributing to divergence among small isolated populations. Inselberg specialist plant populations are discrete because the surrounding matrix of these rock outcrops is inhospitable. The population size of these rock specialists is usually small (assuming a population as the individuals inhabiting the same rock outcrop) because it is determined by the inselberg boundaries (Porembski, 2007; Barbará *et al*., 2009). Pollen flow among populations is possible when they occur in clusters of adjacent inselbergs (archipelagos), and this might increase the effective population size (Millar *et al*., 2014; Wanderley unpl. data). Nevertheless, the inselberg archipelagos are usually highly isolated and can occur under very distinct conditions, such as lowland dry areas or highland humid environments (Porembski, 2007; Barbará *et al.*, 2009; Pinheiro *et al.*, 2014). The impact of the geographical isolation of inselbergs on gene flow is indicated by the reported high levels of genetic differentiation among populations of inselberg specialist plants (Barbará *et al*., 2007; Byrne & Hopper, 2008; Pinheiro *et al.*, 2014). Additionally, interpopulation phenotypic variation in leaf and flower traits have been reported for these plants (Byrne and Hopper, 2008). However, the relative roles of selection and drift as drivers of genetic and morphological variation among inselberg plant populations were not examined.

Ameroglossum (Scrophulariaceae) is a rare genus of ornithophilous shrubs endemic to granitic inselbergs of northeastern Brazil (Wanderley *et al*., 2014a), with only one species described, *A. pernambucense* E.B. Fischer, S. Vogel & A. Lopes. Populations of this genus are small (20 to few hundred individuals) and discrete, commonly occurring in highly isolated inselberg archipelagos. Hummingbirds are the only pollinators of this genus and can promote pollen flow within archipelagos (i.e. among adjacent outcrops ~ 850 m unpl. data). These plants are self-compatible, but hummingbirds are necessary to increase the reproductive success (Wanderley *et al*., 2014 a,b; unpubl. data). The *Ameroglossum* populations occur across a highly heterogeneous landscape, which seems to be accompanied by morphological variation in leaf and flower traits (see Methods), suggesting local adaptation (pers. obs.). Alternatively, small size and spatial isolation could also explain population differentiation induced by genetic drift. Because of the morphological variation among populations of this genus, from now on this taxon will be referred as the *A. pernambucense* complex.

The main goal of this study was to estimate the relative roles of natural selection and genetic drift on genetic and phenotypic differentiation among populations of the *A. pernambucense* complex. To achieve this goal we (1) assessed whether geographical isolation is the main factor constraining gene flow, rather than environmental or temporal barriers. (2) We examined whether morphological differentiation of flowers and leaves among populations is followed by genetic differentiation on six neutral microsatellite loci. (3) We tested the strength of covariation between microsatellite allelic variables and floral and leaf traits with climatic and geographical variables. (4) We looked for evidence that selection is actually acting on specific traits by examining the correlations between leaf area and key climatic variables, and between floral tube length and bill length of local pollinators. Results from this set of studies tested the central hypothesis that divergent selection in steep environmental gradients overcomes the impacts of genetic drift found in isolated small populations.

MATERIALS AND METHODS

STUDY LOCATION

The *A. pernambucense* complex occurs predominantly in the Borborema Plateau, a geological formation located in Northeastern Brazil with uplift ca. 25 Ma. This plateau has a highly heterogeneous landscape composed by a mosaic of phytophisionomies belonging to the semi-arid Caatinga and tropical Atlantic rain forest domains (Andrade-Lima, 1960; Prado, 2003; Corrêa *et al*., 2010; Oliveira & Medeiros, 2012). Only two northernmost populations are known to occur beyond the Borborema Plateau and are located in low land Caatinga (ca. 100-150 m.a.s.l.), at the margins of this plateau (pers. obs.). Environmental conditions where populations of the *A. pernambucense* complex are found can be classified in three rough categories: (1) highland inselbergs at 1000-1215 m.a.s.l. exposed to intense wind and low temperatures at night (~4ºC), and ca. 1000 mm of annual rainfall (2) low-mid elevation seasonally dry inselbergs (150-800 m.a.s.l.) exposed to high solar irradiation, high temperatures during the day, and severe drought periods (~6 months) with ca. 600 mm of annual rainfall; (3) mid elevation (500-600 m.a.sl.) forest-immersed inselbergs partially covered by the Atlantic forest canopy with lower levels of solar irradiation and annual rainfall and drought periods similar to the first category (pers. obs., but see Prado, 2003 for a climatic survey of this region).

In each of these climatic envelopes a different morphotype occurs. The morphotype here named as *pernambucense* inhabits highland inselbergs and refers to *A. pernambucense*, the only species described for the genus, which is characterized by cylindrical stems; the smallest leaves of the complex with phyllotaxy 3-5-verticillate; and short tubular red flowers. Plants inhabiting seasonally dry and forest-immersed inselbergs have larger leaves and flowers than *A. pernambucense* and share quadrangular stems and opposite phyllotaxy. However, plants from forest-immersed inselbergs (*forest-immersed* morphotype) have larger leaves and flowers than those from seasonally dry inselbergs (*dry-season* morphotype), characterizing two other morphotypes, probably subspecies or species related to *A. pernambucense*. All three morphological groups can be easily recognized by leaf and floral traits (pers. orbs.). Classification of these morphotypes are based on field observation and stem cuttings from wild plants grown in greenhouse, whose leaves and flowers produced under similar environmental conditions

maintained the morphological differences above described. However, genetic differences among the three morphotypes have not yet been tested.

POPULATION SAMPLING

For this study, we assumed population to be the cluster of individuals sharing the same inselberg. Overall, 15 populations from distinct inselberg archipelagos were sampled across the whole distribution known for the *A. pernambucense* complex at all three environmental categories described above: three populations from morphotype *pernambucense*; 10 populations from morphotype *dry-season*; and two populations from morphotype *forest-immersed* (Fig. 1). Unbalanced sampling design was due to rarity and difficult accessibility of populations from the first and last morphotypes. In each population, leaf tissue was collected for DNA extraction and mature 1-3 leaves and flowers per plant were sampled for morphometric analysis. Some populations have hundreds of individuals, but most population sampling was limited by a population size inferior to 20 adult plants (Table 1).

GENOTYPING AND GENETIC STRUCTURE

Genomic DNA from leaf samples were extracted following Weising *et al.* (2005). Population genotyping was based in six nuclear microsatellite loci. Primers were designed from a microsatellite-enriched library obtained by shot-gun sequencing on a Roche 454 GS-FLX (Roche Diagnostics). Nuclear microsatellite search and primer design were carried out in 27,424 sequence reads using the online software WebSat (Martins *et al*., 2009). Thirty primer pairs were designed to test allele polymorphisms and the six most polymorphic loci were selected for genotyping (Table 2).

Amplification of the nuclear microsatellite markers by Polymerase Chain Reaction (PCR) was carried out in 15 µl reaction volumes containing 25 ng of genomic DNA (5 ng/µl), 0.2 µl (10 pmol/ µl) of forward and reverse primers, 1.5 µl of 10 x *Taq* Buffer (*Fermentas Life Sciences*), 1.8 µl of dNTP (2.5 mM; *Fermentas Life Sciences*), 0.72 µl of $MgCl_2$ (25mM; *Fermentas Life Sciences*), 1 U of *Taq* DNA polymerase (*Fermentas Life Sciences*), 3 µl of BSA (2 ng/µl) and 3.15 µl of H_2O. Forward primers were labeled with 6 FAM, PET, NED and VIC fluorescent dyes

(*Applied Biosystems*). PCRs were conducted by an initial 94 ºC denaturation period (4 min) followed by 30 cycles at 94 ºC, 57.2-65.3 ºC (depending on the specific annealing temperature of the primers), and 72 ºC. Each cycle step lasted 1 min. Amplified fragments were measured on an ABI 3500xL Genetic Analyser (*Applied Biosystems*). Allele calling was conducted manually in GeneMarker 2.6.2 (SoftGenetics LLC®).

Gene flow among populations was estimated by population pairwise F_{ST} (Weir and Cockerham, 1984) and the genetic variance within and among morphotypes was estimated by hierarchical Analysis of Molecular Variance (AMOVA; Excoffier *et al*., 1992). These analyses were conducted in Arlequin 3.5 (Excoffier *et al*., 2005) with a 10 000 permutation significance test. To validate the assumption of geographical barriers as main factor constraining gene flow we tested for Isolation by Distance (IBD) to examine the association level between genetic structure and geographical isolation using Mantel test. Additionally, we tested for Isolation by Environment (IBE), while controlling for geography using a partial Mantel test. This analysis examined whether environmental barriers affected gene flow more than geographical barriers. Both Mantel tests were performed with 10,000 permutations between the matrices of environmental and/or geographical distance and pairwise F_{ST} of the 15 sampled populations using the VEGAN 2.0-10 (Oksanen *et al.*, 2013) package in R. Environmental variables used to produce the matrix of environmental distance were the same used in the partial Redundancy Analysis (see topic below).

FLOWERING PHENOLOGY

To examine whether temporal barriers created by flowering displacement could contribute to differentiation among populations and morphotypes, and thus confound with the effects of geographical barriers, a phenological survey in six populations, two populations per morphotype, was conducted in 2012 and 2013. Number of individuals monitored per population varied from 21 to 34 depending on number of adult plants in each population (Table 1). Phenological monitoring consisted in monthly counts of all flowers and buds from tagged individuals during the entire flowering season over two consecutive years.

DISCRIMINANT FUNCTION ANALYSIS OF MORPHOLOGICAL AND GENETIC DATA

Ameroglossum leaves are linear (*pernambucense* morphotype) to lanceolate (*dry-season* and *forest-immersed* morphotypes) and flowers are tubular and zygomorphic, with didynamous stamens (Fischer *et al.,* 1999; Wanderley *et al.,* 2014a). To examine patterns of phenotypic variation in the *A. pernambucense* complex, we measured six leaf and six floral traits. Measures taken from leaves were length, width, area, perimeter, length-width ratio and perimeter-area ratio. Floral measures were corolla tube length, distance from the corolla base to stamens insertion, length of the lower and upper stamens pairs, pistil length, and distance from the nectary to the anthers. To test whether these variables captured the morphological differences among morphotypes, a discriminant function analysis (DA) was performed to test the assignment of samples to their morphological groups using the leaf and flower traits measured. To test genetic differences among morphotypes, a second DA was conducted using genetic information of microsatellite markers. Before performing the genetic DA, microsatellite data was first transformed into allelic variables (Westfall and Conkle, 1992). Each observed allele at all six microsatellite loci examined was converted into a variable and each individual sampled was scored for each allelic variable as 0, 0.5 or 1 depending, respectively, whether the allele was absent, in heterozygosis or in homozygosis (Smouse and Williams, 1982). Fifty-four allelic variables were obtained and included in the genetic DA.

PARTIAL REDUNDANCY ANALYSIS

To test the hypothesis that interpopulation environmental differences are driving local adaptation in the *A. pernambucense* complex at both molecular and morphological levels, we performed two partial redundancy analysis (pRDA), using the VEGAN 2.0-10 (Oksanen *et al.*, 2013) package in R. This multivariate analysis allows direct constrains of ordination axes to exploratory variables and is analogous to a linear regression (Borcard *et al*., 1992; Legendre and Legendre, 1998). Therefore, pRDAs examined the extent to which the genetic composition of the populations and their phenotypes are determined by local environment (evidence of selection) and by their geographical location (evidence of genetic drift) in two independent analyses, one for genetic and the other for phenotypic data.

