UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

ESTUDO FILOGEOGRÁFICO E HIBRIDAÇÃO EM UM GRUPO DE ESPÉCIES DE *VRIESEA* (BROMELIACEAE): CONTRIBUIÇÕES PARA A DIVERSIFICAÇÃO DO GÊNERO E HISTÓRIA EVOLUTIVA DA MATA ATLÂNTICA

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Tese submetida ao Programa de Pós-Graduação em Genética e Biologia Molecular da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do Título de Doutora em Ciências (Genética e Biologia Molecular)

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SUMÁRIO

RESUMO

Bromeliaceae é uma das famílias de angiospermas com maior diversidade morfológica e ecológica, podendo habitar desde ambientes extremamente secos até florestas úmidas. *Vriesea* é o segundo maior gênero da subfamília Tillandsioideae, com 231 espécies, e é composto principalmente por espécies de hábito epífito ocupando ambientes mesófilos, sendo a Mata Atlântica o principal centro de diversidade, com 166 espécies. Apesar do polifiletismo do gênero, é possível observar grupos monofiléticos de espécies de ocorrência no leste brasileiro. Um desses grupos é denominado de "grupo das infladas", cujas espécies, dentre outras características, apresentam inflorescência simples, brácteas florais vermelhas e infladas, e flores tubulares com corola amarela. Esta tese é composta por dois manuscritos que buscam entender os padrões históricos, de diversidade e estrutura genética, bem como o fluxo gênico interespecífico e o grau de isolamento reprodutivo em espécies deste grupo. No Capítulo II buscando compreender os padrões históricos, a estrutura e diversidade genética de populações de *V. incurvata* na porção sul da Mata Atlântica, de onde essa espécie é endêmica, foram analisados duas regiões plastidiais e 13 marcadores nucleares microssatélites, além de análises demográficas e de modelagem de nicho ecológico. Os resultados mostraram uma falta de estruturação filogeográfica com um alto compartilhamento de haplótipos entre as populações e uma diversidade genética variando de moderada a alta em ambos os genomas analisados. A modelagem de nicho ecológico indicou que não houve grandes alterações das áreas mais adequadas para a distribuição da espécie nos períodos do Último Máximo Glacial e do Holoceno Médio em relação à distribuição atual. Assim, após a colonização de *V. incurvata*, a porção sul da Mata Atlântica se manteve historicamente estável para sua ocupação, corroborando a estruturação filogeográfica encontrada. No Capítulo III a fim de avaliar o fluxo gênico entre espécies do "grupo das infladas", foram selecionadas três localidades na Mata Atlântica onde ocorrem populações simpátricas das espécies *V. taritubensis* var. *patens, V. simplex*, *V. carinata*, *V. agostiniana*, *V. inflata* e *V. incurvata*, e utilizados 12 marcadores nucleares do tipo microssatélites. Além disso, para analisar as diferenças morfológicas entre as espécies em cada localidade, foram utilizadas 10 variáveis, quatro relacionadas à parte vegetativa e seis a parte reprodutiva das plantas. Os resultados indicaram uma baixa estruturação populacional e uma alta taxa de hibridação entre as espécies de *Vriesea* nas localidades estudadas, sugerindo que as barreiras reprodutivas entre essas espécies são permeáveis. Foram observados alguns indivíduos híbridos com morfologia atípica, no entanto a maioria apresentou características morfológicas semelhantes aos parentais. De modo geral, os resultados obtidos nesta tese contribuem com a compreensão dos processos evolutivos envolvidos na diversificação do gênero *Vriesea*, especificamente em espécies do “grupo das infladas”, bem como com a elucidação dos padrões históricos e evolutivos envolvidos na porção sul da Mata Atlântica.

ABSTRACT

Bromeliaceae is one of the most morphological and ecological diverse family of flowering plants, which inhabits environments from extremely dry to humid forests. *Vriesea* is the second largest genus of Tillandsioideae subfamily with 231 species, and is composed mainly by epiphytic plants in mesophilic environments, being Atlantic Forest the main center of diversity, with 166 species. Despite the polyphyly of this genus, it is possible to observe monophyletic groups of species from eastern Brazil. One of these groups are the "inflated group", which among other characteristics, presents simple inflorescence, red and inflated floral bracts, and tubular flowers with yellow corolla. The present thesis consists of two manuscripts that seek to understand the historical patterns, genetic diversity and structure, as well as the interspecific gene flow and the degree of reproductive isolation in species of this group. In Chapter II, seeking to understand the historical patterns, structure and genetic diversity of *V. incurvata* populations from southern portion of the Atlantic Forest, where it is endemic, two plastid regions and 13 nuclear microsatellite markers were analyzed, in addition to demographic and ecological niche modeling. The results showed a lack of phylogeographic structure with high haplotypes sharing among populations and the genetic diversity ranging from moderate to high in both analyzed genomes. The ecological niche modeling indicated no major changes in the most suitable areas for the distribution of this species in the Last Glacial Maximum and the Mid Holocene periods from the current distribution. Thus, after the colonization of *V. incurvata*, the southern portion of the Atlantic Forest remained historically stable for its occupation, corroborating the finding of no phylogeographic structure. In Chapter III, in order to assess the gene flow between species of the "inflated group", three localities with sympatric populations of *V. taritubensis* var. *patens*, *V. simplex*, *V. carinata*, *V. agostiniana*, *V. inflata* e *V. incurvata* species in the Atlantic Forest were selected and analysed using 12 microsatellite nuclear markers for this purpose. In addition, to analyze the morphological differences between species in each locality, 10 variables were used, four related to the vegetative and six to the reproductive plants structures. The results indicated a low population structure and a high percentage of hybridization among the *Vriesea* species in the studied localities, suggesting that the reproductive barriers can be considered permeable. It was observed some hybrid individuals with atypical morphology, but the majority presented morphological characteristics of purebreds parental. In general, the results obtained in this thesis

contribute to understanding the evolutionary processes involved in *Vriesea* genus diversification, specially in species from “inflated group”. Also, this study contributed to better understand the historical and evolutive patterns involved in the southern portion of Atlantic Forest.

CAPÍTULO I

INTRODUÇÃO GERAL

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1.0 A biodiversidade da Mata Atlântica

A Mata Atlântica é uma das maiores florestas tropicais das Américas, abrangendo 17 estados brasileiros ao longo de toda a costa litorânea, estendendo-se também a planaltos e serras no interior do continente, chegando a regiões do Paraguai e Argentina (Ribeiro *et al*., 2009; SOSMA/INPE, 2020). Atualmente, no Brasil, devido à alta pressão antrópica sofrida desde a época da colonização, estima-se que exista apenas cerca de 12% da floresta em comparação com sua cobertura vegetal original (Morellato e Haddad, 2000; Ribeiro *et al*., 2011a; SOSMA/INPE, 2020). A ampla variação de latitude (3ºS a 30ºS), longitude (35ºO a 60ºO), altitude (0 – 2.900m acima do nível do mar) e precipitação anual (1.000 a 4.200 milímetros), torna a Mata Atlântica um ambiente altamente heterogêneo, formando um mosaico de tipos vegetacionais que inclui desde florestas ombrófilas (densa, aberta e mista), florestas estacionais (decidual e semidecidual), campos de altitude, até mangues e restingas (Câmara, 2003; Ribeiro *et al*., 2009; Ribeiro *et al*., 2011a). Tais características geográficas favorecem a alta diversidade e endemismo de espécies da flora e fauna, tornando esta floresta um dos *hotspots* mundiais para biodiversidade (Morellato e Haddad, 2000; Myers *et al*., 2000; Ribeiro *et al*., 2009).

1.1 A família Bromeliaceae

Bromeliaceae apresenta 3.652 espécies distribuídas em 78 gêneros (Gouda *et al*., [cont. updated]), e é caracterizada por uma recente radiação adaptativa, tornando a família uma das mais diversas morfológica e ecologicamente entre as angiospermas (Benzing, 2000; Givnish *et al*., 2011). As inovações apresentadas por estas plantas ao longo do tempo, tais como o epifitismo, a formação de um "tanque" através da disposição rosulada das folhas, a capacidade dos tricomas foliares de absorver água e nutrientes, a polinização por vertebrados e o surgimento do sistema CAM de fotossíntese (metabolismo ácido das crassuláceas), permitiram as bromélias estabelecerem-se nos mais variados ambientes, de xéricos à mesofíticos, e estão relacionadas às altas taxas de diversificação observadas na família (Givnish *et al*., 2007; 2014; Silvestro *et al*., 2013). A evolução em uma ampla diversidade de cores e formas de estruturas florais atraíram os mais variados polinizadores, como insetos, morcegos e beija-flores (Benzing, 2000). Os beija-flores, no entanto, podem ser considerados os principais agentes polinizadores da família (Benzing, 2000; Dias *et al*., 2014). Em um estudo de polinização com beija-flores, realizado em regiões do sudeste brasileiro, na Mata Atlântica, as bromélias representaram 36% da flora ornitófila e 33% das flores mais ricas em néctar (Buzato *et al.*, 2000). Ecologicamente, além do néctar utilizado como recompensa para os polinizadores, essas plantas possuem frutos carnosos que são consumidos e dispersados por aves, e o tanque formado permite o armazenamento de água e nutrientes, que além de servir para sua própria nutrição, fornece um microambiente favorável que proporciona alimento, abrigo e local para reprodução para diversos animais associados (Reitz, 1983; Benzing, 2000; Dias *et al.*, 2014).

As bromélias são quase exclusivas do Neotrópico, ocorrendo desde o sul dos Estados Unidos (estados do Texas, Virgínia e Califórnia) até o norte da Patagônia, na Argentina (Benzing, 2000). A exceção é a espécie *Pitcairnia feliciana* (A. Chev.) Harms & Mildbr. que ocorre no Oeste da África, provavelmente devido a um evento recente de dispersão à longa distância (Benzing, 2000; Givnish *et al.*, 2004). São considerados quatro centros de diversidade para a família, os Andes, a América Central, o Planalto das Guianas, e o Escudo brasileiro (Givnish *et al.*, 2011). O Brasil apresenta uma grande diversidade de bromélias com 51 gêneros (23 endêmicos) e 1.399 espécies (1.196 endêmicas) (Flora do Brasil 2020). Especificamente, a Mata Atlântica apresenta uma grande riqueza de espécies (Martinelli *et al.*, 2008), sendo as epífitas dentre as Angiospermas com o maior número de registros neste bioma (37,6%) (Ramos *et al.*, 2019).

Tradicionalmente, Bromeliaceae estava dividida em três subfamílias: Pitcairnioideae, Tillandsioideae e Bromelioideae (Smith e Downs, 1974; 1977; 1979). No entanto, análises filogenéticas realizadas com marcadores plastidiais indicaram o reconhecimento de oito subfamílias (Givnish *et al.*, 2007; 2011): Brocchinioideae, Lindmanioideae, Tillandsioideae, Hechtioideae, Navioideae, Pitcairnioideae, Puyoideae, Bromelioideae. Estima-se que a as bromélias surgiram no Escudo das Guianas há cerca de 100 milhões de anos atrás (Ma), durante o Período Cretáceo, com as subfamílias existentes começando a divergir há apenas 19 Ma (Givnish *et al.*, 2011). Os mesmos autores sugerem que as bromélias começaram a ocupar os Andes, a Amazônia, a América Central, o Caribe e o Escudo brasileiro entre 15 e 10 Ma.

A subfamília Tillandsioideae sempre foi reconhecida como monofilética e é a que apresenta a distribuição geográfica mais ampla entre as demais. Pode ser dividida em quatro tribos: Glomeropitcairnieae, Catopsideae, Tillandsieae e Vrieseeae, sendo que estas duas últimas formam o chamado "core Tillandsioideae" (Barfuss *et al.*, 2005; 2016). Givnish *et al.* (2011), baseados em oito marcadores plastidiais, sugerem que o ancestral das linhagem do "core Tillandsioideae" provavelmente surgiu nos Andes há aproximadamente 14,2 Ma, com os gêneros atuais começando a divergir entre si há aproximadamente 8,7 Ma, expandindo-se pela América Central, litoral norte da América do Sul e Caribe. Todavia, Kessous *et al.* (2020), através de uma abordagem biogeográfica, sugerem que um evento vicariante (Mar do Paraná) ocorrido por volta de 10 Ma, teria ocasionado a divergência de grupos do "core Tillandsioideae", com membros da subtribo Cipuropsidinae e tribo Tillandsieae colonizando os Andes, e a subtribo Vrieseinae (Vrieseeae), ao qual *Vriesea* pertence, o Escudo brasileiro. Os autores também sugerem que o ancestral comum de Vrieseinae surgiu nos domínios da Mata Atlântica e Chaco durante o Plioceno (5,3 – 2,6 Ma), com os grupos infragenéricos e complexos de espécies divergindo no Pleistoceno (2,6 – 0,1 Ma).