For the pRDA using genetic data, the dimensionality of the 54 allelic variables was reduced by Principal Component Analysis (PCA), as described in Grivet *et al.* (2008), where the first 15 principal component (PC) axes, which explained 83% of genetic variation among populations, were used as dependent variables. Response variables used in the phenotypic pRDA were the 12 leaf and floral variables mentioned in the previous section. For both genetic and phenotypic pRDAs, six environmental and six geographic variables were used as explanatory variables. A PCA with 19 bioclimatic variables with 30 arc-second resolution (Hijmans *et al.*, 2005; downloaded at www.worldclim.org) and elevation data from the 15 sampled locations was performed to determine the most important variables capturing environmental variation among locations (analysis not shown). The six variables loading highest canonical scores for the five first axes were selected as explanatory variables used on pRDAs. Environmental variables selected were elevation (elev), temperature seasonality (Tseas), mean temperature of coldest quarter (Tcoldq), precipitation seasonality (Pseas), precipitation of driest quarter (Pdq) and precipitation of warmest quarter (Pwq). Geographical explanatory variables were longitude (X), latitude (Y) and their cross-product (X*Y). For each type of pRDA, i.e. with genetic or phenotypic data as response variables, two models were tested. In both models, linear and quadratic forms of the geographical coordinates were included; however, the first model ("mixed model") included non-transformed environmental variables, while the second model ("quadratic model") included the quadratic forms of the environmental variables.

LEAF AND FLORAL TRAIT ASSOCIATIONS WITH ABIOTIC AND BIOTIC VARIABLES

The third approach to test the hypothesis of population differentiation by local adaptation consisted in testing the association levels between specific traits and environmental selective pressures. For this purpose, two sets of Spearman correlations tests were performed. The first set of correlations were among the morphological traits measured in this study and the six environmental variables used in the pRDAs. Because within the sets of both leaf and flowers traits there were highly correlated ($r > 0.8$; correlation matrix not shown) groups of variables, only the most redundant variable of each group were selected. Therefore, *Leaf area*, *leaf area-leaf width ratio* (LL/LW), *floral tube length*, and *stamens length* were the variables used for the first set of correlations tests.

The second set of correlations were between the two floral variables used in the former set of correlations (*floral tube length*, and *stamens length*) and bill length of the most frequent hummingbird recorded pollinating the *A. pernambucense* flowers in each of the observed populations. Floral traits were correlated with bill length of the most frequent pollinator because flower phenotypes are expected to be determined by their main pollinators (Rosas-Guerrero, 2014). Pollinator observation was performed in six of the 15 populations sampled for genetic and morphometric analyses (Table 1). Additional information on pollinator frequency, provided in Wanderley *et al*. (2014a) from LB and PG populations, was also used to test the association between flower phenotype and bill length of main pollinators. Bill length of the hummingbirds observed pollinating the *A. pernambucense* complex flowers were determined from specimens of the Ornithology Collection of *Universidade Federal de Pernambuco*, Brazil.

RESULTS

GENETIC DIVERSITY AND STRUCTURE, AND FLOWERING PHENOLOGY

Microsatellite loci revealed low to moderate polymorphisms per population and the number of alleles per locus varied from one to eight with 3.17 ± 1.41 (mean ± standard deviation) alleles per locus per population. Total number of alleles obtained for all six loci was 54, and the number of alleles per locus varied from six to 13, with an average of 9 ± 2.76 alleles per locus. Because microsatellite data were gathered to infer gene flow and analyze environmental and geographical patterns of genetic variation across populations, an extensive description of population genetic parameters is beyond the scope of this study and was not here included.

All pairwise F_{ST} between populations were significant and moderate to extremely high, ranging from 0.095-0.919 (overall F_{ST} = 0.49; Table 3). Different from expected, AMOVA revealed higher genetic variation within (F_{SM} = 0.42) rather than among morphotypes (F_{MT} = 0.12; Table 4). This is mostly due to high F_{ST} values between some populations of *dry-season* morphotypes such as SM-AJ, whose F_{ST} was extremely high (0.919), and between SM or AJ and all other populations from morphotype *dry-season*, whose pairwise F_{ST} ranged from 0.409 (AJ-LB) to 0.664 (SM-RN; Table 3). The extremely high genetic structuring of SM and AJ, even relative to its closest populations ($F_{ST(SM-AB)}$ = 0.438; $F_{ST(AJ-ESP)}$ = 0.356), is due to homozygosis of all six loci in SM and in five loci in AJ. These two populations have in common the smallest population size (< 15 individuals) of the sampled populations and might have experienced high inbreeding. Furthermore, 10 out of the 15 sampled populations belong to the *dry-season* morphotype. Thus, it is not surprising to find a higher amount of genetic variation within morphotypes. Another contribution for lower genetic variation among morphotypes is the relative low pairwise F_{ST} values ($F_{ST(ESP-AN)}$ = 0.095; $F_{ST(ESP-SER)}$ = 0.172) between ESP (*dry-season*) and AN or SER (*forest-immersed*) populations. Genetic variation was structured significantly among morphotypes (F_{MT} = 0.12, P < 0.001) but the structure of the genetic variation within morphotypes was even higher (F_{SM} = 0.42, Table 4). The high overall F_{ST} = 0.49 was a clear evidence of restricted gene flow among most of the examined populations. The higher effect of geographical isolation, rather than environmental barriers constraining gene flow was supported by a significant IBD (r = 0.42; p < 0.001; Fig. 2) and non-significant IBE (r = 0.028; p = 0.42).

Two years of phenological monitoring showed flowering overlap among all six populations surveyed, revealing lack of temporal barriers to gene flow among populations of the three different morphotypes (Fig. 3). Altogether, this set of results support the role of geographical barriers as the main factor preventing gene flow and causing high population genetic structure in the *A. pernambucense* complex.

DISCRIMINANT FUNCTION ANALYZES

The first DA revealed that the morphological leaf and floral variables measured assigned samples to their morphotypes with 5.21% error rates. Therefore, a substantial proportion of morphological variation among morphotypes was captured by the 12 leaf and flower variables. The second DA revealed genetic differences among morphotypes, assigning individuals of each morphotype with only 2.79% error rates (Fig. 4, Table 5).

PARTIAL REDUNDANCY ANALYSIS

All pRDA models, using either genetic or phenotypic data as response variables, supported the hypothesis of local adaptation leading population differentiation in the *A. pernambucense* complex. Genetic and morphological variation explained uniquely by environmental variables was approximately 2-folds the proportion explained purely by geographical variation in both mixed and quadratic models. However, pure environment and pure geography explained a higher proportion of the genetic variation than the morphological variation. An important portion (35.87 to 60.51%) of both genetic and morphological variation could not be partialled out due to spatial structuring of environmental variables (Table 6).

LEAF AND FLORAL TRAIT ASSOCIATIONS WITH ABIOTIC AND BIOTIC VARIABLES

Significant correlations among most morphological variables and abiotic and biotic data also supported the hypothesis of differentiation due to divergent selection in the *A. pernambucense* complex (Table 7). Both leaf area and leaf area-leaf width ratio (LL/LW) correlated significantly with all six abiotic variables tested. Highest correlation coefficients among all tests were between

leaf area and elevation ($r = -.59$; $p < 0.001$; Fig 5 A), and between floral tube length and the bill length of the most frequent pollinator of the population ($r = 0.85$; $p < 0.001$; Fig. 5 B). This pattern corresponds to leaf and floral traits of the morphotypes. While *pernambucense* occurs at the highest elevations and shows the smallest and most linear leaves (higher LL/LW) of the complex, lower altitude populations of *dry-season* show larger lanceolate leaves. *Forest-immersed* populations, however, show larger leaves than populations from *dry-season* at similar elevations (550-650 m.a.s.l.), what is observed by the outliers around 500 m at the elevation axis of figure 5 A. Larger leaves of *forest-immersed* might be an adaptation to a more humid habitat faced by this morphotype, than by *dry-season* (see Discussion). *Pernambucense* showed the shortest floral tube length (FTL = 2.8 cm) of the complex and was mostly pollinated by the shortest-billed hummingbirds observed pollinating *Ameroglossum* flowers, *Chlorostilbon lucidus*, with ca. 1.9 cm of bill length (BL), and *Amazilia fimbriata* (BL ~ 1.97). The medium-sized d*ry-season* flowers (FTL = 3.82 cm) were mostly pollinated by the medium-billed *Eupetomena macroura* (BL ~ 23.7 cm), while the *forest-immersed* longest flowers (FTL ~ 4.54 cm) were almost exclusively pollinated by the long-billed hummingbird *Phaethornis pretrei* (BL ~ 3.5 cm). See Table S1 [Supplementary Information] for pollinator information.

DISCUSSION

This study provides evidence that differentiation among the highly isolated populations of the *A. pernambucense* complex is largely driven by natural selection due to local adaptation, although isolation by distance also contributes to pairwise population distance. Moreover, this study did not find any evidence that isolation by environment played a major role in population differentiation. First, the Partial Mantel Test did not find evidence supporting the Isolation by Environment hypotheses when examining pairwise population differentiation. Second, the fact that the populations showed high overlap in the timing of flowering means that there was not temporal isolation of gene flow promoted by ecological factors. Additionally, high overall F_{ST} (0.49) and significant IBD provide evidence of low gene flow, mostly determined by geographic barriers. On the other hand, pRDAs revealed that genetic composition and phenotypes of populations are influenced by local selection pressures. Thus, environmental driven population differentiation in the *A. pernambucense* complex may mostly result from selection of locally advantageous traits whose fixation in the population is facilitated by low levels of immigration preventing gene swamping, instead of non-random gene flow among populations from similar environments that could also conduce to local adaptation. Nonetheless, the evidence of the contribution of drift to divergence among sampled populations is not negligible and corresponded to 18.97-24.26% of genetic variation, and to 12.47-13.76% of morphological variation, suggesting that genetic drift might also contribute to differentiation of isolated small populations responding to divergent selection.

Together, DAs, pRDAs and correlations tests showed that the morphotypes of the *A. pernambucense* complex form three different genetic groups, whose morphological and genetic variation are associated to environmental variables. Therefore, we consider that the morphotypes *pernambucense*, *dry-season* and *forest-immersed* are ecotypes (although we do not discard the possibility of three different subspecies or close related species in this complex, taxonomic treatments are beyond the scope of this research). Elevation showed the highest correlation coefficients with both leaf area and LL/LW. This pattern corresponds to the leaf shape of these morphotypes. The smallest leaves of the highland ecotype *pernambucense* is in accordance with our hypothesis of local adaptation because adapted leaves are expected to decrease in size across altitudinal gradients due to decreasing average temperatures (Milla and Reich, 2011; Chitwood *et*

al., 2012). However, because leaf size is also expected to increase with precipitation (Chitwood *et al.*, 2012; Ramírez-Valiente *et al*., 2014), larger leaves of *forest-immersed* than *dry-season* populations might be an adaptation to a more humid habitat faced by the former. Nonetheless, the more striking evidence of divergence by selection was revealed by the association between the bill length of local main pollinators and floral tube length, the strongest correlation observed (r = 0.85). Pollinator-associated floral shifts are common and usually reveal floral phenotype responses to variation in pollinator assemblages across heterogeneous landscapes (Boyd, 2002; Pérez-Barrales *et al*., 2007; Anderson and Johnson, 2008) and this seems to occur in the *A. pernambucense* complex.

Although *dry-season* and *forest-immersed* ecotypes were statistically supported as two different genetic groups, the existing overlap between these two groups (Fig. 4 B) is due to historical gene flow among ESP and the two *forest-immersed* ecotype populations. Additionally, pairwise F_{ST} values among these populations (ESP, AN and SER) are lower than any pairwise comparison of each of these three populations with all other examined populations, either from *dry-season* or *pernambucense* ecotypes. Higher gene flow among ESP, AN and SER, especially between ESP and AN, is likely due to their geographic proximity (~11 km). However, current gene flow between ESP and AN or SER is unlikely because there is no evidence of pollinator partition between these population pairs allowing pollen flow (Table S1). While the only pollinator observed in ESP was *Eupetomena macroura*, a habitat generalist hummingbird, this bird was never observed in AN and SER, whose main pollinator was *Phaethornis pretrei*, a species typical from forested areas (Table S1; Feinsinger and Colwell, 1978). Therefore, the evidence of historical gene flow mostly between ESP and AN likely dates the last interglacial maxima (~120 ka), where the current relictual Atlantic rainforest from the scarps of the Borborema Plateau, known as *Brejos de Altitude*, where AN and SER are located, likely extended over the west in areas now dominated by the semi-arid Caatinga where ESP occurs (Silva and Castletti, 2003). If this supposition is correct, then, current ecological factors are perhaps differentiating ESP from AN and SER towards morphotype *dry-season* by convergence (e.g. Foster *et al.*, 2007). Because of the geographic proximity between AJ and ESP (~13 km), both populations might share a similar history of environmental changes from the last interglacial maxima, although the former showed no evidence of higher gene flow with ESP, AN and SER, likely due to high inbreeding revealed by high homozygosis.