1.2 Gênero *Vriesea* Lindl.

Vriesea é o segundo maior gênero de Tillandsioideae com 231 espécies (Gouda et al. [cont. updated]). O gênero é composto principalmente por plantas com hábito epífito ocupando ambientes mesófilos, mas também podem ser terrestres e rupícolas e ocorrer em campos rupestres, campos de altitude e *inselbergs* (Costa *et al.*, 2014). A distribuição geográfica das espécies varia desde casos de ampla distribuição até microendemismos (Costa *et al.*, 2014). Dois centros de diversidade são reconhecidos para o gênero, o primeiro do norte da América do Sul, América Central e o Caribe (Smith e Downs, 1977), e o segundo no leste do Brasil, onde 87% das espécies ocorrem (Costa *et al.*, 2015; Gomes-da-Silva e Souza-Chies, 2017). Apresenta maior riqueza na Mata Atlântica, com 166 espécies (Martinelli *et al.*, 2008), sendo o gênero epifítico de Bromeliaceae com mais registros de ocorrência deste bioma (37%) (Ramos *et al.*, 2019), podendo também ser encontradas nos biomas Cerrado, Caatinga, Amazônia e Pampa (Flora do Brasil 2020).

O gênero foi historicamente dividido em duas seções: *Vriesea* sect. *Vriesea* com flores de antese diurna, brácteas florais coloridas (do vermelho ao amarelo), pétalas linguladas, e estames exsertos; e *V.* sect. *Xiphion* com flores de antese noturna, brácteas florais verdes e castanhas, pétalas obovais e estames inclusos (Smith e Downs, 1977; Costa *et al.*, 2014).

Essas duas seções foram originalmente descritas com características claramente associadas à síndromes de polinização por beija-flores (*V.* sect. *Vriesea*) ou morcegos (*V.* sect. *Xiphion*); no entanto, esta classificação formal das espécies também utilizou a posição dos estames (exsertos ou inclusos) levando a equívocos taxonômicos em algumas espécies com morfologia floral intermediária (Costa *et al*., 2014; Neves *et al*., 2020). Além disso, as duas seções não puderam ser recuperadas em grupos monofiléticos, tanto em filogenias morfológicas (Costa *et al*., 2015), quanto naquelas baseadas em marcadores moleculares (Barfuss *et al*., 2016; Gomes-da-Silva e Souza-Chies, 2017; Machado *et al*., 2020). Na filogenia realizada com caracteres morfológicos (Costa *et al*., 2015), apesar da evidente homoplasia em caracteres descritos tradicionalmente para delimitar as duas seções, foi possível observar em clados diferentes as espécies atribuídas a uma ou outra seção. Neves *et al*. (2020) ressaltaram a importância da síndrome de polinização (beija-flor x morcego) na diferenciação de espécies de *Vriesea*, o que foi suportado tanto por análises genéticas quanto por traços florais distintos nas diferentes síndromes, sugerindo o polinizador como o agente condutor do isolamento ecológico no gênero. Os mesmos autores também sugeriram uma possível transição entre as diferentes síndromes nas espécies que apresentam traços florais mistos.

A recente diversificação de *Vriesea* é evidenciada pelo seu polifiletismo, baixa resolução infragenérica, bem como a difícil delimitação entre algumas espécies (Barfuss *et al*., 2005, 2016; Costa *et al*., 2014, 2015; Gomes-da-Silva e Souza-Chies, 2017; Machado *et al.*, 2020). No entanto, é possível observar alguns grupos monofiléticos, sendo formados por espécies que ocorrem no leste brasileiro, bem como complexos de espécies inseridos dentro destes grupos (Costa *et al*., 2009; 2014; 2015; Kowalski e Tardivo, 2015; Gomes-da-Silva e Souza-Chies, 2017; Neves *et al*., 2018; Machado *et al*., 2020; Uribbe *et al*., 2020). Um dos grupos monofiléticos observados é o "grupo das infladas", que compreende espécies com características como: inflorescência simples, brácteas florais em sua maioria infladas, involutas ou não, flores tubulares e com corola amarela, e a presença de estreitamento entre a lâmina e a bainha foliar (Costa *et al*., 2014, 2015). Um dos complexos observados dentro deste grupo é o complexo *V. incurvata*, distribuído na Mata Atlântica, do Rio Grande do Sul (RS) ao Rio de Janeiro (RJ), e é composto pelas espécies *V. incurvata* Gaudich. (Figura 1A), *V. sucrei* L. B. Sm. & Read, *V. taritubensis* var. *taritubensis* E. Pereira & I. A. Penna, *V. taritubensis* var. *brevisepala* E. Pereira & I. A.

Penna, e *V. taritubensis* var. *patens* B. Neves & A. F. Costa (Figura 1B) (Neves *et al*., 2018). A filogenia molecular de espécies do complexo, baseada em marcadores plastidiais, sugere que as espécies *V. incurvata* (Figura 1A) e as variedades de *V. taritubensis* sofreram um evento vicariante na região da Serra da Bocaina (estado de São Paulo-SP), por volta de 5 Ma, no Plioceno, ocasionando uma quebra do fluxo gênico, o que levou a diferenciação e estruturação destas espécies, com as variedades de *V. taritubensis* ocupando do norte de SP ao RJ, e *V. incurvata* do centro-sul de SP ao RS (Aguiar-Melo *et al*., 2019; Neves *et al*., 2019). Uma das características morfológicas que melhor distingue essas espécies é a morfologia da bráctea floral, com *V. incurvata* apresentando brácteas elípticas e mais largas em relação as variedades de *V. taritubensis*, que variam de brácteas lanceoladas à elípticas (Neves *et al*., 2019). *Vriesea taritubensis* var. *patens* (Figura 1B) foi recentemente descrita sendo endêmica do Parque Estadual da Serra do Mar - Núcleo Santa Virgínia em SP, se diferenciando das demais espécies do complexo por apresentar inflorescência mais longa e estreita, muitas vezes patente e sigmoide, com numerosas flores eretas (Neves *et al*., 2018).

Outras espécies que podemos observar dentro do “grupo das infladas” são, entre outras: *V. inflata* (Wawra) Wawra (que dá nome ao grupo), *V. carinata* Wawra, *V. simplex* (Vellozo) Beer e *V. agostiniana* E. Pereira (Figura 1) (Costa *et al*., 2014; 2015; Neves *et al*., 2020). Estas espécies apresentam folhas variando do verde claro ao escuro, podendo apresentar bainha foliar levemente arroxeada, *V. simplex* também pode apresentar coloração avermelhada na face abaxial da lâmina; já as brácteas florais no geral são vermelhas, algumas apresentando ápice amarelo ou verde (Smith e Downs, 1977). *Vriesea inflata* (Figura 1C) ainda apresenta o pedúnculo da inflorescência um pouco mais curto que as demais, brácteas florais densamente imbricadas e infladas, e ocorre do Espírito Santo (ES) ao Paraná (PR) (Smith e Downs, 1977). Já *V. carinata* (Figura 1D) apresenta brácteas florais geralmente mais estreitas e encurvadas no ápice, distribuindo-se da Bahia (BA) ao RS (Smith e Downs, 1977). *Vriesea simplex* (Figura 1E) e *V. agostiniana* (Figura 1F) apresentam a inflorescência pêndula (Smith e Downs, 1977), e as características que melhor as diferencia é o tamanho e a forma da bráctea floral, com *V. agostiniana* apresentando bráctea com ápice encurvado e carenadas ao longo de todo o dorso, e *V. simplex* apresentando ápice ereto e carenada somente próximo ao ápice (Smith e Downs, 1977; Kessous *et al*., 2018). *Vriesea simplex* distribui-se nos estados da BA, ES, RJ e SP

(Flora do Brasil 2020), já *V. agostiniana* está restrita a poucas populações em SP e RJ (Kessous *et al*., 2018).

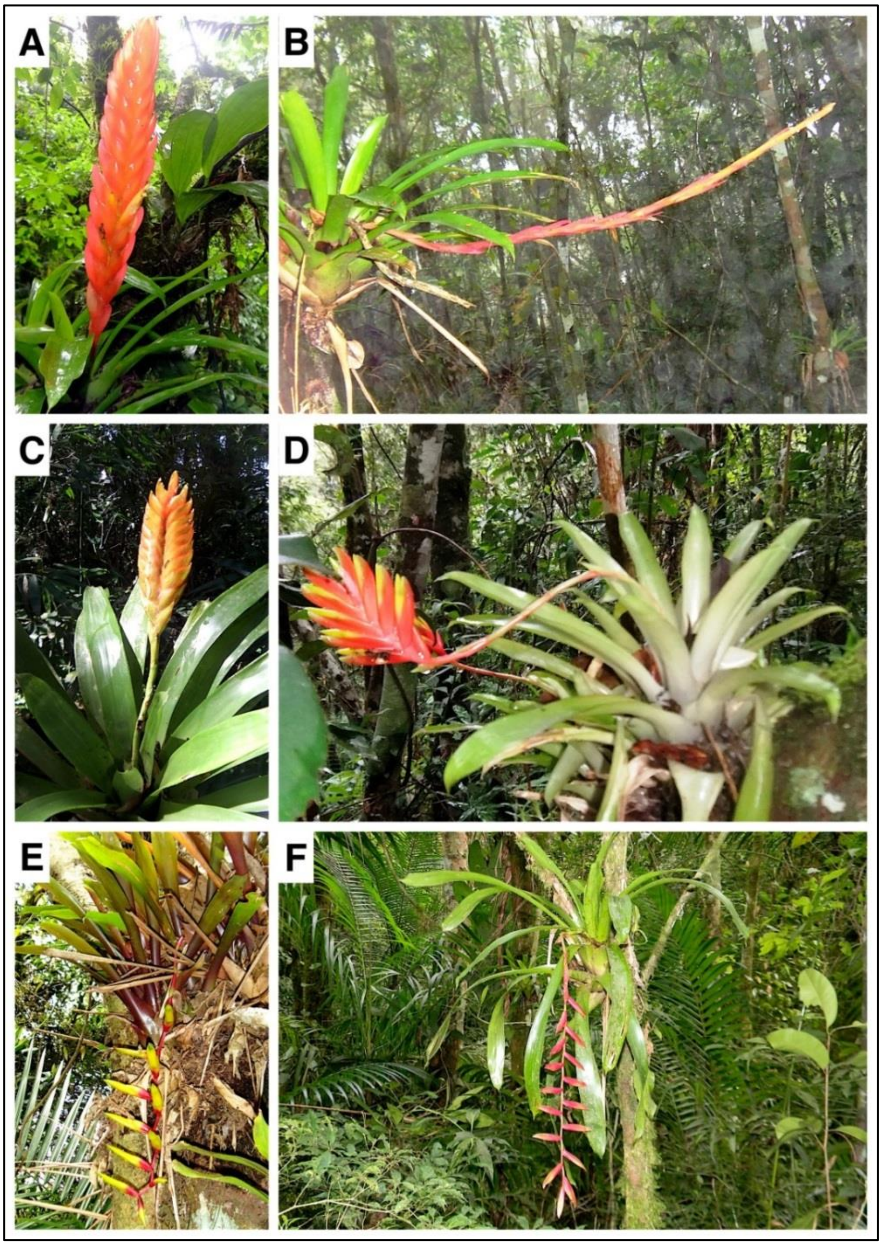

Figura 1. Espécies de *Vriesea* Lindl.: **A:** *V. incurvata* Gaudich.; **B:** *V. taritubensis* var. *patens* B. Neves & A. F. Costa; **C:** *V. inflata* (Wawra) Wawra; **D:** *V. carinata* Wawra; **E:** *V. simplex* (Vellozo) Beer e **F:** *V. agostiniana* E. Pereira. Registro: Camila de Aguiar Melo (A-E); Igor Kessous (F).

Como já mencionado, a delimitação de muitas espécies de *Vriesea* ainda é pouco compreendida (Costa *et al*., 2014; 2015). Assim, quanto mais estudos com caracteres morfológicos e abordagens evolutivas diferentes, como filogeografia e hibridação, em diferentes grupos, complexos e espécies, maior será a compreensão dos processos evolutivos envolvidos na diversificação do gênero, bem como os mecanismos que delimitam estas espécies.