Several recent studies have been reporting the role of ecological factors as major drivers of population differentiation when compared to geographical ones, indicating that adaptive evolutionary mechanisms are more important than genetic drift when taxa are facing environmental heterogeneous landscapes (Keller *et al.*, 2009; Sork *et al.*, 2010; Andrew *et al*., 2012; Ortego *et al*., 2012; Gugger *et al.*, 2013; Wang *et al*., 2013). However, these studies dealt with species from continuous habitats where population size is not restricted to small patches of suitable habitat, as inselberg specialist plant populations are. Because the impacts of genetic drift over a population are inversely related to its size and degree of isolation (Wright, 1931; Slatkin, 1987; Ellstrand and Elam, 1993), evidence of population divergence by local adaptation in the *A. pernambucense* complex is quite surprising.

Some life history strategies might help to explain population divergence by natural selection in the *A. pernambucense* complex. First, tests of pollen flow using fluorescent dye particles as pollen analogous revealed sufficient pollen flow within and among populations (ca. 850 m away) from the same inselberg archipelago to potentially prevent or reduce genetic drift. However, because populations of the *A. pernambucense* complex, even when considering the whole archipelago, can still be considered small, evidence of pollinator activity increasing population size was not enough to discard drift as the major evolutionary mechanism driving population differentiation in this taxon (unpubl. data). Secondly, pollination experiments performed in four *dry-season* populations and one *pernambucense* population revealed no differences in number of fruits and seeds produced after manual self- and cross-pollinations, suggesting a full self-compatible breeding system in the *A. pernambucense* complex. However, fruit and seed set after spontaneous self-pollination was lower than after manual pollinations, indicating dependence on pollinators to increase reproductive success. Although reproductive dependence on pollinators might increase allogamy, because populations are small and isolated, crosses occur more frequently within than between populations, allowing high levels of inbreeding. (Wanderley *et al.*, 2014a; unpubl. data). However, endogamic depression is less frequent in self-compatible small populations due to purging of deleterious alleles by selection (Barrett and Charlesworth, 1991a). Furthermore, self-compatibility is expected to facilitate local adaptation by reducing gene flow between environmental contrasting populations and rapidly fixing locally adapted traits (Levin, 2010). Therefore, pollinator activity potentially reducing drift, genetic purging and the possibility of rapid fixation of locally beneficial traits by self-compatibility are possible factors favoring

differentiation by local adaptation, rather than by genetic drift, in the *A. pernambucense* complex populations.

Because gene flow among the studied populations is constrained by geographical barriers, but genetic and phenotypic composition are more related to ecological than geographical factors, we conclude that divergent selection can be the primary driver leading differentiation of discrete populations. Although we found some overlap between *forest-immersed* ecotype and an adjacent *dry-season* population, our results suggest that ecological factors are morphologically structuring leaves and flowers in three different genetic groups. Nevertheless, evidence of genetic drift was not negligible and might be a secondary factor driving differentiation of discrete populations. Altogether, our results suggest that small and highly isolated populations can be locally adapted despite their susceptibility to the effects of genetic drift.

ACKNOWLEDGMENTS

The authors are thankful to Fundação de Amparo à Ciência e Tecnologia de Pernambuco (FACEPE) and the Brazilian Research Council (CNPq) for fellowships and a research grant (CNPq - 470806/2011-7).

TABLE 1 - Studied populations of the three morphotypes observed in the *Ameroglossum pernambucense* complex and sampling size for microsatellite genotyping, leaf and flowers morphometry, flowering phenological survey and time of pollinator observation.

Population	Elevation (m)	Morphotype	*N* (genotyping)	*N* (morphometry)	*N* (Phenology)	*N time-hours* (Pollinator observation)
P	1215	*pernambucense*	16	15	34	14.23
Rei	1086	*pernambucense*	5	16	34	15.08
SJTP	1182	*pernambucense*	14	4	-	-
QA	514	*dry-season*	18	25	-	10.0
Spe	808	*dry-season*	12	20	-	-
SM	796	*dry-season*	16	9	-	-
AB	860	*dry-season*	14	12	-	-
LB	443	*dry-season*	14	11	-	-
PG	677	*dry-season*	17	4	-	-
ESP	640	*dry-season*	18	16	24	11.5
AJ	650	*dry-season*	7	8	21	-
TAC	162	*dry-season*	12	10	-	-
RN	150	*dry-season*	7	13	-	-
AN	629	*forest-immersed*	15	12	23	13.2
SER	549	*forest-immersed*	11	17	29	10.0
		Total	196	192	165	74.01

TABLE 2 - Description of six nuclear microsatellite *loci* and respective primer pairs used to genotype 15 populations of the *Ameroglossum pernambucense* complex.

Locus	Motif	Size (bp)	Primer	Ta (°C)
amg 3	$(TA)_7$	235	f - 5' CAGCCAACCAATCTGGAGTT 3'	65.3
			r – 5' CATGCGACATAAGGTGGTACA 3'	
amg 12	$(AC)_9$	154	f - 5' TTGTAATAGTGAAGGCGTCCAA 3'	64.2
			r – 5' ATGCAAGAAACCCTGTTCCA 3'	
amg 14	$(AAT)_8$	219	f - 5' ATATTTAGCTCCACCAATGCC 3'	61
			r – 5' GCCAATTTCCAGTTCAGGAT 3'	
amg 15	$(GA)_9$	153	f - 5' CAATCCCTCACATTACTCCCA3'	58.5
			r – 5' CCCTAAGCTCCGATTCATCA3'	
amg 21	$(AT)_7$	167	f - 5' TGAGACACATTGCTCCTTGG 3'	57.2
			r – 5' TCCGCAAATGGTGATGTTTA 3'	
amg 23	$(AT)_{11}$	202	f - 5' CGGCTTTAATGTTGATCGGT 3'	58.5
			r – 5' GATTGATGCTTCGTCCCTTC 3'	

TABLE 3 - Pairwise F_{ST} among 15 populations from three morphotypes of the *Ameroglossum pernambucense* complex. Morphotypes *pernambucense*, *dry-season*, and *forest-immersed* are respectively abbreviated as FI-, DS- and pern-morpho.

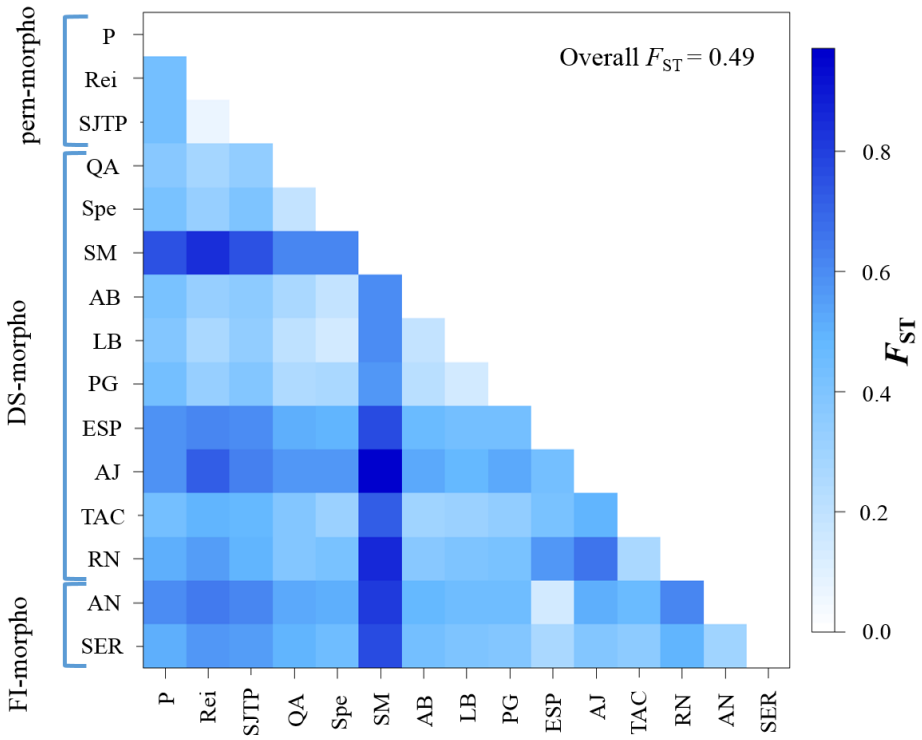

TABLE 4 - AMOVA design and results of genetic variation from six microsatellite loci among and within three morphotypes of the *Ameroglossum pernambucense* complex.

F_{ST} = genetic structure among all populations; F_{SM} = genetic structure among populations within morphotypes; F_{MT} = genetic structure of populations among morphotypes.

Source of variation	d.f.	Sum of squares	Variance components	Variation (%)
Among morphotypes	2	107.86	0.30	12.58
Within morphotypes	12	289.83	0.89	36.80
Within populations	377	461.77	1.22	50.62
Total	391	859.45	2.42	
Fixation indices	*P*			
F_{ST} = 0.49	>0.001			
F_{SM} = 0.42	>0.001			
F_{MT} = 0.12	0.004			

TABLE 5 - Discriminant function analysis (DA) among three morphotypes from 15 populations of the *Ameroglossum pernambucense* complex using 12 leaf and floral traits and 54 allelic variables obtained from six nuclear microsatellite loci. Errors rates of DAs using morphological and genetic data are, respectively, at the left and right sides of the slashes signs. Morphotypes *pernambucense*, *dry-season*, and *forest-immersed* are respectively abbreviated as FI-, DS- and pern-morpho.

DA morphology/genetic	Pern-morpho	DS-morpho	FI-morpho	Total	Error (%)
Pern-morpho	35/33	0/0	0/0	35/33	0/0
DS-morpho	0/0	120/119	8/3	128/122	6.25/2.46
FI-morpho	0/0	4/2	25/22	29/24	13.79/8.33
Total	35/33	124/121	33/25	192/179	**5.21/2.79**

TABLE 6 - Partitioning of the genetic and morphological variation from 15 populations of the *Ameroglossum pernambucense* complex, constrained either by environmental and geographical variables. Values in the columns represent the amount of genetic and morphological variation explained uniquely by environment or geography, as well as their joint effect. Results are shown for models including linear, quadratic and cross-products forms of geographical coordinates (latitude and longitude) and linear forms of the environmental variables (mixed model), and models including the same transformations of geographical locations and the quadratic forms of the environmental variables (quadratic models). Environmental variables used for the models were: elevation, temperature seasonality, temperature of coldest quarter, precipitation seasonality, precipitation of driest quarter, precipitation of warmest quarter. Elevation measures were obtained from field and the other environmental variables were downloaded from www.worldclim.org.

Model	Constrained inertia (mixed model/ quadratic model)	Proportion (%)
pRDA using genetic variation from microsatellite loci		
Pure environment	19.06/21.15	37.4/39.8
Pure geography	9.67/12.87	18.97/24.26
Environment + geography	22.23/19.03	43.62/35.87
pRDA using morphological variation from leaf and floral traits		
Pure environment	102.1/103.3	26.77/27.02
Pure geography	52.48/47.67	13.76/12.47
Environment + geography	226.82/231.33	59.47/60.51

TABLE 7 - Spearman correlation tests between morphological traits of the *Ameroglossum pernambucense* complex and environmental variables. Except for variable *Bill length*, where the number of analysed populations was eight, the other correlations were performed on data obtained from 15 populations. Variables *Tseas, Tcoldq, Pseas, Pdq* and *Pwq* were obtained at www.worldclim.org.

Morphological traits	Elev	Tseas	Tcoldq	Pseas	Pdq	Pwq	Bill length
Leaf area	-0.59***	-0.54***	0.46***	-0.24***	0.18**	0.28***	-
Leaf length/L. width	0.57***	0.38***	-0.57***	-0.22***	0.18**	0.24***	-
Floral tube length	-0.37***	-0.48***	0.27***	-0.42***	0.09 *ns*	0.06 *ns*	0.85***
Stamens length	0.3***	-0.26***	-0.23***	-0.12**	-0.17*	0.18**	-0.01 *ns*

Elev, elevation; **Tseas**, temperature seasonality, **Tcoldq**, temperature of coldest quarter; **Pseas**, precipitation seasonality; **Pdq**, precipitation of driest quarter; **Pwq**, precipitation of warmest quarter, **Bill length**, bill length of the most frequent pollinator of a population.

*, P < 0.05; **, P = 0.01, ***, P > 0.001; *ns* = non-significant.

TABLE S1 -Pollinator observation time, pollinator frequency and floral tube length from eight populations of the *Ameroglossum pernambucense* complex. Complete names of hummingbirds, as well as their respective bill lengths are presented in the lower part.

Population	Observation time (h)	Hummingbirds (no. of visits)	Floral tube length (mean – SD*)
QA	10	Em (14); Gh (1)	34.1 – 29.4
PG**	52,5	Af (152); Pp (41)	38.5 – 3.01
LB**	10	Em (9); Av (6)	39.7 – 2.87
SP	14.23	Af (6); Pp (3), Cl (2), Em (2)	28.9 – 2.9
Rei	15.08	Cl (13); Af (4); Pp (1)	27.6 – 2.3
ESP	11.5	Em (4)	38.8 – 2.7
AN	13.2	Pp (13); Af (2)	45.6 – 4.78
SER	10	Pp (24); Af (1)	45.3 – 3.63

Hummingbird acronyms	Bill length (mm)
Af - *Amazilia fimbriata*	19.73
Av – *A. versicolor*	17.1
Cl- *Chlorostilbon lucidus*	19
Em - *Eupetomena macroura*	23.7
Gh - *Glaucis hirsuta*	31
Pp - *Phaethornis pretrei*	35

*Standard deviation; ** Pollinator frequency is from Wanderley *et al*. (2014a)

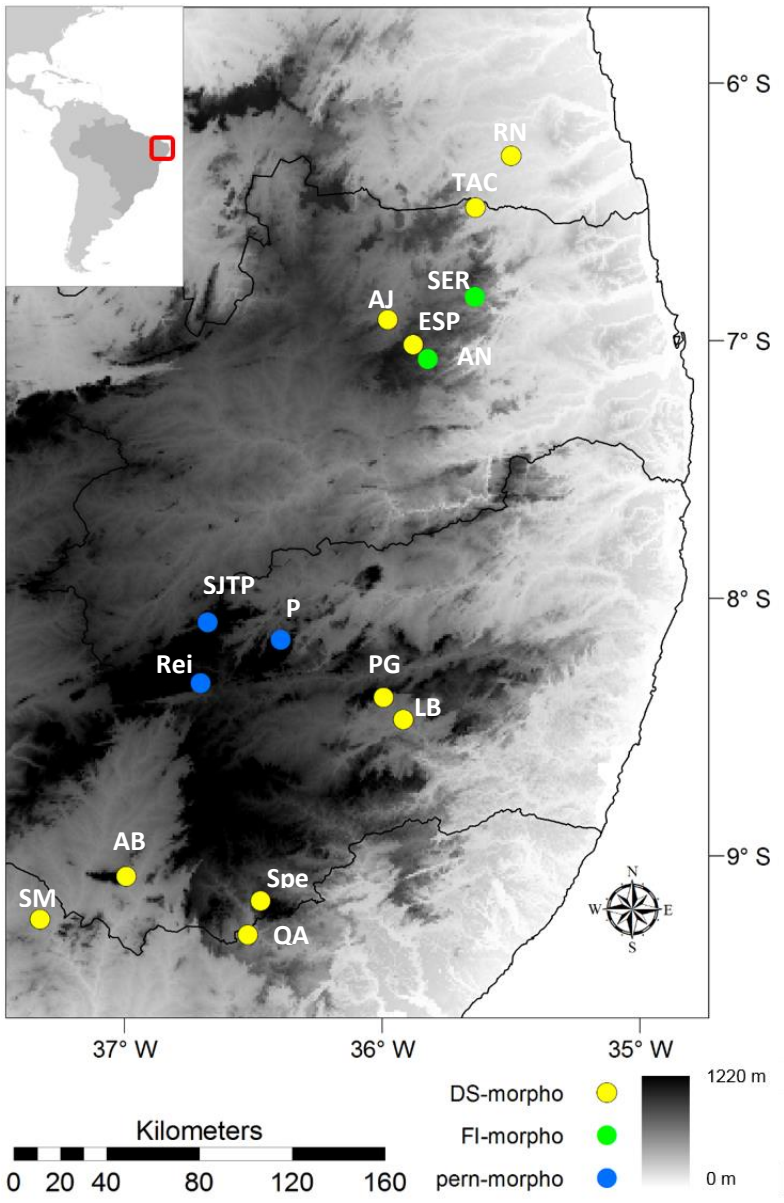

FIGURE 1 - Distribution map of sampled populations from three morphotypes of the *Ameroglossum pernambucense* complex. Morphotypes *pernambucense*, *dry-season*, and *forest-immersed* are respectively abbreviated as FI-, DS- and pern-morpho.

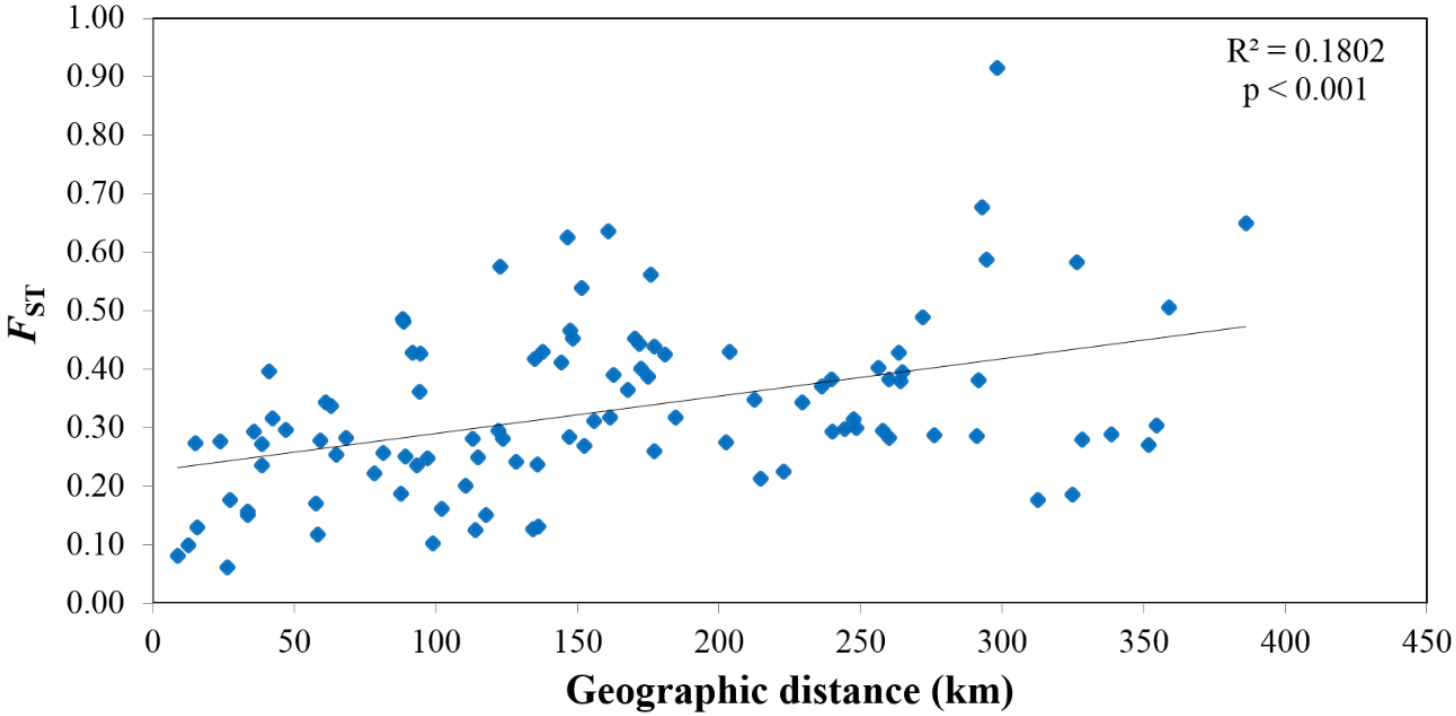

FIGURE 2 - Isolation by distance (IBD) obtained from six microsatellite loci from 15 populations of the *Ameroglossum pernambucense* complex.

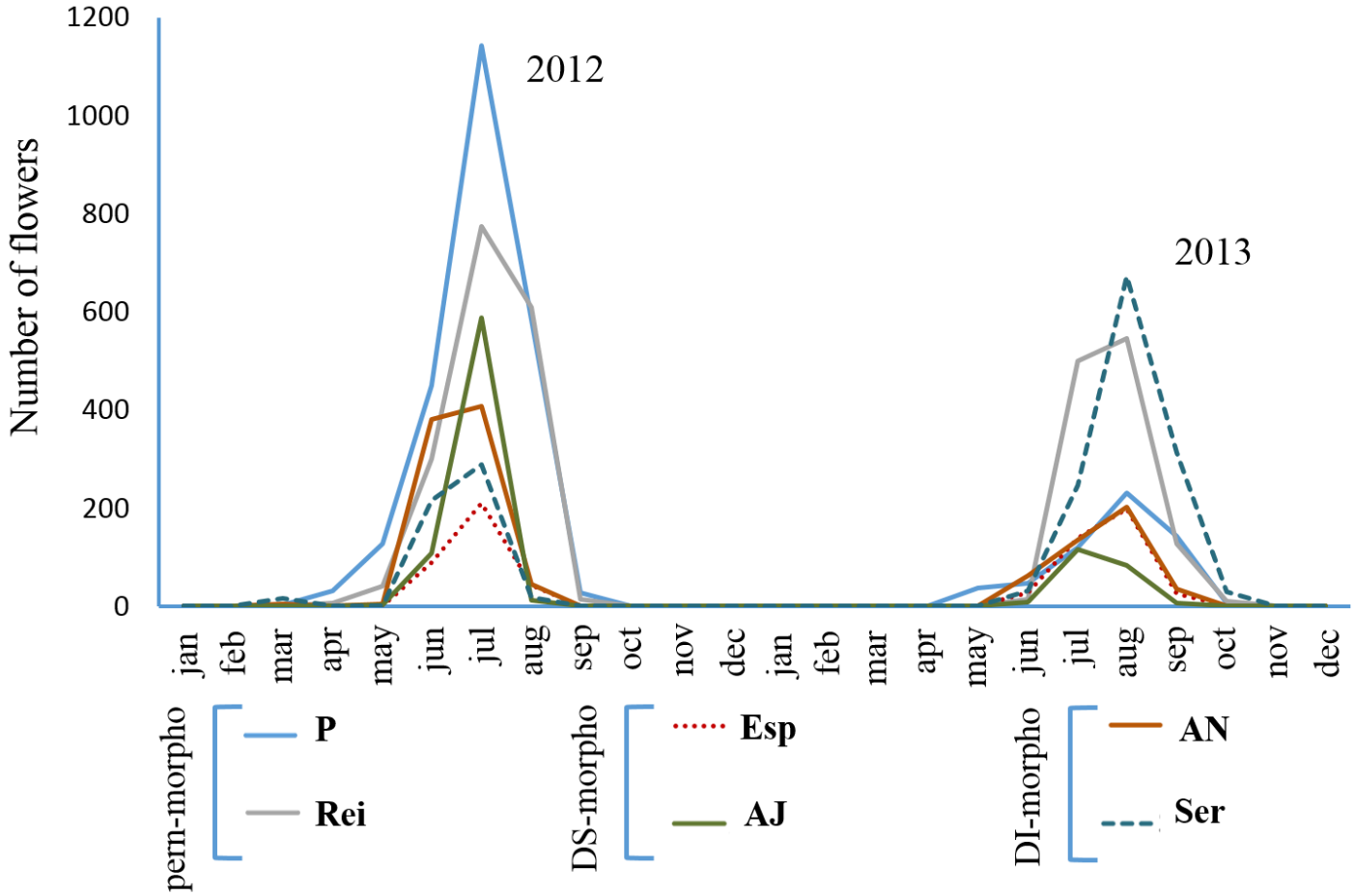

FIGURE 3 - Flowering phenology from six populations belonging to three morphotypes of the *Ameroglossum pernambucense* complex during two years (2012 and 2013). Morphotypes *pernambucense*, *dry-season*, and *forest-immersed* are respectively abbreviated as FI-, DS- and pern-morpho.