1.3 Filogeografia em bromélias e padrões históricos da Mata Atlântica

A filogeografia é uma disciplina integrativa que está baseada nos processos que envolvem a origem e distribuição da biodiversidade, bem como de mudanças climáticas do passado que influenciaram na estruturação genética atual de populações de animais e plantas (Avise, 2009; Turchetto-Zolet *et al.*, 2013). Estudos com abordagens filogeográficas podem ser encontrados em diferentes gêneros de bromélias (*Catopsis* – Kartzinel *et al.*, 2015; *Aechmea* – Goetze *et al.*, 2016; *Vriesea* – Zanella, 2013; *Dyckia* – Melo, 2016). No entanto, trabalhos com exclusivo enfoque filogeográfico contemplando a distribuição total das espécies ainda são escassos (Palma-Silva *et al.*, 2016; Castello *et al.*, 2020). Mais especificamente na Mata Atlântica, tais estudos foram realizados apenas com *Vriesea gigantea* Gaudich. (Palma-Silva *et al.*, 2009), e mais recentemente com espécies do complexo *V. incurvata* (Aguiar-Melo *et al.*, 2019; Neves *et al.*, 2019) e do complexo *Pticairnia flammea* (Mota, 2019).

A história evolutiva da Mata Atlântica é complexa, esse bioma parece ter sofrido grandes impactos com as mudanças climáticas do Pleistoceno, com expansão da floresta nos períodos interglaciais e retração nos períodos glaciais em zonas de estabilidade florestal (os refúgios) (Behling e Negrelle, 2001; Ledru *et al.*, 2005; Carnaval e Moritz, 2008; Batalha-Filho e Miyaki, 2011). Os padrões evolutivos observados em relação às porções norte e sul da floresta ainda são fonte de muito debate, com alguns estudos indicando estabilidade da porção norte durante as glaciações, e maior fragmentação da porção sul com a expansão da vegetação campestre (Carnaval e Moritz, 2008; Carnaval *et al.*, 2009; Martins *et al.*, 2009; D'Horta *et al.*, 2011), e outros indicando que a porção sul não foi tão severamente impactada, sugerindo áreas estáveis que serviram de refúgio para espécies de plantas e animais (Thomé *et al.*, 2010; Brunes *et al.*, 2015; Peres *et al.*, 2015; Bünger *et al.*, 2016; Costa *et al.*, 2018; Aguiar-Melo *et al.*, 2019). Além disso, em muitos trabalhos, foram identificadas regiões, ao longo da floresta, que estão envolvidas em quebras do fluxo gênico, gerando estruturação e diferenciação entre populações de animais e plantas (Pellegrino *et al.*, 2005; Palma-Silva *et al.*, 2009; Batalha-Filho *et al.*, 2010;

Ribeiro *et al.*, 2011b; Brunes *et al.*, 2015; Menezes *et al.*, 2016; Turchetto-Zolet *et al.*, 2016). Essas regiões proporcionam descontinuidades filogeográficas, e provavelmente são oriundas de vários eventos, como as mudanças climáticas do Pleistoceno, dinâmica geomorfológica e sistemas fluviais (Thomé *et al.*, 2010; Ribeiro *et al.*, 2011b; Menezes *et al.*, 2016). Neste sentido, quanto mais estudos com enfoque evolutivo nos mais variados organismos que habitam as diferentes porções desta floresta, maior será a clareza da história evolutiva deste bioma.

1.4 Barreiras reprodutivas e Hibridação em bromélias

O fluxo gênico é o movimento de genes de uma população para outra, podendo ocorrer dentro ou entre espécies (Slatkin, 1985; Ridley, 2007). O isolamento reprodutivo ocorre através da evolução de barreiras naturais que impedem ou reduzem o fluxo gênico entre grupos de populações, ocasionando o acúmulo de diferenças genéticas, podendo levar a especiação destes grupos (Rieseberg e Willis, 2007). As barreiras ao fluxo gênico entre espécies são classificadas de dois modos: redução da probabilidade de os gametas serem combinados para a formação de um zigoto viável (pré-zigótica), ou redução da viabilidade ou potencial reprodutivo de híbridos interespecíficos (pós-zigótica) (Tiffin *et al.*, 2001). Nas plantas, estas barreiras reprodutivas podem ser divididas em pré-polinização (mecanismos pré-zigóticos) e pós-polinização (mecanismos pré e pós-zigóticos) (Tiffin *et al.*, 2001; Baack *et al.*, 2015). As barreiras pré-polinização incluem o isolamento ecogeográfico, a diferenciação nos períodos de floração, assim como a especialização em diferentes polinizadores com a diferenciação de cores e traços florais (Rieseberg e Willis, 2007; Baack *et al.*, 2015). Os mecanismos pré-zigóticos na pós-polinização incluem a heteroespecificidade do pólen (interações polén-pistilo, polén-óvulo e tamanho do tubo polínico) que reduz a chance do óvulo ser alcançado em comparação com pólen conespecífico, impedindo ou reduzindo as taxas de germinação interespecíficas (Tiffin *et al.*, 2001; Baack *et al.*, 2015). Já os mecanismos pós-zigóticos incluem a inviabilidade, esterilidade do híbrido, e a falha ou redução da reprodução bem sucedida nas gerações seguintes, causado por incompatibilidades genéticas ou baixa aptidão em habitats das espécies parentais (Tiffin *et al.*, 2001; Rieseberg e Willis, 2007; Goodwillie e Ness, 2013). O isolamento reprodutivo, normalmente, envolve múltiplas fases de vida das plantas, e na maioria dos casos, uma variedade de barreiras reprodutivas contribui para o isolamento total ou parcial (Rieseberg e Willis, 2007; Cozzolino e Scopece, 2008; Lowry *et al*., 2008).

O estudo experimental das barreiras reprodutivas é de extrema importância para decifrar o papel do fluxo gênico interespecífico na especiação em Bromeliaceae (Palma-Silva *et al*., 2016). Em um estudo realizado com 42 espécies de bromélias simpátricas da Estação Biológica Santa Lúcia (ES) na Mata Atlântica, foi observada uma variada sobreposição dos períodos de floração (de 26 a 62%) e o compartilhamento de visitantes florais (principalmente beija-flores) sugerindo a falta de barreiras pré-polinização (mecanismos pré-zigóticos) nestas espécies (Wendt *et al*., 2008). Já em outro estudo, na mesma localidade, foi realizado o cruzamento e a análise de crescimento do tubo polínico em 13 espécies de bromélias (Matallana *et al*., 2016). Os autores observaram a rejeição de pólen heteroespefíco em 73,5% dos casos, confirmando a existência de mecanismos pré-zigóticos (pós-polinização), contudo a penetração do óvulo foi observada nos outros 26,5%, sugerindo que os mecanismos pré-zigóticos (pós-polinização) podem também ser ineficientes e levar a formação de híbridos naturais. Apesar da formação de híbridos ser observada, a divergência dos sistemas de reprodução observada entre as espécies *V. scalaris* E.Morren. (predominantemente autocompatível) e *V. simplex* (hercogâmica) pode funcionar como uma barreira pré-zigótica (pré-polinização) permitindo a coexistência e a manutenção destas espécies quando em simpatria (Neri *et al*., 2017a). Os mesmos autores também observaram uma baixa viabilidade das sementes F1 em cruzamentos artificiais, sugerindo a importância das barreiras pós-polinização (pós-zigóticas).

Uma vez que o isolamento reprodutivo entre espécies não esteja completo e haja permeabilidade das barreiras reprodutivas, a hibridação pode ocorrer (Lowry *et al*., 2008). Diferentes consequências evolutivas podem ser observadas a partir do processo de hibridação, como a criação de novas linhagens, podendo ocorrer no mesmo nível de ploidia (homoploidia) ou pela duplicação do genoma (alopoliploidia), manutenção ou aumento da diversidade genética intraespecífica, origem e transferência de adaptações genéticas, reforço ou a quebra de barreiras reprodutivas, e mesmo a extinção de populações ou espécies (Rieseberg, 1997; Abbott *et al*., 2013; Todesco *et al*., 2016; Runemark *et al*., 2019). A hibridação pode ocorrer em diferentes contextos espaciais e temporais, como por exemplo, contato secundário após um período de evolução independente (formação de zonas híbridas em limites parapátricos abruptos) ou contato contínuo com seleção divergente (troca de genes entre populações localmente adaptadas), além de poder ocorrer em um complexo mosaico de habitats, combinando características de zonas híbridas com aquelas de adaptação local (Abbott *et al*., 2013). Neste sentido, a perturbação do habitat, causada por ações antrópicas, é um dos fatores que ajudam a promover a hibridação entre espécies, proporcionando contato entre populações que até então estavam isoladas (Wiegand 1935; Lamont *et al*., 2003; Gilman e Behm, 2011; Harrison e Larson, 2014).

Casos de hibridação natural já foram observados em diferentes gêneros de bromélias, como *Pticairnia* L'Hér. (Wendt *et al* 2000; 2001), *Tillandsia* L. (Gardner, 1984; Gonçalves e Azevêdo-Gonçalves, 2009) *Puya* Molina (Schulte *et al*., 2010) *Dyckia* Schult. & Schult.f. (Hirsch *et al*., 2020) e *Vriesea*. (Matos *et al*., 2016; Zanella *et al*., 2016; Neri *et al*., 2017b). Muitas vezes, espécies irmãs ocorrem na mesma região, compartilhando polinizadores (Wendt *et al*., 2008; Matallana *et al*., 2016), e, sendo Bromeliaceae um exemplo de radiação adaptativa recente (Givnish *et al*., 2007; 2011), muitas linhagens ainda não estão completamente isoladas reprodutivamente, o que pode permitir a ocorrência de hibridação (Palma-Silva *et al*., 2016).

1.5 Objetivos

A família Bromeliaceae abriga diversos complexos de espécies que divergiram recentemente e que ocupam biomas neotropicais que carecem de estudos para a melhor compreensão da sua história evolutiva. Nesse contexto, esta tese tem como objetivos gerais utilizar espécies do gênero *Vriesea* pertencentes ao grupo das infladas para melhor compreender o grau de isolamento reprodutivo entre elas, bem como colaborar com a compreensão dos padrões históricos envolvidos na diversificação das espécies e do bioma Mata Atlântica.

1.5.1 Objetivos Específicos

- Analisar os padrões de fluxo gênico e a história demográfica de *V. incurvata* ao longo de toda sua distribuição (região sul da Mata Atlântica) utilizando marcadores plastidiais e nucleares;

- Estimar o fluxo gênico interespecífico, bem como a estruturação populacional através de marcadores nucleares do tipo microssatélite entre espécies simpátricas em três localidades da Mata Atlântica: Parque Estadual da Serra do Mar – Núcleo Santa Virgínia-SP (*V. carinata*; *V. taritubensis* var. *patens*; *V. simplex* e *V. agostiniana*); Estação Biológica da Boraceia - SP (*V. inflata* e *V. incurvata*); e Estrada Dona Francisca em Joinville – SC (*V. carinata* e *V. incurvata*);

- Analisar características morfológicas das espécies para identificar as diferenças entre estas dentro de cada localidade, assim como para com os supostos híbridos;

- Inferir o quanto as barreiras reprodutivas estão sendo eficientes entre as espécies de *Vriesea* do grupo das infladas nas localidades analisadas.

CAPÍTULO II

Ecological niche modeling and a lack of phylogeographic structure in *Vriesea incurvata* suggest historically stable areas in the southern Atlantic Forest

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RESEARCH ARTICLE

Ecological niche modeling and a lack of phylogeographic structure in *Vriesea incurvata* suggest historically stable areas in the southern Atlantic Forest

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PREMISE: Despite the efforts to understand the processes that shape neotropical biodiversity, the complexity of certain biomes, such as the Atlantic Forest (AF), prevents the generalization of patterns. Initially, ecological niche modeling (ENM), with phylogeographic studies, identified past stable areas in the central and northern portions of the AF, while the southern portion was thought to be highly fragmented. Here, we examined the phylogeography, historical patterns, genetic diversity, and population structure of *Vriesea incurvata*, an endemic species of the southern portion of the AF.

METHODS: We evaluated 149 individuals using two plastid DNA regions (cpDNA) and 13 nuclear microsatellite markers (nuSSRs) to access the historical patterns, genetic diversity, and structure of *V. incurvata* populations. We also conducted historical demography and ENM analyses.

RESULTS: We found moderate to high genetic diversity and low population structure for both genomes. The cpDNA network revealed high haplotype sharing. The ENM suggested no drastic changes in suitable areas for *V. incurvata* occurrence, corroborating the finding of no phylogeographic structure.