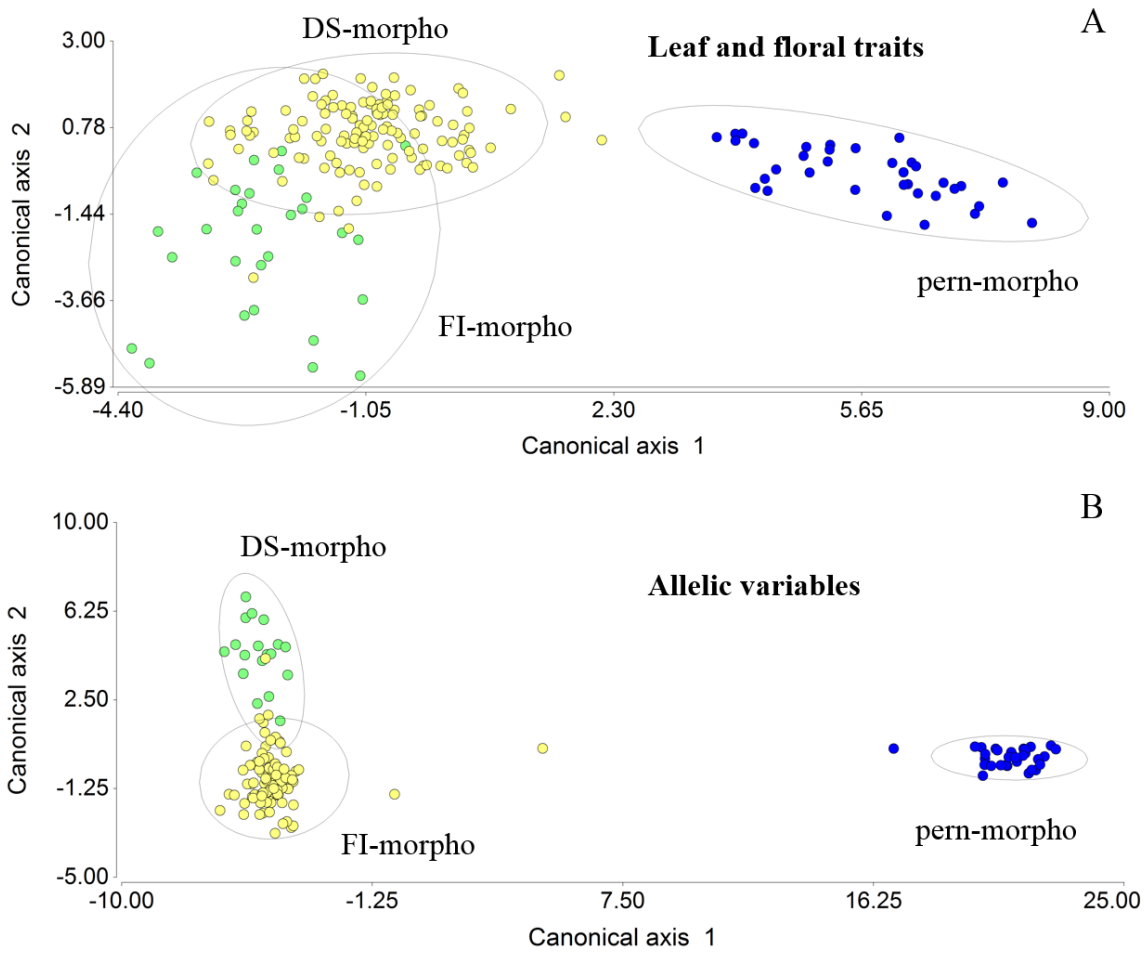

FIGURE 4 - Phenotypic and genetic clustering of three morphotypes observed in 15 populations of the *Ameroglossum pernambucense* complex using discriminant function analysis. Phenotypes were measured from 14 leaf and floral traits (A), and genotypes were defined from 54 allelic variables (B) obtained from six nuclear microsatellite loci.

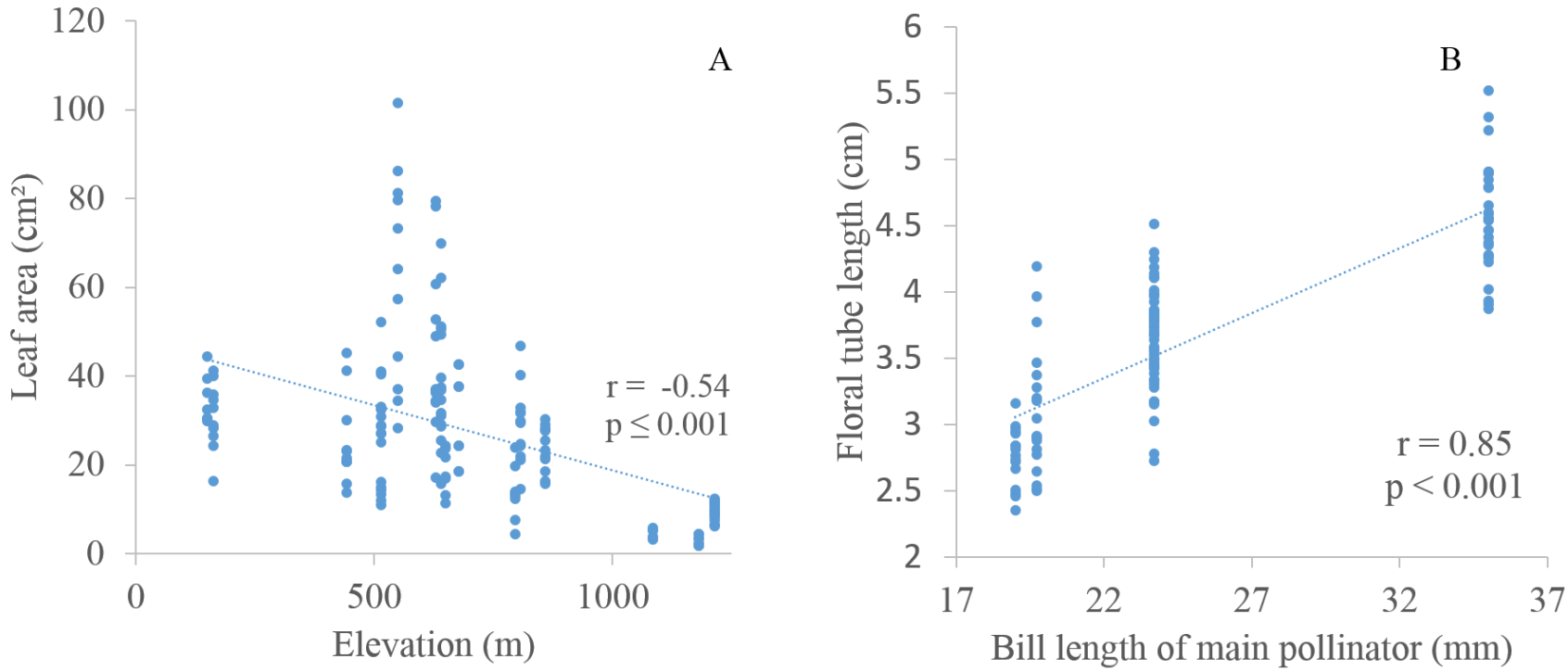

FIGURE 5 - Spearman correlation tests between leaf area and elevation (A), and between floral tube length and bill length of the main pollinator in the *Ameroglossum pernambucense* complex. Data used for A was from 15 populations and data used in B was from 8 of these populations.

5 FUNCTIONAL DECOUPLING BETWEEN FLOWERS AND LEAVES OF THE *A. PERNAMBUCENSE* COMPLEX, AN ORNITHOPHILOUS PLANT DISTRIBUTED ACROSS A POLLINATOR AND CLIMATIC HETEROGENEOUS LANDSCAPE

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ABSTRACT

Decoupling between floral and leaf traits are expected in plants with specialized pollination systems to assure a precise flower-pollinator fit, irrespective to leaf variation associated to environmental heterogeneity (functional modularity). Nonetheless, developmental interactions of floral traits are also expected to decouple flowers from leaves (developmental modularity). This study tested the functional modularity of the hummingbird-pollinated flowers of the *Ameroglossum pernambucense* complex while controlling for developmental modularity. At the within population level, functional decoupling between flowers and leaves was examined by testing whether the strength of covariation between two key floral traits related to flower-pollinator fit (tube length – TL and anther-nectary distance - AN) was higher than the covariation of these traits with a floral trait not related to pollination (sepal length – SL - control for functional decoupling), and with a leaf trait (leaf length – LL). The stability of TL and AN was also compared to SL and LL stabilities. If functionally decoupled, the highest covariation and trait stabilities should be observed for TL and AN. At the among population level, functional decoupling was examined by testing whether TL and AN variation was independent from SL and LL, and whether the variation of local pollinators better explained TL and AN variation than SL and LL variation. All the analyses supported flower functional decoupling. 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.

Keywords: functional and developmental modularity; pollinator specialization; geographic variation; local adaptation; inselbergs.

INTRODUCTION

The body of complex organisms is formed by different parts (e.g. organs and limbs). Because traits within the same 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 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 (or decoupling) between morphological structures can be due to developmental relatedness and/or selection pressures, both leading to higher trait covariation within than among these structures (Wagner & Altenberg, 1996; Klingenberg, 2008).

Plants with specialized pollination systems (i.e. pollinated by one or few groups of pollinators), usually exhibit flowers that tightly fit pollinator morphology to assure a precise pollen deposition and pollen collection on a specific part of the pollinator's body (functional modules). Furthermore, because specialized flowers rely on specific pollinators with similar size, the traits directly related to an accurate pollination are expected to have a higher stability (i.e. low variance) - allowing a precise contact of anthers and stigmas within a particular body region of the pollinators – than leaf or other vegetative traits. Therefore, flowers with specialized pollination systems are expected to be functional modules, which can be predicted by higher covariation among floral traits directly related to pollination than among these floral traits and vegetative traits (subjected to a distinct set of selection agents), and by higher stability of floral than the vegetative traits (Berg, 1959, 1960). Although 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), decoupling between floral and vegetative parts, and higher stability of floral traits have also been commonly observed in plants with non-specialized pollination systems (Armbruster *et al.*, 1999; Pérez-Barrales *et al*., 2007). Because decoupling from vegetative traits tend to be weaker in non-specialized than in specialized flowers, the flowers of the former group have been recognized as mainly developmental modules, whereas the flowers of the latter group as primarily functional modules (Armbruster *et al.*, 1999; Herrera *et al.*, 2002;

Ordano *et al.*, 2008). Therefore, appropriately testing for flower functional decoupling (i.e. functional modularity of flowers), requires controlling for flower developmental decoupling from vegetative traits. This is possible to achieve by examining patterns of stability and covariation among floral traits directly related to pollination, floral traits not related to pollination and vegetative traits (e.g. Conner & Via, 1993; Baranzelli *et al.*, 2014; Cosacov *et al.*, 2014).

The study of functional decoupling between flowers and leaves or other vegetative traits is important to understand the mechanisms underlying local adaptation, and therefore potential for speciation, in species distributed across highly heterogeneous landscapes because independent flower and leaf responses to local selection agents (biotic and or abiotic) is expected to be an adaptive feature (Chalcoff *et al*. 2008). This feature is particularly critical in plants with specialized pollination systems if varying local selection pressures on leaf morphology affects the fit between flowers and the local pollinators (Cosacov *et al.*, 2014). Functional decoupling between flowers and leaves are 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 are indicative of lack of: pleiotropism, genetic linkage and/or developmental interactions underlying trait variation between modules. Testing functional decoupling uniquely at the among population level may be misleading because uncoupled traits at the within population level can covary at the among population level due to independent selection for similar changes in trait size (Armbruster, 1991; Chalcoff *et al.*, 2008). Conversely, once functional decoupling is observed at the within population levels, testing it at the among population level 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 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 a early stages of speciation (Armbruster, 1991; Chalcoff *et al.*, 2008; Cosacov *et al.*, 2014).

The *Ameroglossum pernambucense* complex is a group of ornithophilous shrubs 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 Northeastern

Brazil (Wanderley, 2014a,b). The plants of this complex are self-compatible, although hummingbirds (the only pollinators) are necessary to increase fruit and seed formation (Wanderley *et al.*, 2014a; unpl. data). Because of the island-like condition of the inselbergs, most populations of the *A. pernambucense* complex occur in archipelagos of adjacent inselbergs highly isolated from populations of others inselberg archipelagos (unpl. data). This taxon is treated as a species complex because its populations can vary in plant size, phyllotaxy, and leaf and flower shapes (Wanderley, 2014a,b). The landscape within the distribution range of this complex is a mosaic of highly heterogeneous environments, where the lower areas (200-500 m.a.s.l.) of this region are dominated by the semi-arid Caatinga (a savanna-like ecosystem) and the higher lands (>500 m.a.s.l.) are mainly dominated by Antlantic rainforest enclaves (Andrade-Lima, 1960). In a previous study, evidence of population differentiation due to local adaptation was reported for the *Ameroglossum pernambucense* complex based on geographical and environmental patterns of genetic (nuclear microsatellites) and morphological (leaf and flower traits) variation, suggesting that this complex is composed by at least three different ecotypes. A further analyses of this same study revealed that leaf dimensions were moderately correlated (r = -0.59 - 0.28) with environmental variables (elevation, temperature and precipitation), whereas floral tube length was highly correlated (0.85) to the bill length of the main local pollinators (Wanderley *et al.*, unpl. data). Therefore, functional decoupling between flowers and leaves is expected in the *A. pernambucense* complex because it allows flower adaptation to local pollinators irrespective to the local environmental factors affecting leaf dimensions.

The central goal of this study was to test whether phenotypic floral and leaf variation in the *A. pernambucense* complex are uncoupled. Nonetheless, because developmental modularity alone is expected to decouple flower and leaf variation, decoupling between these structures does not necessarily reflect a functional fit between flowers and local pollinators (Klingenberg, 2008). Therefore, functional decoupling between flowers and leaves was tested controlling for developmental modularity. Specifically it was tested whether floral traits directly related to pollinator fit (floral tube length – TL, and anthers-nectary distance - AN) are more tightly associated between them, than with a floral trait not related to pollinator fit (sepal length - SL) – control for developmental modularity – and to a leaf trait (leaf length - LL). Within and among population functional decoupling were tested using several approaches. The central hypothesis of this study was that the flowers and leaves of the *A. pernambucense* complex are functionally

decoupled. Both within and among population tests of functional decoupling supported this prediction. Additionally, the significant differences in TL and AN (independent from SL and LL covariation) among populations whose the main local pollinators differed in bill length is an evidence that the observed among population functional decoupling is due to local adaptation (divergent selection) caused by current selection agents.

MATERIALS AND METHODS

STUDY PLANTS 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 exserted stamens with filaments adnate to the corolla at the base. The gynoecium has a conical ovary and a filiform exserted style. The 5-lobed calyx has a largest upper lob, 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 attributable to this structure during pollination (Fischer *et al.*, 1999; pers. obs.). These flowers can offer copious amounts of nectar (up to 107.9 µl and 24.8 mg of sugar per flower), and are exclusively pollinated by hummingbirds. Although self-spontaneous, when exposed to pollinators, flowers produced significantly higher fruit and seed set. Therefore, pollinator selection on floral traits is expected (Wanderley *et al.*, 2014a; unpl. data). The leaves of this complex are lanceolate, cauline, opposite-decussate or 3-6-verticilate (pers. obs.).

In a previous study it was suggested that the *A. pernambucense* complex is composed by at least three distinct ecotypes with varying sizes of leaves and flowers, and apparently adapted to different environmental conditions (Fig 1; Wanderley *et al.*, unpl. data). The first ecotype (*pernambucense* ecotype) is the species described as *A. pernambucense*, which have 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 ºC), with average annual rainfall ~1000 mm. The main pollinators observed in two populations of this ecotype are the short-billed hummingbirds *Chlorostilbon lucidus* (Shaw, 1812; bill length ~1.9 cm) and *Amazilia fimbriata* (Gmelin, 1788; 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). The populations of this ecotype 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* (Gmelin, 1788). The third ecotype of the *A. pernambucense* complex (*forest-immersed* ecotype) has the longest floral tubes (~4.5 cm) and the largest leaves (~14.35 cm long). The populations of this ecotype occur in mid elevation (500-600 m.a.sl.) forest-immersed inselbergs partially covered by the Atlantic forest canopy with lower levels of solar irradiation, but with average annual rainfall similar to the first category. The main pollinator observed in two populations of this ecotype is the long-billed (bill length ~3.5 cm) hummingbird *Phaethornis pretrei* (Lesson and Delattre, 1839). The pollinator information provided above are based on systematic pollinator observations in seven populations of all three ecotypes (Wanderley *et al.*, 2014a, unpl. data).

Flowers and leaves were sampled in 13 populations, including the seven populations where pollinator information is available, of the three ecotypes along the entire geographic range known for the *A. pernambucense* complex. In each population 8 to 25 plants (total = 184 individuals) were sampled and 1-3 fully developed flowers and leaves were collected per individual. The overall number of leaves and flowers collected were, 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 sampled populations of the second ecotype (Table 1). Nonetheless, because this study is centered in investigating functional decoupling within the *A. pernambucense* complex, instead of within its ecotypes, this unbalanced sampling design does not affect the analyses. The samples from the 13 populations used in this is study is a subset of a larger population sampling across 15 population of the *A. pernambucense* used in a previous study focused on understanding the relative roles of natural selection and genetic drift underlying genetic and morphological variations in this complex.

TRAIT MEASURES AND DATA ANALYSES

Three floral traits and one leaf trait were measured: floral tube length (TL), the distance between the anthers and the nectary (AN), the upper largest lobe of the calyx (sepal length - SL) and leaf length (LL). Because 1-3 flowers and leaves per individual were measured, the individual means of each trait were used in the analyses described below. These traits were chosen to test functional decoupling between floral and leaf traits because they comprise two floral traits directly related to flower-pollinator fit (TL and AN), one floral trait not related to pollination

(SL) and a leaf trait (LL). TL and AN variation are expected to be highly coordinated because TL 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 low stability (high variance) of one or both of these traits might prevent efficient pollen transfer. On the other hand, from a functional viewpoint, high covariation between neither of these two traits and SL or LL are necessary for a precise pollination. Therefore, if the flowers of the *A. pernambucense* complex are functional modules, high covariation is expected between TL and AN, whereas low covariation is expect between these two traits and SL and LL. The distance between stigma and the nectary was not selected instead of AN, 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 to the strength of this contact. The filaments, on the other hand, are more sessile and less flexible. Therefore, because of the style flexibility, the strength of natural selection for a tight covariation between stigma and TL, as well as for low variance of the stigma-nectary distance (stability) might be lower than the strength of natural selection for a tight TL-AN covariation and AN stability. Indeed, these predictions were supported during the preliminary analyses of this study (data not reported).

The first analysis of this study aimed 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 test. To satisfy the assumption of homoscedasticity, data was square root transformed. After transformation, only AN did not pass in 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 AN was used in the ANOVA.

Several statistical analysis were performed to test functional decoupling between flowers and leaves. At the within population level, analyses were as follows. First, within population Pearson correlation tests between each possible pair of measured traits (i.e. TL-AN, TL-SL, TL-LL, AN-SL, AN-LL, SL-LL) were performed. After this, 13 correlation coefficients, one per population, were obtained for each pair of traits. These correlation coefficients were grouped into four different correlation categories: Fl-Fl, included the coefficients of TL-AN correlation tests; Fl-Se, included the population means of the coefficients obtained from TL-SL and AN-SL

correlations tests; Fl-Le, included the population means of the coefficients from TL-LL and AN-LL correlations tests; and Se-Le, included the correlation coefficients between SL and 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 non-significant coefficients were used in the further analysis. The first test of functional decoupling at the within population level was done comparing by one-way ANOVA the means of the absolute values (i.e. the modulus) of the correlation coefficients obtained in Fl-Fl, Fl-Se, Fl-Le, Se-Le. Because the correlation coefficients obtained were not fully independent, the significance of differences between these means was tested after 20000 data permutations. In case of functional decoupling, Fl-Fl should have the highest of correlation coefficient mean (Berg, 1959, 1960). Conversely, similar Fl-Fl and Fl-Se means, and higher than Fl-Le and Se-Le 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 20000 permutations of the dataset. In case of functional modularity, TL and AN should have the lowest CV means, i.e. the highest stability, whereas in case of developmental modularity TL, AN and SL would have similar CV means, all higher than the LL 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. $\alpha = 0.05$/number of comparisons).

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 TL and AN (see Results) remained significant when controlling for SL and LL covariation independently. These ANCOVAs used the same dataset used in the first ANOVA to explore the variation of the four measured traits across the sampled populations. If among population differences in TL and AN dimensions 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 SL and LL. Because seven of the 13 examined populations have pollinator information (Wanderley *et al.* 2014, unpl. data), evidence of among population functional decoupling driven by divergent selection was obtained by observing whether TL and AN means of the populations pollinated by hummingbirds with

different bill sizes were significantly different, after removing SL and LL covariance. The Tukey test was used for the ANCOVAs post-hoc multiple comparisons among each population pair. Finally, functional decoupling at the among population level due to current adaptation to local pollinators was tested by comparing four simple linear regression models. These models tested whether among population variations of the bill length of the main local pollinators better predicted among population variation of TL and AN than among population variation of SL and LL. Under a functional decoupling scenario, higher regression coefficients are expected for the models TL or AN = a + b*bill length + Error, than the models SL or LL = a + b*bill length + Error. This last analysis was performed using only data from seven of the 13 sampled populations, to which pollinator information was available (Wanderley *et al.* 2014, unpl. data). To achieve homoscedasticity and distribution normality of the error all four measured traits were square root transformed.

RESULTS

The first one-way ANOVA revealed among population significant differences for all examined traits (Fig. 2; Table 2). Although populations of the ecotypes *pernambucense* and *forest-immersed* were significantly different for all traits, populations of the *dry-season* ecotype were more heterogeneous. 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 this ecotype (Table 2.). All populations, but QA, mainly pollinated by hummingbirds with different bill sizes also showed significant differences in TL, AN and SL means. Although QA, LB and ESP are mainly pollinated by the same hummingbird (*Eupetomena macroura*; Wanderley *et al.*, 2014, unpl. data), AN and TL means of QA was significantly different from LB and ESP means. However, this result is likely due to outliers (Fig. 3). Among population patterns LL variation were slightly different from the variation patterns of TL, AN and SL (Fig. 2; Table 2).

FUNCTIONAL DECOUPLING AT THE WITHIN POPULATION LEVEL

The Fl-Fl correlation coefficients were always significant and showed the highest values across all the 13 populations (r = 0.86 ± 0.13; mean of the absolute correlation coefficients ± standard deviation). Fl-Se correlation coefficients across populations ranged from 0.03 to 0.81 (r = 0.28 ± 0.23), while Fl-Le ranged from 0.04 to 0.56 (r = 0.26 ± 0.16), and Se-Le correlations coefficients ranged from 0.03 to 0.57 (r = 0.3 ± 0.18). The correlation matrices of the 13 studied populations are shown in TableS1. The ANOVA comparing these correlation coefficient means was significant ($F_{3, 48}$ = 33.49; P = 0), where Fl-Fl was the only significant different mean (Fig 3 A). The lowest CV means were of TL and AN, respectively, 8.09 ± 2.34 and 7.27 ± 2.28. The TL CVs across the sampled populations ranged from 2.62 to 11.72, and for AN the CVs ranged from 3.32 to 10.58. The CV ranges and means (within brackets) for SL and LL were, respectively, 8.36-17.44 (13.1 ± 2.63) and 12.15-23.57 (17.73 ± 3.43). The CVs of all traits per population are shown in Table S2. The ANOVA comparing the CV means was also significant ($F_{3, 48}$ = 41.81; P =0). TL and AN CVs means were significantly lower than SL and LL means, and the CV mean of SL was

significantly lower than the CV mean of LL ($p = 0.001$; Fig. 3 B). Therefore, functional decoupling at the within population level between flower and leaves was supported by these two tests.