CONCLUSIONS: Contrary to some studies, our results indicate that the southern AF was a historically stable climate region for *V. incurvata* occupation after southward colonization by the species. Past climatic changes probably did not cause structuring among its populations.

KEY WORDS Bromeliaceae; cpDNA; genetic diversity; historical patterns; neotropics; nuSSR; phylogeography; *Vriesea*.

The Atlantic Forest (AF) covers the eastern slopes of a huge mountain chain along the coastline from southern to northeastern Brazil, is the third largest biodiversity hotspot of the world, with approximately 20,000 plant species, of which 8000 are endemic (Morellato and Haddad, 2000; Myers et al., 2000; Ribeiro et al., 2011). It holds a considerable proportion of South America's biodiversity, but, currently, only 7.5% of its primary vegetation remains, and it continues to suffer from significant anthropogenic impacts (Myers et al., 2000; Ribeiro et al., 2009). The increasing fragmentation of the AF has propelled research and proposals to mitigate the effects of devastation (Morellato and Haddad, 2000). However, the design of efficient strategies requires knowledge of current biodiversity patterns and, more importantly, a better understanding of its evolutionary history (Álvarez-Presas et al., 2014). Much effort has been put into understanding the complex and high levels of biodiversity in the AF through phylogeographic, biogeographic, and paleomodeling studies, which may provide valuable insights into the historical processes underlying diversification in this region (Martins, 2011; Turchetto-Zolet et al., 2013; Leal et al., 2016; Antonelli et al., 2018; Pinheiro et al., 2018). Nevertheless, neotropical biodiversity data present numerous uncertainties, which hamper the generalization of patterns (Antonelli et al., 2018). Thus, further contributions in the area of phylogeography and biogeography of AF organisms are very important to fill this gap.

Palynological and paleodistribution modeling studies have suggested that AF was severely affected by climate changes during

the Pleistocene (Behling and Negrelle, 2001; Ledru et al., 2005; Carnaval and Moritz, 2008), which makes this a suitable region for studying the phylogeography of species endemic to this region (Cabanne et al., 2016). Initially, ecological niche models, together with phylogeographic studies, identified favorable environments for the occurrence of different species (stable forest areas) that seem to be important drivers of biodiversity in the northern portion of AF, whereas the southern portion of the forest would have been heavily fragmented by the spread of grassland vegetation (Carnaval and Moritz, 2008; Carnaval et al., 2009; Martins et al., 2009; Palma-Silva et al., 2009; d'Horta et al., 2011). However, further studies proposed that, during glaciations, the southern portion of the AF was not as severely impacted as previously suggested (Thomé et al., 2010; Brunes et al., 2015; Peres et al., 2015; Bünger et al., 2016; Cabanne et al., 2016; Costa et al., 2018). Therefore, the distinct patterns recovered for the southern and northern portions of the AF are still a subject of debate. In this sense, studies on taxa restricted to each region may be important to unveil the specific diversification dynamics found in each area and consequently develop a more complete scenario of processes responsible for the biodiversity of the biome as a whole.

The Bromeliaceae, comprising 3590 species and 75 genera, is one of the most morphologically and ecologically diverse of the flowering plant families native to the tropics and subtropics of the New World (Gouda et al., 2019). These taxa have an incredible diversity of adaptations and have been able to fill numerous niches (Givnish et al., 2011; Zanella et al., 2012). Phylogeographic studies on bromeliad species have been important in elucidating evolutionary processes in different biomes, such as the Caatinga, Cerrado, and AF (Palma-Silva et al., 2009; Goetze et al., 2016, 2018; Gonçalves-Oliveira et al., 2017; Leal et al., 2018). *Vriesea* Lindl. (subfamily Tillandsioideae) is one of the most diverse genera in the Bromeliaceae, with 226 species described (Costa et al., 2015; Gouda et al., 2019). Approximately 74% of all *Vriesea* species occur in the AF, the main diversity center of the genus (Costa et al., 2015), and are very representative of this biome.

The *Vriesea incurvata* complex is a monophyletic group distributed in the AF between approximately 22°S and 29°S (Rio Grande do Sul to Rio de Janeiro Brazilian States); it includes the morphologically similar species *V. incurvata* Gaudich, *V. sucrei* L.B.Sm. and Read, and *V. taritubensis* E.Pereira I.A.Penna (with three varieties) (Neves et al., 2018). *Vriesea incurvata* was first described as occurring throughout the distribution area of the entire complex (Smith and Downs, 1977; Martinelli et al., 2008), but a recent taxonomic study indicated that populations farther north (north São Paulo and Rio de Janeiro Brazilian states) are in fact a different taxon assigned as *V. taritubensis* (Neves et al., 2018). B. Neves et al. (unpublished manuscript) concluded, based on analyses of chloroplast regions, that *V. taritubensis* and *V. incurvata* likely diverged approximately 4.5 Ma (millions of years ago) during the late Quaternary (Pliocene) period and started to diversify in the region of the Bocaina Mountain range (22°68′S; 44°31′W) toward both extremities of AF, with *V. incurvata* colonizing strictly the southern portion of the forest.

The aim of the present study was to examine the phylogeographic and demographic historical patterns and the genetic diversity of *V. incurvata*, endemic to the southern portion of AF. Specifically, we addressed the following questions: (1) What are the patterns of gene flow and the historical demography for this species? (2) Are the observed patterns consistent with other studied taxa from the AF? (3) How is the genetic diversity of *V. incurvata* distributed across the AF? (4) Is there population structure in this species? If yes, what are the patterns? The results will be discussed in the context of both historical and contemporary gene flow.

MATERIALS AND METHODS

Study species

Vriesea incurvata is an epiphytic plant with simple inflorescences with red floral bracts and yellow flowers (Martinelli, 1994; Neves et al., 2018). Anthesis is diurnal, it blooms all year, with peak flowering from January to March (Machado and Semir, 2006; Neves et al., 2018). It is self-compatible with high fruiting success in flowers from cross-pollination (Martinelli, 1994). *Vriesea incurvata* inhabits humid places near waterfalls and streams, and the color of the flower bracts is the most important attractant for its pollinators, such as the hummingbird *Phaethornis eurynome* Lesson (Martinelli, 1994; Machado and Semir, 2006; Neves et al., 2018). The seeds have plumose appendages, which facilitate long-distance dispersal (Smith and Downs, 1977; Magalhães and Mariath, 2012).

Population sampling and DNA isolation

A total of 149 individuals of *V. incurvata* were sampled in nine localities across most of the species' geographic distribution corresponding to the south portion of the AF (Fig. 1A). The specifications and identification codes of each locality and the number of individuals collected from each site are summarized in Table 1. The voucher specifications are presented in Appendix 1. Fresh leaves were collected and stored in silica gel for drying, and total genomic DNA was isolated using the cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1990).

Molecular markers, sequencing, and genotyping

We used plastid DNA regions (cpDNA) and nuclear simple sequence repeats (nuSSRs) to investigate genetic diversity and population structure of *V. incurvata*. Two cpDNA regions, the *trnL-trnF* spacer and the *matK* gene, were chosen based on the extent of their polymorphism and were amplified and sequenced from 52 individuals from all nine localities sampled (Table 2). Polymerase chain reaction (PCR) and sequencing strategies for *trnL-trnF* and *matK* followed the protocol of Zanella et al. (2016). PCR amplifications were performed in a Veriti 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA), and the products were sequenced from both ends using the BigDye Kit (Applied Biosystems) at Macrogen Inc. (South Korea). All sequences were visually checked in the Chromas 2.33 sequence viewer (Chromas Technelysium, South Brisbane, Australia) and edited to obtain the consensus sequence using the tool Muscle (Edgar, 2004) implemented in MEGA version 5.2 (Tamura et al., 2011). *Vriesea incurvata* sequences generated in this study were deposited in GenBank (accessions KT346053 to KT346104 for *trnL-trnF*, KT346177 to KT346228 for *matK*). Polymorphisms at mononucleotide microsatellites were excluded due to ambiguous alignment and higher mutation rates. For statistical analyses, the sequences of the two plastid regions were concatenated.

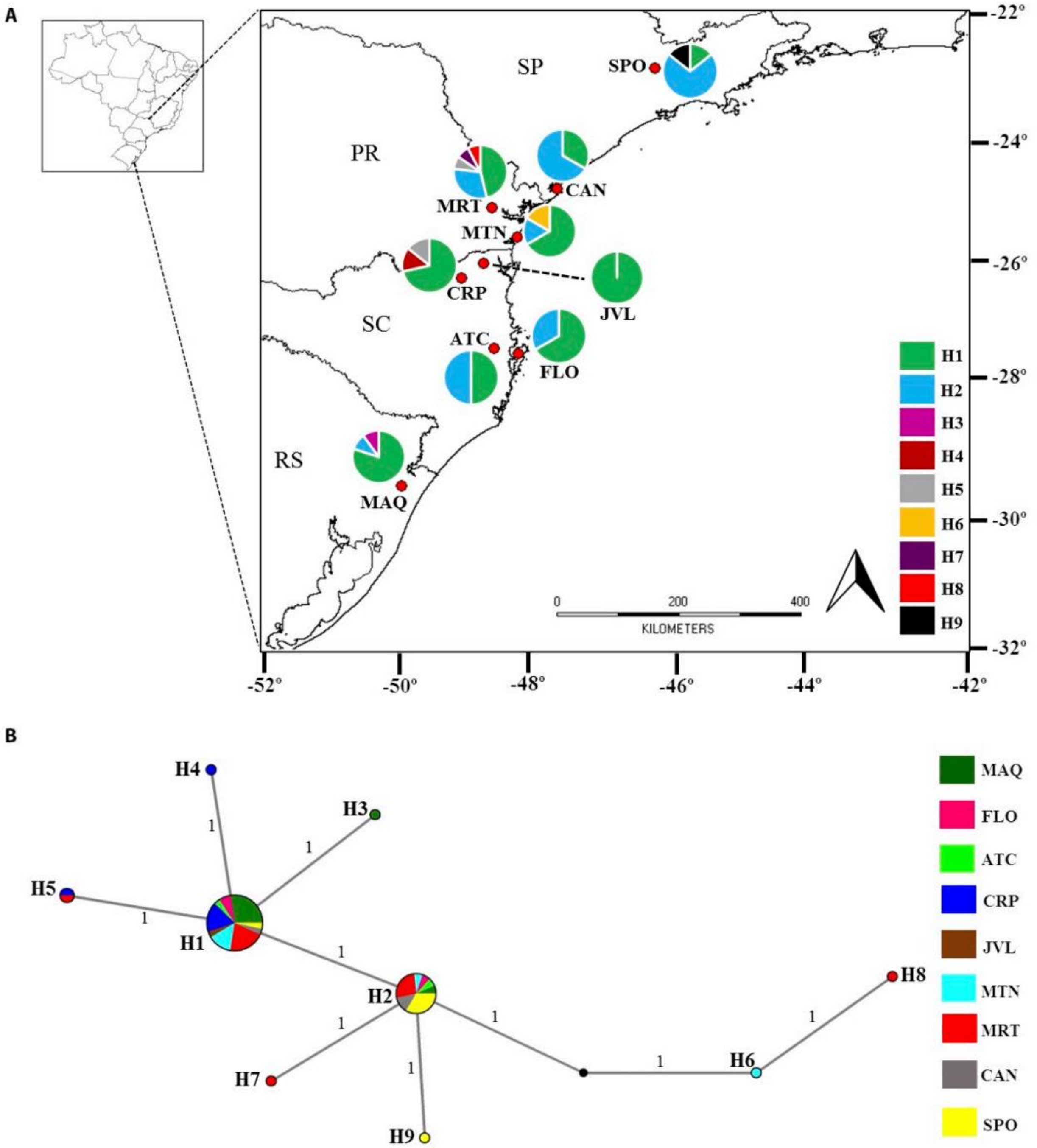

FIGURE 1. (A) Geographic distribution of nine haplotypes recovered from cpDNA (*matK* + *trnL-trnF*) and identified in nine *Vriesea incurvata* localities sampled in the Atlantic Forest. (B) Median-joining network showing the genealogical relationship for nine cpDNA haplotypes identified in *V. incurvata*. Each circle represents one haplotype, with diameter proportional to frequency and colors representing localities with the haplotype (see key on right for locality abbreviations, which are defined in Table 1).

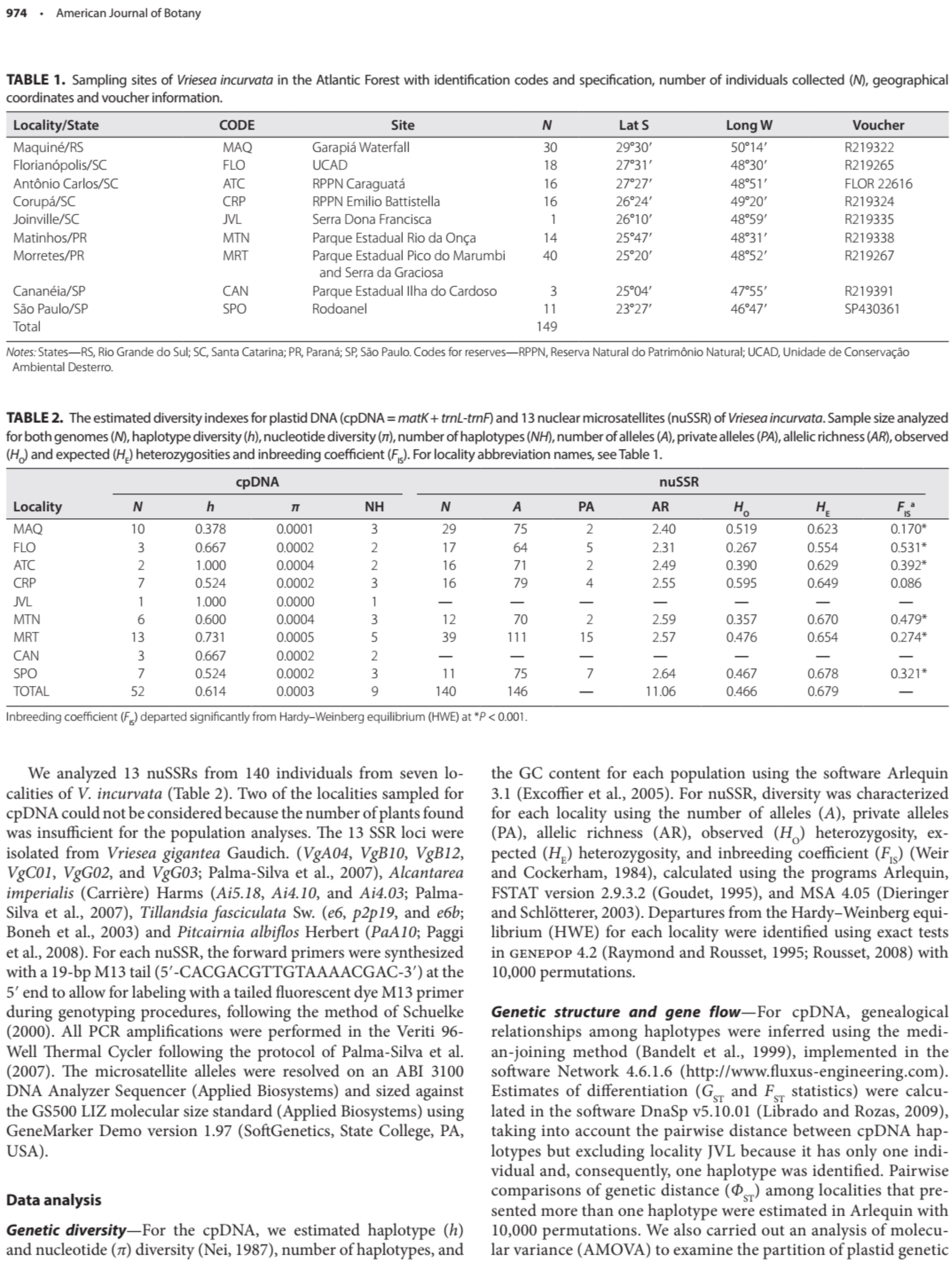

TABLE 1. Sampling sites of *Vriesea incurvata* in the Atlantic Forest with identification codes and specification, number of individuals collected (*N*), geographical coordinates and voucher information.

Locality/State	CODE	Site	*N*	Lat S	Long W	Voucher
Maquiné/RS	MAQ	Garapiá Waterfall	30	29°30′	50°14′	R219322
Florianópolis/SC	FLO	UCAD	18	27°31′	48°30′	R219265
Antônio Carlos/SC	ATC	RPPN Caraguatá	16	27°27′	48°51′	FLOR 22616
Corupá/SC	CRP	RPPN Emilio Battistella	16	26°24′	49°20′	R219324
Joinville/SC	JVL	Serra Dona Francisca	1	26°10′	48°59′	R219335
Matinhos/PR	MTN	Parque Estadual Rio da Onça	14	25°47′	48°31′	R219338
Morretes/PR	MRT	Parque Estadual Pico do Marumbi and Serra da Graciosa	40	25°20′	48°52′	R219267
Cananéia/SP	CAN	Parque Estadual Ilha do Cardoso	3	25°04′	47°55′	R219391
São Paulo/SP	SPO	Rodoanel	11	23°27′	46°47′	SP430361
Total			149			

Notes: States—RS, Rio Grande do Sul; SC, Santa Catarina; PR, Paraná; SP, São Paulo. Codes for reserves—RPPN, Reserva Natural do Patrimônio Natural; UCAD, Unidade de Conservação Ambiental Desterro.

TABLE 2. The estimated diversity indexes for plastid DNA (cpDNA = *matK* + *trnL-trnF*) and 13 nuclear microsatellites (nuSSR) of *Vriesea incurvata*. Sample size analyzed for both genomes (*N*), haplotype diversity (*h*), nucleotide diversity (*π*), number of haplotypes (*NH*), number of alleles (*A*), private alleles (*PA*), allelic richness (*AR*), observed (H_O) and expected (H_E) heterozygosities and inbreeding coefficient (F_{IS}). For locality abbreviation names, see Table 1.

	cpDNA				nuSSR						
Locality	*N*	*h*	*π*	NH	*N*	*A*	PA	AR	H_O	H_E	$F_{IS}{}^a$
MAQ	10	0.378	0.0001	3	29	75	2	2.40	0.519	0.623	0.170*
FLO	3	0.667	0.0002	2	17	64	5	2.31	0.267	0.554	0.531*
ATC	2	1.000	0.0004	2	16	71	2	2.49	0.390	0.629	0.392*
CRP	7	0.524	0.0002	3	16	79	4	2.55	0.595	0.649	0.086
JVL	1	1.000	0.0000	1	—	—	—	—	—	—	—
MTN	6	0.600	0.0004	3	12	70	2	2.59	0.357	0.670	0.479*
MRT	13	0.731	0.0005	5	39	111	15	2.57	0.476	0.654	0.274*
CAN	3	0.667	0.0002	2	—	—	—	—	—	—	—
SPO	7	0.524	0.0002	3	11	75	7	2.64	0.467	0.678	0.321*
TOTAL	52	0.614	0.0003	9	140	146	—	11.06	0.466	0.679	—

Inbreeding coefficient (F_{IS}) departed significantly from Hardy–Weinberg equilibrium (HWE) at $^*P < 0.001$.

We analyzed 13 nuSSRs from 140 individuals from seven localities of *V. incurvata* (Table 2). Two of the localities sampled for cpDNA could not be considered because the number of plants found was insufficient for the population analyses. The 13 SSR loci were isolated from *Vriesea gigantea* Gaudich. (*VgA04*, *VgB10*, *VgB12*, *VgC01*, *VgG02*, and *VgG03*; Palma-Silva et al., 2007), *Alcantarea imperialis* (Carrière) Harms (*Ai5.18*, *Ai4.10*, and *Ai4.03*; Palma-Silva et al., 2007), *Tillandsia fasciculata* Sw. (*e6*, *p2p19*, and *e6b*; Boneh et al., 2003) and *Pitcairnia albiflos* Herbert (*PaA10*; Paggi et al., 2008). For each nuSSR, the forward primers were synthesized with a 19-bp M13 tail (5′-CACGACGTTGTAAAACGAC-3′) at the 5′ end to allow for labeling with a tailed fluorescent dye M13 primer during genotyping procedures, following the method of Schuelke (2000). All PCR amplifications were performed in the Veriti 96-Well Thermal Cycler following the protocol of Palma-Silva et al. (2007). The microsatellite alleles were resolved on an ABI 3100 DNA Analyzer Sequencer (Applied Biosystems) and sized against the GS500 LIZ molecular size standard (Applied Biosystems) using GeneMarker Demo version 1.97 (SoftGenetics, State College, PA, USA).

Data analysis

Genetic diversity—For the cpDNA, we estimated haplotype (*h*) and nucleotide (*π*) diversity (Nei, 1987), number of haplotypes, and the GC content for each population using the software Arlequin 3.1 (Excoffier et al., 2005). For nuSSR, diversity was characterized for each locality using the number of alleles (*A*), private alleles (PA), allelic richness (AR), observed (H_O) heterozygosity, expected (H_E) heterozygosity, and inbreeding coefficient (F_{IS}) (Weir and Cockerham, 1984), calculated using the programs Arlequin, FSTAT version 2.9.3.2 (Goudet, 1995), and MSA 4.05 (Dieringer and Schlötterer, 2003). Departures from the Hardy–Weinberg equilibrium (HWE) for each locality were identified using exact tests in GENEPOP 4.2 (Raymond and Rousset, 1995; Rousset, 2008) with 10,000 permutations.

Genetic structure and gene flow—For cpDNA, genealogical relationships among haplotypes were inferred using the median-joining method (Bandelt et al., 1999), implemented in the software Network 4.6.1.6 (http://www.fluxus-engineering.com). Estimates of differentiation (G_{ST} and F_{ST} statistics) were calculated in the software DnaSp v5.10.01 (Librado and Rozas, 2009), taking into account the pairwise distance between cpDNA haplotypes but excluding locality JVL because it has only one individual and, consequently, one haplotype was identified. Pairwise comparisons of genetic distance (Φ_{ST}) among localities that presented more than one haplotype were estimated in Arlequin with 10,000 permutations. We also carried out an analysis of molecular variance (AMOVA) to examine the partition of plastid genetic diversity within and among localities using Arlequin with 10,000 permutations.

For nuSSR, we assessed nuclear genetic differentiation using estimates of F_{ST} (Weir and Cockerham, 1984), the unbiased estimator of relative differentiation G_{ST} (Nei and Chesses, 1983), and the standardized genetic differentiation measure G'_{ST} (Hedrick, 2005) calculated using FSTAT. Pairwise comparisons of F_{ST} between localities were estimated with 10,000 permutations for each of the seven localities using the software Arlequin. AMOVA was carried out to examine the partition of nuclear genetic diversity within and among localities in Arlequin with 10,000 permutations. To investigate the occurrence of population structure, we performed a Bayesian clustering algorithm implemented in STRUCTURE version 2.3.4 (Pritchard et al., 2000). For each K (from 1 to 10), we performed 10 replicates, using the admixture model, assuming independent allele frequencies and using a burn-in period of 500,000 and a run length of 1,000,000 to confirm the stabilization of summary statistics (Pritchard et al., 2000). We determined the most likely number of populations, K, by using the method ΔK described by Evanno et al. (2005), implemented in Structure Harvester 0.6.94 (Earl and vonHoldt, 2012). We also estimated the optimal number of clusters using the silhouette method of the PAM algorithm using the factoextra package in R version 3.5.1 (R Core Team, 2018). We did a discriminant analysis of principal components (DAPC) (Jombart et al., 2010) implemented in the R package adegenet version 2.1.0 (Jombart, 2008; Jombart and Ahmed, 2011) to identify population structure.

To estimate the relative contribution of pollen versus seed flow to total gene flow, we compared G_{ST} values from biparentally inherited nuSSRs with those from uniparentally inherited plastid markers according to the equation proposed by Petit et al. (2005).

The hypothesis that individuals from each locality are differentiated due to isolation by distance was tested by calculating the correlation between geographic and genetic distance matrices (F_{ST}) with a standardized Mantel test using GENEPOP. The significance was assessed through a randomization test using 10,000 Monte Carlo simulations. Pairwise effective migration rates ($N_e m$) were estimated among localities, following a coalescent theory and maximum-likelihood-based approach using Migrate 3.0.3 software (Beerl and Felsenstein, 1999). The computations were carried out under a Brownian motion model, and mutation rates (μ) among the loci were estimated from the data.