FUNCTIONAL DECOUPLING AT THE AMONG POPULATION LEVEL

The ANCOVAs revealed that most among population variation in TL and AN observed in the former ANOVA, remained significant after removing both SL and LL covariances, although LL covariation with TL and AN was not significant (Table 3). SL covariation with TL and AN, however, was significant ($R^2_{TL\text{-}SL} = 0.57$, $P_{TL\text{-}SL} = 0.0001$; $R^2_{AN\text{-}SL} = 0.64$, $P_{AN\text{-}SL} = 0.0001$). Differences in TL and AN means of the populations with different main pollinators remained significant after controlling for SL and LL covariances. All regression models were significant, nonetheless, the highest regression coefficients were obtained for the models with the square roots of TL and AN, respectively 0.7 and 0.69 (Table 4). Therefore, ANCOVAs and regression analyses also supported functional decoupling between flowers and leaves of the *A. pernambucense* complex at the among population level, with evidence of divergent selection due to adaptation of local pollinator as major force governing the decoupling patterns.

DISCUSSION

All analyses used in this study supported functional decoupling between the flowers and leaves of the *A. pernambucense* complex at both within and among population levels, supporting the expectation of functional modularity of pollination related traits in plants with specialized pollination systems (Berg, 1959, 1960). The Fl-Fl correlation coefficient mean, more than two-folds higher than any other correlation category mean, strongly supported flower functional decoupling in this complex. Furthermore, the lowest TL and AN CV means also corresponded to the expected for a functional module. The ANCOVAs, in turn, revealed that most of the significant among population variation in TL and AN detected by the first ANOVA of this study remained significant after controlling for covariation with SL and LL, including most of the populations with pollinator information. The linear regression models revealed TL and AN as the variables with better fit to the among population variation of local main pollinators. Therefore, the populations of this complex are likely able to adapt their flowers to local pollinators morphology without be constrained by variations in leaf morphology because of local selection pressures on leaves and possibly other vegetative traits. Nonetheless, some evidence of developmental decoupling was also obtained. From a functional viewpoint, SL and LL stability and/ or covariation with TL and AN are equally irrelevant to a precise pollination system. Nevertheless, the higher mean CV of SL than LL, and the significant among population covariation of SL, but not of LL, with TL and AN, revealed by the ANCOVAs, likely reflect also developmental decoupling in the examined flowers. This is in accordance to the expected buffering of floral traits from variation in vegetative traits, even for plants with unspecialized pollination systems, as reported in previous studies (Armbruster *et al.*, 1999; Pérez-Barrales *et al*., 2007).

Although the coefficient of 0.55 for the linear regression model with LL as dependent variable may suggest moderate coupling between flowers and leaves, the evidence of strong functional decoupling at the within population level suggest, an alternative explanation for this regression coefficient; a pollinator-climate association. The populations with the shortest TL and AN (ecotype *pernambucense*) are pollinated by the hummingbirds with the shortest bills observed pollinating the *A. pernambucense* complex populations, and also have the smallest leaves (small LL). However, these populations are located at the highest elevations recorded for this species complex. Leaf size is generally negatively related to altitude (Milla & Reich, 2011). Similarly,

populations of the ecotype *forest-immersed* have the longest TL, AN and LL, 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. Humidity tends to be positively associated to leaf size, whereas solar irradiation is negatively related to it (Pélabon *et al.*, 2011). Finally, the populations of the *dry-season* ecotype where pollinators were observed present intermediate TL, AN and LL, 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. Thus, independent selection pressures leading to similar size change of TL and AN (pollinators) and LL (mostly climate) might explain the moderate regression coefficient obtained in the LL model. Futhermore, if the observed association between LL and the bill length of the main local pollinator was due to flower-leaf coupling, then a similar regression coefficient should be observed between SL and bill length. However, this coefficient was the lowest observed among the four models, 0.46. Another evidence of flower-leaf decoupling is also observed by the type of the relationship between these traits within different populations. Besides the usually weak positive association between FL/AN and LL (Table S1), in some populations these associations are negative. Thus, this is an indicative of how local independent selection agents on flowers and leaves can create different types of association between these structures in each population.

The combined results of both within and among population decoupling, and TL/AN-pollinator covariation in seven populations, altogether, are an interestingly evidence of how functional decoupling between floral and vegetative traits may favor local differentiation. In a previous study it was reported that the morphological floral and leaf variation among populations of the *A. pernambucense* complex were likely due to local selection agents - pollinators for flowers, and precipitation and temperature variables for leaves (Wanderley *et al.*, unpl. data). Nonetheless, it remained unclear whether selection on floral trait variation by pollinators could constrain selection on leaf trait variation by climatic variables because of lack of functional decoupling between these organs. Therefore, the results here presented suggest that not only selection for local floral phenotypes do not constrain selection for leaf phenotypes, but also that this feature is shaping the current patterns of among population variations of flowers and leaves across at least seven population of the *A. pernambucense* complex pollinated by morphologically different hummingbirds. Among population covariation between specialized flowers morphology and

pollinator morphology is not an obvious expectation. For instance, Herrera *et al.* (1993; 2002) observed 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 pollinators. 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 demography 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*, while these traits were decoupled from corolla size, a trait not directly related to pollination efficiency in this species. Moreover, the variation of the key floral traits for the pollination of the species examined by Cosacov *et al.* (2014) was also decoupled from climatic variation associated to vegetative traits.

CONCLUSIONS

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. More importantly, the results of this study, together with a previous research (Wanderley *et al.*, unpl. data), suggest that functional decoupling is allowing independent among population variation of floral and leaf traits due to current local selection agents in the *A. pernambucense* complex. These findings support previous evidence of population differentiation due to local adaption in this species complex (Wanderley *et al.*, unpl. data). Finally, these results shed light on the role of functional decoupling between flowers and leaves or other vegetative traits to increase chances of local adaptation in species distributed across highly heterogeneous biotic and abiotic landscapes.

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TABLES

TABLE 1 - Sampled populations of the *Ameroglossum pernambucense* complex. Populations highlighted in bold are those where pollinator information is available (Wanderley *et al.*, 2014a, unpl. data).

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

TABLE 2 - Results of the one-way ANOVA comparing the variation of three floral traits and one leaf trait among 13 populations of the *Ameroglossum pernambucense* complex. Post-hoc multiple comparisons (Tukey test) are also presented. Populations with common letters were not significantly different in trait size ($\alpha = 0.005$).

Square root of tube length (TL) F = 45.69; d.f. = 12; *P* < 0.0001 Tukey test: LSD = 0.104, d.f. = 171							**Square root of anther-nectary distance (AN)** F = 38.85; d.f. = 12; *P* < 0.0001 Tukey test: LSD = 0.11, d.f. = 171							
Population							**Population**							
Rei	A						Rei	A						
P	A						P	A	B					
QA		B					QA		B	C				
SM		B	C				SM			C	D			
TAC		B	C	D			TAC			C	D	E		
ESP			C	D			LB			C	D	E	F	
Spe			C	D			ESP				D	E	F	
LB			C	D			AB					E	F	
RN			C	D			Spe						F	
AJ				D	E		AJ						F	
AB				D	E		RN						F	G
SER					E	F	SER							G
AN						F	AN							
Square root of sepal length (SL) F = 40.37; d.f. = 12; *P* < 0.0001 Tukey test: LSD = 0.096, d.f. = 171							**Square root of leaf length (LL)** F = 34.87; d.f. = 12; *P* < 0.0001 Tukey test: LSD = 0.472, d.f. = 171							
Population							**Population**							
P	A						Rei	A						
Rei	A						P	A	B					
SM		B					SM	A	B	C				
AJ		B	C				AJ		B	C	D			
Spe			C	D			LB			C	D	E		
QA			C	D	E		TAC			C	D	E		
TAC			C	D	E		Spe				D	E		
ESP			C	D	E		RN				D	E		
AB				D	E		QA					E	F	
AN				D	E		AB					E	F	G
RN				D	E		ESP						F	G
LB					E	F	AN							G
SER						F	SER							

TABLE 3 - Results of the ANCOVAs comparing the among population variation of two floral traits directly related to pollination (floral tube length – TL, and anthers-nectary distance -AN) of the *Ameroglossum pernambucense* complex. Two traits not related to pollination were used as covariates, sepal length (SL) and leaf length (LL).

Square root of floral tube length (TL)																			
Covariate: sepal length (SL) F = 17.66; d.f. = 12; *P* < 0.0001 Tukey test: LSD = 0.1, d.f. = 170										Covariate: leaf length LL F = 23.23; d.f. = 12; *P* < 0.0001 Tukey test: LSD = 0.1, d.f. = 170									
Population										**Population**									
Rei	A									Rei	A								
P	A	B								P	A								
QA		B	C							QA		B							
TAC			C	D						SM		B	C						
LB			C	D						TAC		B	C						
SM				D	E					ESP			C						
ESP				D	E					Spe			C						
Spe				D	E	F				LB			C						
RN				D	E	F				RN			C	D					
AB				D	E	F				AB			C	D					
AJ					E	F	G			AJ			C	D					
SER						F	G			SER				D					
AN							G			AN				D					
Square of root of anther-nectary distance (AN)																			
Covariate: SL F = 17.42; d.f. = 12; *P* < 0.0001 Tukey test: LSD = 0.11, d.f. = 170										Covariate: LL F = 19.12; d.f. = 12; *P* < 0.0001 Tukey test: LSD = 0.11, d.f. = 170									
Population										**Population**									
Rei	A									Rei	A								
P	A	B								P	A								
QA	A	B								QA	A	B							
TAC		B	C							SM		B	C						
SM		B	C	D						TAC		B	C						
LB		B	C	D						LB			C	D					
ESP			C	D	E					ESP			C	D					
AB			C	D	E	F				AB			C	D					
RN				D	E	F				Spe				D	E				
Spe				D	E	F				AJ				D	E	F			
AJ					E	F	G			RN				D	E	F			
SER						F	G			SER					E	F			
AN							G			AN						F			

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 anthers-nectary distance – AN), and two traits not related to pollination (sepal length – SL, leaf length – LL). The dataset used in this analysis are from seven populations of the *Ameroglossum pernambucense* complex.

Response variable	**N**	**Adjusted R^2**	**d.f.**	**F**	***P*-value**
TL	112	0.7	1	255.0	< 0.0001
AN	112	0.69	1	249.52	< 0.0001
SL	112	0.46	1	94.23	< 0.0001
LL	112	0.55	1	138.69	< 0.0001

TABLE S1. - Within population correlation matrices between pairs of floral and leaf traits in 13 populations of the *Ameroglossum pernambucense* complex. In each matrix the values below the diagonal are the Pearson correlation coefficients between trait pairs, whereas above the diagonal are the *P-values* of these correlation tests. TL = floral tube length; AN = anthers-nectary distance; SL = sepal length; LL = leaf length.

Population: P					Population: Spe				
	TL	**AN**	**SL**	**LL**		**TL**	**AN**	**SL**	**LL**
TL	1	0.02	0.85	0.28	**TL**	1	<0.0001	0.02	0.78
AN	0.59	1	0.66	0.37	**AN**	0.78	1	0.01	0.38
SL	0.05	-0.12	1	0.12	**SL**	0.53	0.57	1	0.43
LL	0.3	0.25	0.42	1	**LL**	0.07	0.21	0.19	1
Population: Rei					Population: SM				
	TL	**AN**	**SL**	**LL**		**TL**	**AN**	**SL**	**LL**
TL	1	0.01	0.16	0.87	**TL**	1	<0.0001	0.88	0.7
AN	0.6	1	0.06	0.43	**AN**	0.93	1	0.81	0.66
SL	0.37	0.48	1	0.02	**SL**	-0.06	0.09	1	0.16
LL	0.05	0.21	0.57	1	**LL**	0.15	0.17	-0.52	1
Population: QA					Population: AB				
	TL	**AN**	**SL**	**LL**		**TL**	**AN**	**SL**	**LL**
TL	1	<0.0001	0.14	0.36	**TL**	1	<0.0001	0.73	0.06
AN	0.93	1	0.16	0.31	**AN**	0.95	1	0.8	0.05
SL	0.31	0.29	1	0.06	**SL**	0.11	0.08	1	0.79
LL	0.19	0.21	0.38	1	**LL**	-0.56	-0.57	-0.09	1

TABLE S1 - cont.