Demographic analyses—For cpDNA data, neutrality tests were performed using Tajima's D (Tajima, 1989), considering the segregating sites and Fu's F_S (Fu, 1997), based on the haplotype distribution for all localities with the exception of JVL (only included in the total of individuals). Tests were carried out with 10,000 simulation steps using Arlequin, considering all individuals in the eight localities with more than one haplotype. Bayesian skyline plot (BSP) analysis (Drummond et al., 2005) was carried out for the total number of individuals ($n = 52$) using Beast version 1.7.5 (Drummond et al., 2012) with the following prior conditions: lognormal relaxed clock (uncorrelated), with a substitution rate previously estimated for cpDNA in the subfamily Bromelioideae (cpDNA: $7.64 \times 10^{-4} \pm 4.5 \times 10^{-6}$; D. Silvestro, University of Gothenburg, personal communication). The HKY + I nucleotide substitution model was used. Substitution models were selected based on the Akaike information criterion (AIC) implemented in the program jModelTest 2.1.3 (Darriba et al., 2012). Markov chains were run for 50,000,000 steps, with sampling performed every 1000 steps. The BSP computation and convergence checks were completed in the program Tracer 1.6 (available at http://beast.bio.ed.ac.uk/Tracer). An effective sample size (ESS) of more than 200 was used as a threshold (Drummond and Rambaut, 2007).

For nuSSR data, a heterozygosity excess test was used to test for genetic bottlenecks using the software BOTTLENECK 1.2.02 (Piry et al., 1999) and the parameters described by Goetze et al. (2018).

Time of divergence—The time of the cpDNA haplotype divergence was estimated using a Bayesian approach implemented in the software Beast, using *Alcantarea imperialis* (GenBank accession AY614032.1 [*matK*] and AY614276.1 [*trnL-trnF*]) and *Catopsis juncifolia* Mez and Wercklé (GenBank accession AY614027.1 [*matK*] and AY614271.1 [*trnL-trnF*]) as an outgroup. Priors used included the birth–death speciation model and the lognormal relaxed clock (uncorrelated). The same substitution rate and substitution model was used as in the BSP analysis (see above). Markov chains were run for 30,000,000 steps, with sampling done every 1000 steps. The results were viewed in Tracer to check for convergence to a stationary distribution and for an ESS of more than 200 (Drummond and Rambaut, 2007). TreeAnnotator 1.7.5, part of the Beast software package, was used to summarize the trees, and the statistical support for all branches was measured as Bayesian posterior probability (PP). The software FigTree 1.4.2 was used to draw the tree (Rambaut, 2014).

Ecological niche modeling—To infer suitable regions of *V. incurvata* in the late Quaternary period, we carried out ecological niche modeling (ENM) for current species distribution under two time periods (mid-Holocene [MH]) at 6000 years before the present (yBP) and Last Glacial Maximum (LGM; at 21,000 yBP), using a model of maximum entropy implemented in Maxent 3.4.1 (Phillips et al., 2006). We employed all of the collection sites georeferenced by Neves et al. (2018) using herbarium records and existing databases (i.e., GBIF, SpeciesLink, and Jabot). We removed double records and localities nearby, resulting in 64 records that matched the species distribution (Appendix S1). Of these, eight records (except for the SPO) correspond to the populations of this study.

The distribution models were developed using a set of 19 bioclimatic RASTER layers, with a 2.5-arc-min resolution (4.5 km at the equator) (Hijmans et al., 2005) derived from the CCSM4 (Community Climate System Model maintained by National Center for Atmospheric Research, USA) general circulation model (GCM). The RASTER layers for present and past climatic periods are hosted by the WorldClim website version 1.4 (Hijmans et al., 2005). The grid layers were cut to include most of the area of AF and the entire geographical distribution for *V. incurvata* and extracted through the RASTER package (Hijmans, 2017) implemented in R. We first ran the analysis in Maxent with all of its bioclimatic variables (19). Pearson's correlation between the variables was calculated using the RASTER package. We selected bioclimatic variables with low correlation ($R \leq 0.75$) and that presented a high percentage of importance to the model in a preliminary run (Nakazato et al., 2010; Segatto et al., 2017). We used cross-validation to evaluate the species' distribution model, using 10 replicates. All other parameters were set to the default settings. Cross-validation modeling is recommended for small data sets, as in the case of *V. incurvata* (Phillips, 2017). To analyze the models, we checked the area under the receiver operating characteristic curve (AUC) in Maxent results, indicating one reasonably well performance of above 0.95 (Nakazato et al., 2010). The final figures were edited in QGIS 3.2.1 Bonn (Qgis Development Team, 2018).

RESULTS

Genetic diversity

The two regions of cpDNA were concatenated, resulting in a length of 2716 bp and GC content of 32.65%. Seven polymorphic sites were observed (four transitions, two transversions, and one indel), leading to nine haplotypes for the 52 analyzed individuals. The haplotype diversity ranged from 0.378 to 1.000, while nucleotide diversity varied from 0.0001 to 0.0005 (Table 2). The number of haplotypes varied from one to five per locality sampled (Table 2).

The 13 nuSSR markers were polymorphic in the samples from seven localities evaluated and included a total of 146 alleles (Table 2). The number of alleles ranged from 64 (FLO) to 111 (MRT) in each locality. Allelic richness varied from 2.31 (FLO) to 2.64 (SPO). Three localities had two private alleles each (MQN, ATC, and MTN). Localities FLO, CRP, and SPO had five, four, and seven private alleles, respectively, and MRT had 15 (Table 2). The observed and expected heterozygosities per locality ranged from 0.267 to 0.595 and from 0.554 to 0.678, respectively (Table 2). Inbreeding coefficients (F_{IS}) departed significantly from HWE for all the localities analyzed, except for CRP (Table 2).

Population structure and gene flow

The cpDNA network revealed nine haplotypes separated from each other by one mutational step, except for two steps from H2 to H6 (Fig. 1B). The two most frequent haplotypes, H1 and H2, were shared among almost all localities along the geographic distribution of *V. incurvata* (Fig. 1A). Haplotype H5 was shared between CRP and MRT. The locality MRT showed two private haplotypes (H7 and H8). Haplotypes H3, H4, H6, and H9 appeared exclusively in MAQ, CRP, MTN, and SPO, respectively. The genetic differentiation results from the cpDNA were F_{ST} = 0.017 and G_{ST} = 0.052. The pairwise Φ_{ST} estimates were low or nonsignificant between most localities, except between MAQ, CRP, and MTN when compared with SPO ($P > 0.05$) (Appendix S2). AMOVA revealed that most of the genetic variation at cpDNA was within localities (92.25%) and the F_{ST} was 0.078 and nonsignificant ($P = 0.118$) (Table 3).

The genetic differentiation results from the nuSSR were F_{ST} = 0.051, G_{ST} = 0.050, and G'_{ST} = 0.058, suggesting low genetic structure. AMOVA revealed more variation within localities (95.24%) than among them (4.76%): F_{ST} = 0.048 and was significant ($P <$ 0.001) (Table 3). The Evanno method based on structure analyses indicated that two is the most likely number of clusters, corroborating PAM algorithm analysis (Appendix S3). In general, the sampled localities showed a high degree of admixture, with the exception of MAQ (cluster 2) and MTN/SPO (cluster 1) (Fig. 2). The DAPC analysis corroborated the genetic groups found by Bayesian analysis but with a clearer structuring and identified three genetic groups (Fig. 3). The pairwise F_{ST} estimates varied from 0.004 (between MAQ and CRP and between CRP and MRT) to 0.112 (between SPO and FLO), with most of the comparisons being highly significant ($P < 0.001$) (Appendix S2). As expected, due to the similar values of G_{ST} between the markers, the ratio of pollen flow to seed flow estimated according to Petit et al. (2005) was close to zero (−0.66). The Mantel test results indicated isolation by distance among localities ($r^2 = 0.3021$; $P < 0.05$). Additionally, the maximum-likelihood-based estimates of effective migration rates ($N_e m$) were high, ranging from 0.506 (MAQ to SPO) to 3.964 (MRT to MTN) (Appendix S4).

TABLE 3. Analyses of molecular variance (AMOVA) based on cpDNA (*matK* + *trnL-trnF*) and 13 nuclear microsatellites (nuSSRs).

Source of variation	Percentage of variation	F_{ST}
cpDNA		
Among localities	7.75	0.078
Within localities	92.25	
nuSSR		
Among localities	4.76	0.048*
Within localities	95.24	

Notes: F_{ST}, fixation index; *$P < 0.001$.

Demographic history

For cpDNA, the results for Tajima's *D* and Fu's F_S were not significant for any of the localities tested, suggesting demographic stability (Appendix S5). However, when all individuals were considered, Fu's F_S indicated population expansion. The BSP analyses indicated a slight reduction in population size, followed by a more recent expansion (around 0.1 Ma), although this result should be interpreted with caution because the size of the estimated confidence limits does not indicate statistical significance (Appendix S6). BOTTLENECK analyses with nuSSRs did not detect a significant excess of heterozygosity; therefore, no reductions in population sizes through time were found (data not shown).

Haplotype divergence time

The divergence of the cpDNA haplotypes of *V. incurvata* started at approximately 2.04 Ma (95% highest posterior density: 0.49–5.73 Ma), in the early Pleistocene, but most of the actual haplotypes diversified in the last 1 Ma (Fig. 4).

Ecological niche modeling

The AUC values indicated a good predictive model performance of 0.985 for all analyzed periods (present, MH, and LGM). The most important bioclimatic variables with a lower correlation between them were minimum temperature of coldest month, mean temperature of warmest quarter, annual precipitation, precipitation of driest month, precipitation seasonality (coefficient of variation), and precipitation of coldest quarter. The respective percentages of contribution of these bioclimatic variables for each period (present, MH, and LGM), according to the Maxent results, are presented in Appendix S7. The species' known distribution ranges in the AF (from approximately 23°S to 29°S from SP to RS of the Brazilian Federative States; Fig. 5A) coincide with the most suitable areas for the present time (Fig. 5B). During the MH (Fig. 5C), the suitable areas were very similar to those observed in the present period, as the climate was probably similar. The suitable areas predicted for *V. incurvata* were slightly larger under the LGM conditions (Fig. 5D), when sea levels were lower as compared with during the other two periods. Interestingly, the LGM distribution model revealed some points of suitable areas where no records of

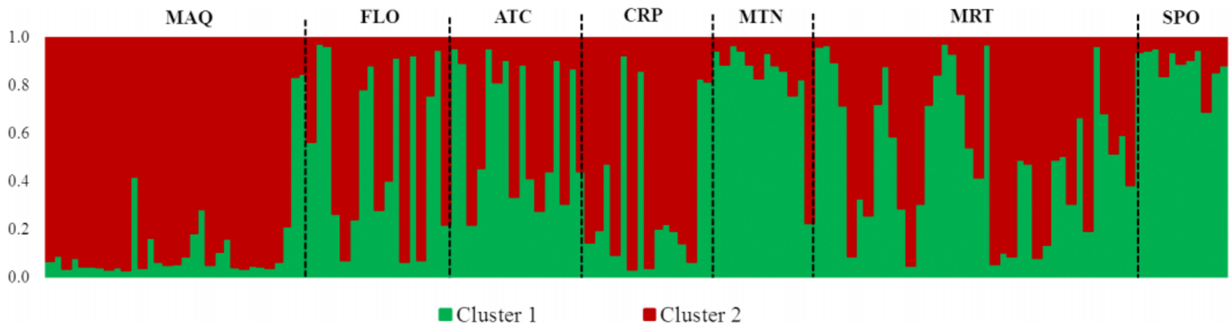

FIGURE 2. Population structure in *Vriesea incurvata* using Bayesian assignment analysis for a $K = 2$ population model based on 13 nuSSR loci. Cluster 1 corresponds to southeast populations and cluster 2 to southern populations. For locality abbreviation names, see Table 1.

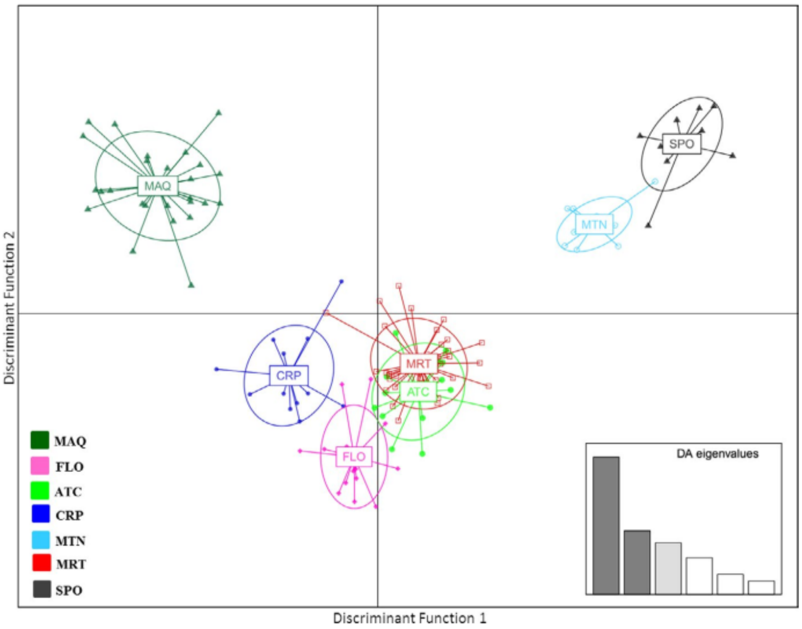

FIGURE 3. Discriminant analysis using principal components (DAPC) of the multilocus microsatellite genotypes of seven localities sampled for *Vriesea incurvata*; each locality is coded in a different color. For locality abbreviation names, see Table 1.