Population: LB				
	TL	**AN**	**SL**	**LL**
TL	1	<0.0001	0.42	0.23
AN	0.9	1	0.87	0.05
SL	0.27	-0.06	1	0.25
LL	-0.39	-0.6	0.38	1

Population: TAC				
	TL	**AN**	**SL**	**LL**
TL	1	<0.0001	<0.001	0.52
AN	0.95	1	0.01	0.67
SL	0.83	0.8	1	0.65
LL	0.23	0.16	0.17	1

Population: ESP				
	TL	**AN**	**SL**	**LL**
TL	1	<0.0001	0.1	0.18
AN	0.94	1	0.19	0.13
SL	0.43	0.35	1	0.72
LL	0.35	0.4	0.1	1

Population: RN				
	TL	**AN**	**SL**	**LL**
TL	1	<0.0001	0.15	0.68
AN	0.84	1	0.23	0.88
SL	0.42	0.36	1	0.53
LL	-0.13	0.05	-0.19	1

Population: AJ				
	TL	**AN**	**SL**	**LL**
TL	1	<0.0001	0.6	0.27
AN	0.93	1	0.42	0.58
SL	0.22	0.33	1	0.37
LL	-0.45	-0.23	0.37	1

Population: AN				
	TL	**AN**	**SL**	**LL**
TL	1	<0.0001	0.85	0.18
AN	0.92	1	0.59	0.13
SL	0.06	-0.17	1	0.93
LL	0.41	0.46	-0.03	1

Population: SER				
	TL	**AN**	**SL**	**LL**
TL	1	<0.0001	0.35	0.89
AN	0.93	1	0.27	0.57
SL	0.24	0.29	1	0.05
LL	0.04	0.15	0.49	1

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

Population	**TL**	**AN**	**SL**	**LL**
AB	11.72	10	8.36	15.7
AJ	2.62	3.32	12.82	13.93
AN	10.49	9.96	9.22	20.84
ESP	6.7	7.14	13.05	19.15
LB	7.35	6.36	10.2	18.37
P	9.63	4.33	17.44	12.15
QA	8.62	9.51	12.09	21.26
Rei	8.08	6.67	13.08	20.63
RN	8.11	7.16	15.06	14.1
SER	8.02	8.19	13.84	18.01
SM	6.41	5.25	14.35	23.57
Spe	6.65	6.04	14.9	18.28
TAC	10.75	10.58	15.88	14.45

FIGURES

FIGURE 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.

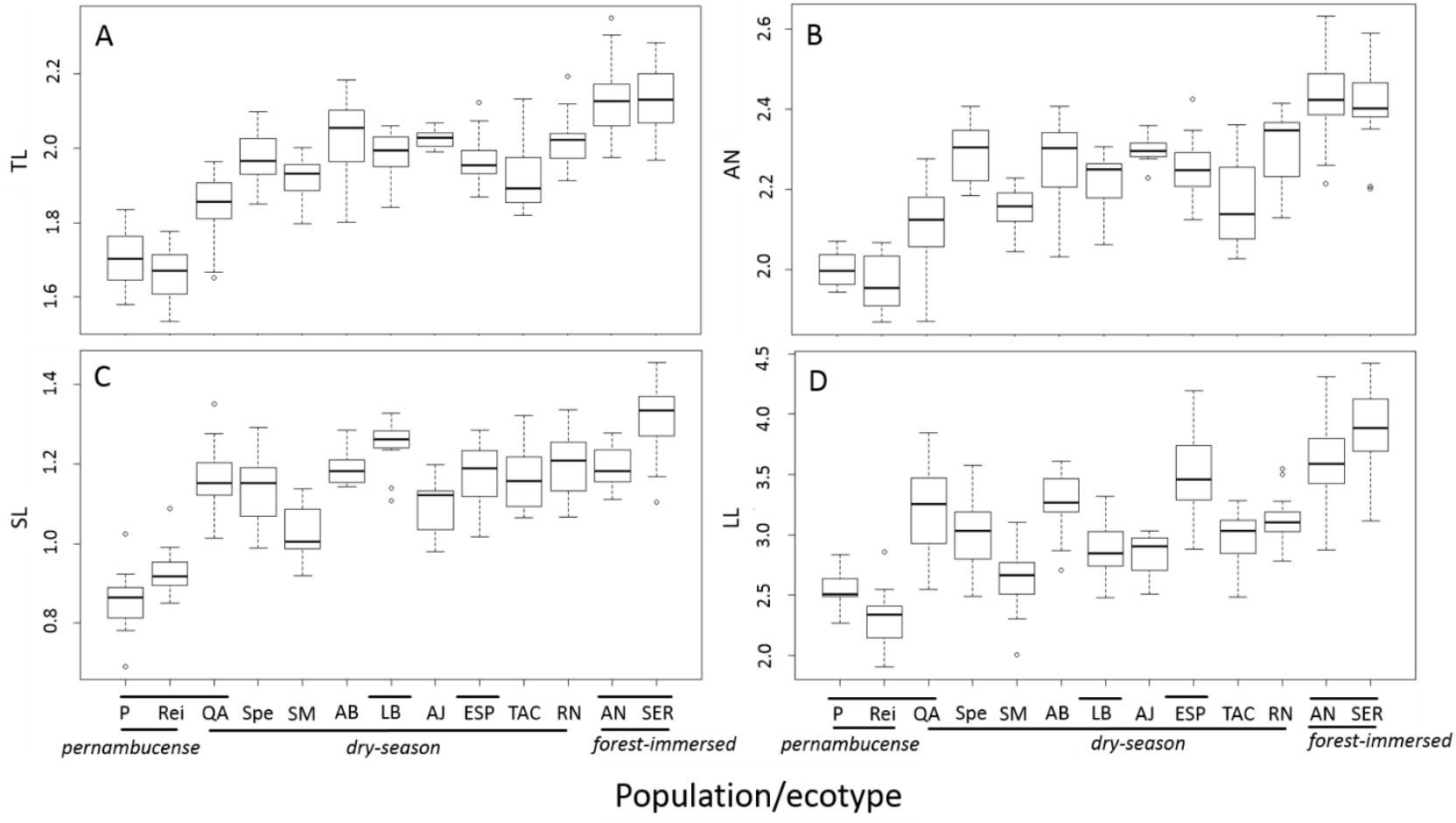

FIGURE 2 - Among population variation of three floral traits (floral tube length – TL, anther-nectary distance – AN, and sepal length – SL) and one leaf trait (leaf length – LL) of the *Ameroglossum pernambucense* complex. Bars below population names divide the populations belonging to three different ecotypes. Bars above population names indicate the populations with pollinator information. The data of the four traits presented were square root transformed.

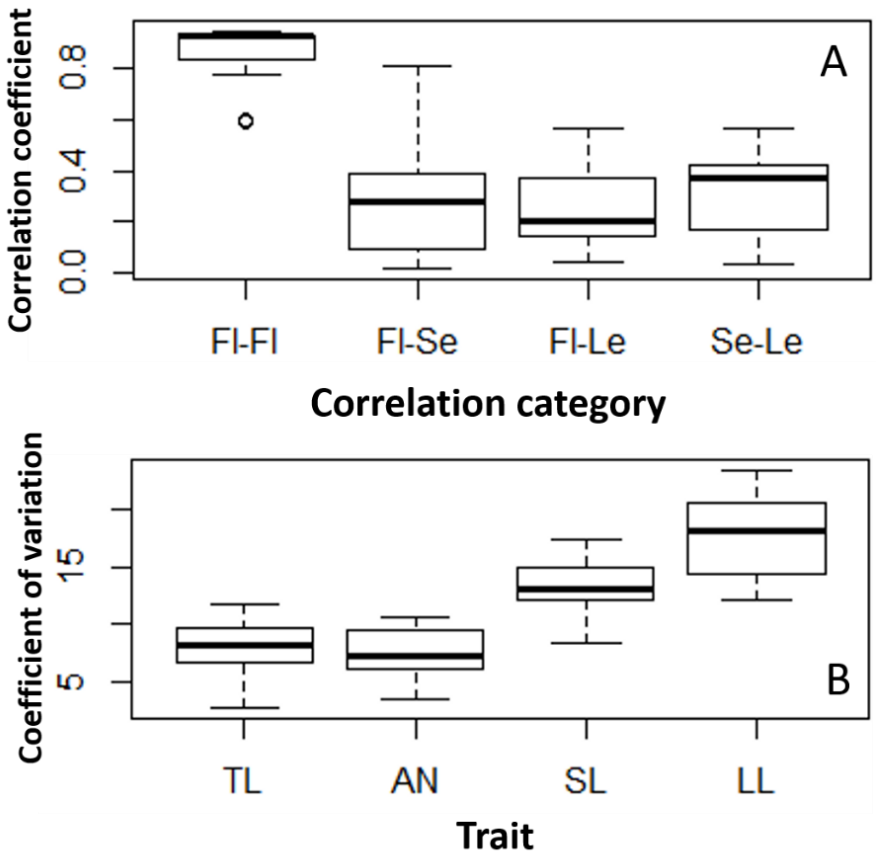

FIGURE 3 - Pearson correlation coefficients for within population correlations between floral and leaf traits of 13 populations of the *Ameroglossum pernambucense* complex (A). Fl-Fl represents correlation tests between to floral traits directly related to pollination (floral tube length – TL, and anthers-nectary distance, AN); Fl-Se represents population means of correlation tests between TL and AN with sepal length (SL); Fl-Le includes the population means of correlation test between TL and AN with leaf length (LL); and Se-Le represents correlation tests between SL and LL. (B) Within population coefficients of variation for each of these traits.

6 CONCLUSÕES

Apesar do pequeno tamanho e isolamento ao longo da paisagem, as populações dos arquipélagos estudados não pareceram sofrer limitação de polinizadores. Não houve evidência de que os polinizadores limitam criticamente as possibilidades de parceiros sexuais dentro dos arquipélagos. Portanto, é pouco provável que haja perda de adaptabilidade devido a endogamia e deriva causadas por deficiência de polinizadores, embora fatores demográficos, tais como históricos de dispersão e vicariância, possam ainda levar a isso. Esses resultados estão de acordo com evidências recentes de que os polinizadores possuem um papel chave na manutenção do potencial adaptativo de pequenas populações de arquipélagos.

Embora diferenciação por deriva genética seja favorecida em condições de isolamento geográfico e reduzido tamanho populacional, a maior associação da composição genética e dos fenótipos das populações examinadas com o clima, e não com sua posição geográfica, indica que as populações do complexo *A. pernambucense* podem adaptar-se localmente. Portanto, isso sugere que populações ocorrentes em pequenos habitats isolados ao longo de paisagens heterogêneas podem responder aos agentes locais de seleção.

O suporte obtido para a hipótese de desacoplamento funcional revelou a possibilidade das plantas estudadas poderem adaptar suas flores aos polinizadores locais independentemente dos ajustes das folhas às pressões climáticas locais. De fato, isso foi suportado pela observação da covariação das dimensões florais com a morfologia dos polinizadores independente das variações de estruturas funcionalmente distintas (sépalas e folhas). Isso permite concluir que o padrão observado de variação interpopulacional das flores desacoplada da variação das folhas é possível pela alta modularidade funcional das flores. Mais do que isso, as evidências indicam que o desacoplamento observado ao longo das populações é provavelmente moldado por agentes locais

de seleção às flores (polinizadores) e folhas (clima), e não flutuações randômicas características de evolução não adaptativa.

Em suma, os resultados dessa pesquisa permitem concluir que em pequenas populações de arquipélagos isolados em paisagens heterogêneas os polinizadores podem contribuir para a manutenção do potencial adaptativo. A capacidade de adaptação dessas populações, por sua vez, foi evidenciada pela estruturação ambiental da composição genética e dos fenótipos foliares e florais. Por fim, o desacoplamento funcional entre flores e folhas permite que essas estruturas se adaptem independentemente aos agentes de seleção locais bióticos e bióticos, o que foi observado.

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