V. incurvata exist, such as in the west of Paraná Brazilian State, near Paraguay. The same was observed for the present and MH times.

DISCUSSION

Stability of the southern portion of the Atlantic Forest and lack of phylogeographic structure in *Vriesea incurvata*

The ancestor of the core tillandsioid lineages, from which the genus *Vriesea* derives, probably arose in the Andes approximately 14.2 Ma. After that, the modern genera started to diverge from each other at approximately 8.7 Ma, expanding to Central America, the northern littoral area of South America, and the Caribbean (Givnish et al., 2011). The expansion and diversification of the *Vriesea* lineages throughout eastern South America are evident, as the species are distributed in almost every ecosystem in the AF, with high rates of endemism (Martinelli et al., 2008; Costa et al., 2014). Currently, the *V. incurvata* complex is abundant and widely distributed throughout the southeastern and southern AF (Neves et al., 2018), with *V. incurvata* colonizing the southern portion of the AF after the divergence from *V. taritubensis* in the Pliocene (B. Neves et al., unpublished manuscript). In the present study, we evaluated the phylogeographic patterns of *V. incurvata* as a contribution to telling the history of the southern AF. The northern and southern parts of the AF have been identified as two distinct bioclimatic domains (Prance, 1982; Carnaval et al., 2014), and phylogeographic studies with different species, support this proposal (Cabanne et al., 2013; Brunes et al., 2015; Leite et al., 2016). Contradicting the intense instability attributed to the southern portion of the AF, the results of our ENM for *V. incurvata* suggested that the area potentially occupied by this species remained similar in the evaluated periods, which may be related to climate stability (Fig. 5). The most suitable areas during the LGM occurred in the south and southeast of Brazil, mainly from 23°S to 27°S from the Santa Catarina to São Paulo states (Fig. 5D), making evident that the appropriate ranges for *V. incurvata* occupation in the continental shelf during the LGM are now submersed, as similarly observed in other studies (Pinheiro et al., 2011; Thode et al., 2014; Leite et al., 2016; Segatto et al., 2017). Our *V. incurvata* results for MH showed that the suitable areas are very similar to those observed in the present (Fig. 5B, C), suggesting, when we analyzed the *V. incurvata* data, that the southern portion of the AF probably was a stable region.

The Bayesian phylogenetic tree results indicate that diversification of the *V. incurvata* haplotypes started in the late Pliocene/early Pleistocene ca. 2.04 Ma (Fig. 4). In addition to this recent diversification, the results show a lack of phylogeographic structure. The network from cpDNA showed high haplotype-sharing between

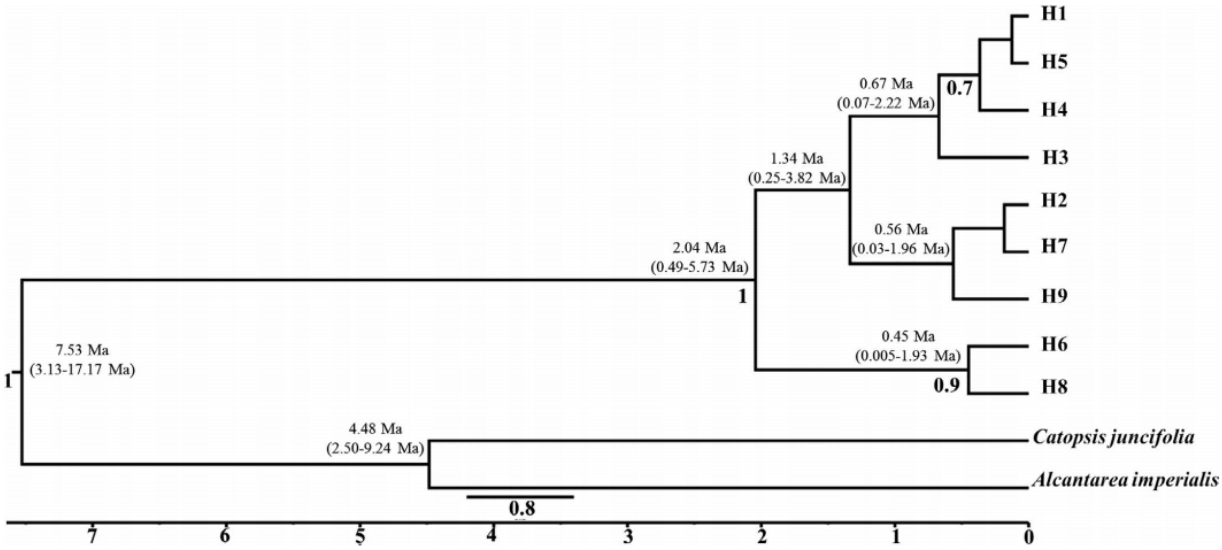

FIGURE 4. Bayesian phylogenetic tree of cpDNA (*matK* + *trnL-trnF*) haplotypes with posterior probabilities (>0.7) shown below the branches, and ages indicated for selected nodes. The time scale is in millions of years ago (Ma).

localities (Fig. 1A, B) even for geographically distant locations (Fig. 1A). Additionally, the AMOVA based on the cpDNA indicated that the highest percentage of genetic variation was observed within localities (92.25%), and the estimated F_{ST} was 0.078 and not significant (Table 3). This result corroborates the lack of phylogeographic structure found and the low F_{ST} and G_{ST} values (0.017 and 0.052, respectively). Most of the pairwise Φ_{ST} estimates were also low and not significant (Appendix S2). Moreover, we found moderate to high genetic diversity of cpDNA, mainly in samples located from 25°S to 27°S, from Paraná to the north of the Santa Catarina states (MTN, MRT, CRP) (Table 2; Fig. 1A). Goetze et al. (2017) also reported that populations of *Aechmea* Ruiz and Pav. (Bromeliaceae) species from the same region harbor the greatest number of haplotypes. Except for birds (Batalha-Filho et al., 2012; Cabanne et al., 2013), few studies on AF organisms suggest a lack of or weak phylogeographic structure. Instead, phylogeographic breaks and, consequently, the formation of structured groups have been reported for a number of organisms, including amphibians (Brunes et al., 2015; Menezes et al., 2016), bees (Batalha-Filho et al., 2010), reptiles (Pellegrino et al., 2005), bats (Martins et al., 2009), and plants (Ribeiro et al., 2011; Turchetto-Zolet et al., 2016). The causes of such breaks have been widely debated and attributed mainly to Pleistocene climatic cycles, geomorphological dynamics, and river systems (Thomé et al., 2010; Ribeiro et al., 2011; Menezes et al., 2016). Most of the phylogeographic studies of the AF biota show recurrent breaks; however, such breaks can be variable according to the region, the species, and its single characteristics (Cabanne et al., 2008; Carnaval et al., 2009; Palma-Silva et al., 2009; Thomé et al., 2010; D'Horta et al., 2011; Batalha-Filho, 2012; Turchetto-Zolet et al., 2013, 2016). Unlike in most studies, we did not find genetic structuring throughout the *V. incurvata* geographic distribution (Fig. 1). However, the separation of *V. incurvata* and *V. taritubensis* found by B. Neves et al. (unpublished manuscript) may be associated with vicariant events previously described for other species in that region near latitudes 22°S and 23°S (Palma-Silva et al., 2009; Martins, 2011). Thus, we propose that, after the southward colonization of *V. incurvata*, populations did not undergo division or structuring due to oscillations in the late Quaternary period (Behling, 1998). Instead, the results of the ENM indicated that the appropriate areas for *V. incurvata* did not change significantly in the Pleistocene, and its geographic distribution was not fragmented, which explains the lack of phylogeographic structure found in the haplotype network and the low values of F_{ST} and G_{ST}. Climatic stability in the Quaternary period was reported for passerine species (Batalha-Filho et al., 2012) and *Eugenia* sect. *Phyllocalyx* Nied. (Myrtaceae) (Bünger et al., 2016) in the southern portion of AF, revealing no changes in population size during the LGM.

The Fu's tests based on the total data set had negative and significant values (Appendix S5), suggesting a demographic expansion of *V. incurvata*. Also, the BSP results (Appendix S6) indicated a recent and continual expansion (0.1 Ma), despite the large confidence interval found. The extension of AF during LGM is a controversial topic, because different studies have found contrasting patterns that indicate retraction, stability, or expansion of forest areas in different portions of the biome (Behling, 2002; Pessenda et al., 2009; Leite et al., 2016). The persistence of forest areas in the southern AF (Cabanne et al., 2013) and the expansion of suitable climatic conditions along the continental shelf during LGM (Leite et al., 2016; present study) should have allowed forests and forest-adapted species to expand, corroborating the evidence of expansion detected for *V. incurvata*. Thus, taking into account climatic data (Maxent analysis), the environment during the Pleistocene was not fragmented; that is, the area of occurrence of the species remained practically unaltered. However, the genetic data show that there was a population expansion; *V. incurvata* was able to disperse and increased demographically.

Genetic diversity and recent gene flow

Overall genetic diversity from the nuSSRs across the sampled localities of *V. incurvata* was moderate to high when compared with

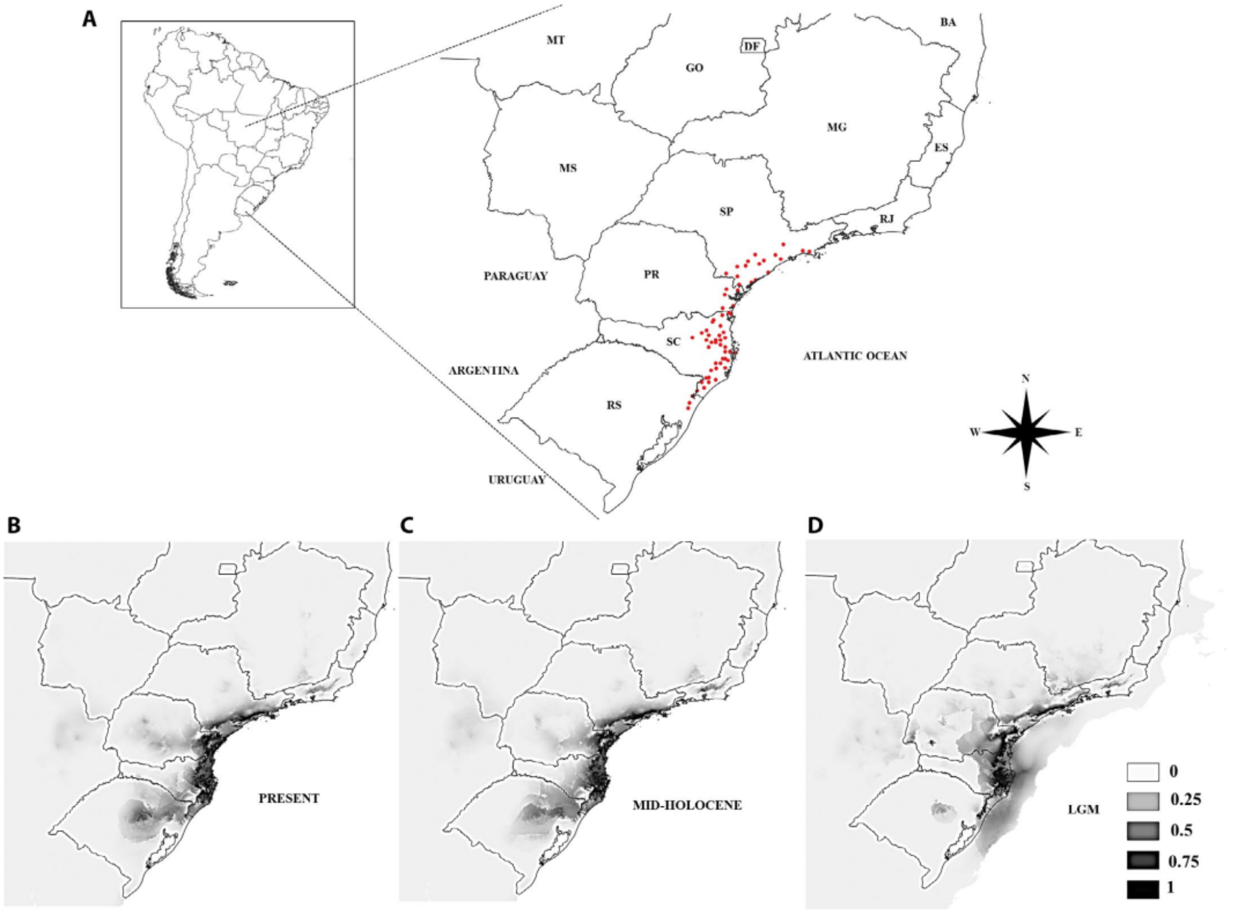

FIGURE 5. Ecological niche modelling (ENM) predictions for *Vriesea incurvata*. (A) Geographical coordinates sites along the Atlantic Forest, represented by red dots, used in the analysis. Predictions for (B) the present climatic conditions; (C) Mid-Holecene (~6000 years before the present [yBP]) and (D) LGM (Last Glacial Maximum ~21,000 yBP). The dark areas represent the higher probabilities of occurrence. RS, Rio Grande do Sul; SC, Santa Catarina; PR, Paraná; SP, São Paulo; MS, Mato Grosso do Sul; MT, Mato Grosso; GO, Goiás; DF, Distrito Federal; MG, Minas Gerais; BA, Bahia; RJ, Rio de Janeiro; ES, Espírito Santo.

other bromeliads (Table 2; Palma-Silva et al., 2009; Lavor et al., 2014; Gonçalves-Oliveira et al., 2017; Goetze et al., 2018; Soares et al., 2018). Individuals from all localities had a high number of alleles (e.g., MRT with 111 alleles), and observed heterozygosities (H_O) varied from 0.267 to 0.595 (Table 2). These results agree with the cpDNA results discussed in the previous topic because the stable southern AF region might be reflected in the high genetic diversity observed today. Several historical and contemporary factors may influence the genetic diversity in natural populations. Species' establishment and/or persistence in a particular region depends on the suitability of that habitat, which is strongly related to historical factors such as climate and geological events and may therefore imprint a signal in the species genetic pool over time (Hewitt, 2000; Carvalho et al., 2017). Carvalho et al. (2017), studying *Euterpe edulis* Mart., a plant from AF, found that genetic diversity was higher in sites with historically stable climate. Other research noted high genetic diversity in areas considered a refuge for natural populations in the AF during the Quaternary climatic oscillations (Carnaval et al., 2009; Leite et al., 2016). In agreement with these results, the results of the BOTTLENECK analysis (data not shown) suggested that populations have not experienced a recent or a strong bottleneck. Among the important factors that increase/maintain genetic variation within populations are gene flow from multiple source populations, explosive population growth, and a lack of bottleneck during colonization events (Ilves et al., 2015; Jermakowicz et al., 2017). On the other hand, all localities except CRP significantly departed from the HWE (Table 2) because of the high proportion of homozygotes, which is common in bromeliads (Palma-Silva et al., 2009; Zanella et al., 2011, 2012; Soares et al., 2018). Heterozygote deficiency may result from many factors, such as genetic drift and inbreeding, and may be closely related with the breeding system of the species (Hamrick and Godt, 1996). *Vriesea incurvata* is a self-compatible species (Martinelli, 1994) and, therefore, can have a mixed mating system, as is true of other *Vriesea* species (Paggi et al., 2007; Lavor et al., 2014), which potentiates the crossing among relatives and may explain the homozygotes excess.

In addition to the high/moderate diversity indexes encountered, the nuSSR data for plants of *V. incurvata* from the various localities had high connectivity and low genetic structure, reflecting high gene flow. The indices that estimate structure for nuSSR were all low, although many were significant (see Results, Table 3, and Appendix S2), likely because the effective number of migrants was high (Appendix S4), offering evidence of low differentiation among the localities. The structure results corroborated the low genetic structure encountered, suggesting a $K = 2$ value and demonstrating weak subdivision of the populations along *V. incurvata* distribution (Fig. 2). In the same sense, the DAPC results revealed three genetic groups and genetic mixture among the localities, indicating that, although weak, there is a recent tendency of structuring. Both results point to differentiated genetic profiles for *V. incurvata* at the edges of its distribution (SPO and MAQ) and a mixture of the two gene pools in the other localities, with the exception of MTN (Figs. 2, 3). The slight association between genetic and geographical distribution detected also corroborates these results. The successional stage of the forest is very important in maintaining the gene flow among populations or localities. Although the samples collected here were mostly from protected areas (Table 1), they all constitute small fragments with some degree of anthropogenic impact. In addition, the high gene flow we found supports the idea that these fragments are somehow connected; however, the fact that a recent weak structuring has been detected should not be ignored and requires further attention. Cascante-Marín et al. (2014), studying the bromeliad *Guzmania monostachia* (L.) Rusby ex Mez in Costa Rica, detected significant genetic variation when comparing patches and continuous forest. The same authors concluded that the forest fragmentation limits the gene flow and consequently influences the local genetic structuring. We highlight the importance of the preservation of the remaining AF for the maintenance of genetic diversity and gene flow of *V. incurvata* and other species. Moreover, the type and behavior of the pollinators may be related to the high connectivity found. Machado and Semir (2006) reported the hummingbird *Phaethornis eurynome* as the most frequent floral visitor of *V. incurvata*. Hummingbirds such as *P. eurynome* have been reported to be long-distance foragers, increasing cross-pollination rates (Singer and Sazima, 2000). In relation to the contribution of pollen/seed flow, the ratio estimated was near zero; thus, we suggest that gene flow in *V. incurvata* is equally effective through pollen and seeds. This pattern is not commonly observed in bromeliads, because the most usual pattern is a higher gene flow via pollen (Palma-Silva et al., 2009; Paggi et al., 2010; Goetze et al., 2018). In the case of *V. incurvata,* the anemochory dispersion of plumose seeds seems to be very effective because events of long-distance dispersal facilitate gene flow between populations, counteract population subdivisions, and prevent the loss of genetic diversity (Jermakowicz et al., 2017). In *V. incurvata*, the efficient mechanisms of dispersion via pollen and seed are in accord with the high number of migrants encountered ($N_em > 1$; Appendix S4), the lack of phylogeographic structure, and the predicted stability for the southern AF, which favors colonization, establishment of the species, and its maintenance.

CONCLUSIONS

In conclusion, *V. incurvata* in the southern portion of the AF has levels of genetic diversity and structure that have not been shaped by climatic oscillations of the late Quaternary period. No historical structuring was found, and a weak recent genetic structure was detected. High gene flow among localities was also revealed. *Vriesea incurvata*, despite occurring in the most threatened and fragmented biome of the world, shows no strong signs of genetic erosion, and pollinators and dispersion mechanisms are very effective.

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AUTHOR CONTRIBUTIONS

C.M.Z., M.G., and C.P.S. collected the samples; C.M.Z. did lab work; C.A.M., C.M.Z., M.G., L.D.H., and B.N. analyzed data. All authors contributed to the discussion of the results and writing the manuscript. F.B., C.P.S., and C.M.Z. led the design of this study and F.B. led the group.

SUPPORTING INFORMATION

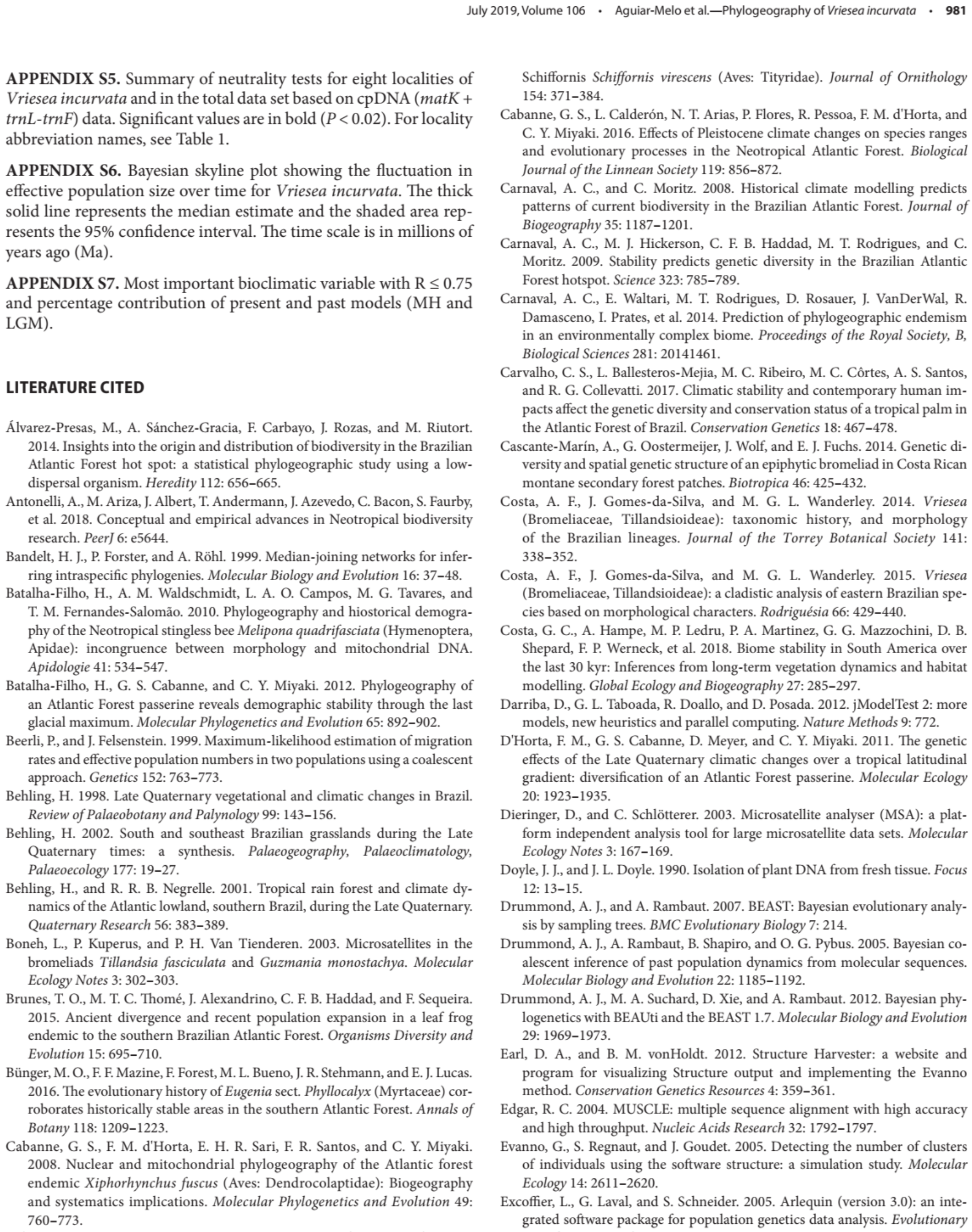
Additional Supporting Information may be found online in the supporting information tab for this article.

APPENDIX S1. Localities sampled and geographic coordinates for the Atlantic Forest in the ecological niche modelling analyses based on data of Neves et al. (2018).

APPENDIX S2. Pairwise Φ_{ST} for localities based on cpDNA (*matK* + *trnL-trnF*) data set below diagonal and pairwise F_{ST} based on 13-nuSSR data set above. For locality abbreviation names, see Table 1.

APPENDIX S3. (A) Magnitude of ΔK from STRUCTURE analysis of K (mean ± SD across 10 replicates), calculated using the ΔK method of Evanno et al. (2005), and (B) the optimal number of clusters using the silhouette method and PAM algorithm for *Vriesea incurvata* microsatellite data set.

APPENDIX S4. Bidirectional migration rates (effective number of migrants, N_em) between seven localities of *Vriesea incurvata* for 13 nuSSR data. For locality abbreviation names, see Table 1.



APPENDIX S5. Summary of neutrality tests for eight localities of *Vriesea incurvata* and in the total data set based on cpDNA (*matK* + *trnL-trnF*) data. Significant values are in bold ($P < 0.02$). For locality abbreviation names, see Table 1.

APPENDIX S6. Bayesian skyline plot showing the fluctuation in effective population size over time for *Vriesea incurvata*. The thick solid line represents the median estimate and the shaded area represents the 95% confidence interval. The time scale is in millions of years ago (Ma).

APPENDIX S7. Most important bioclimatic variable with R ≤ 0.75 and percentage contribution of present and past models (MH and LGM).

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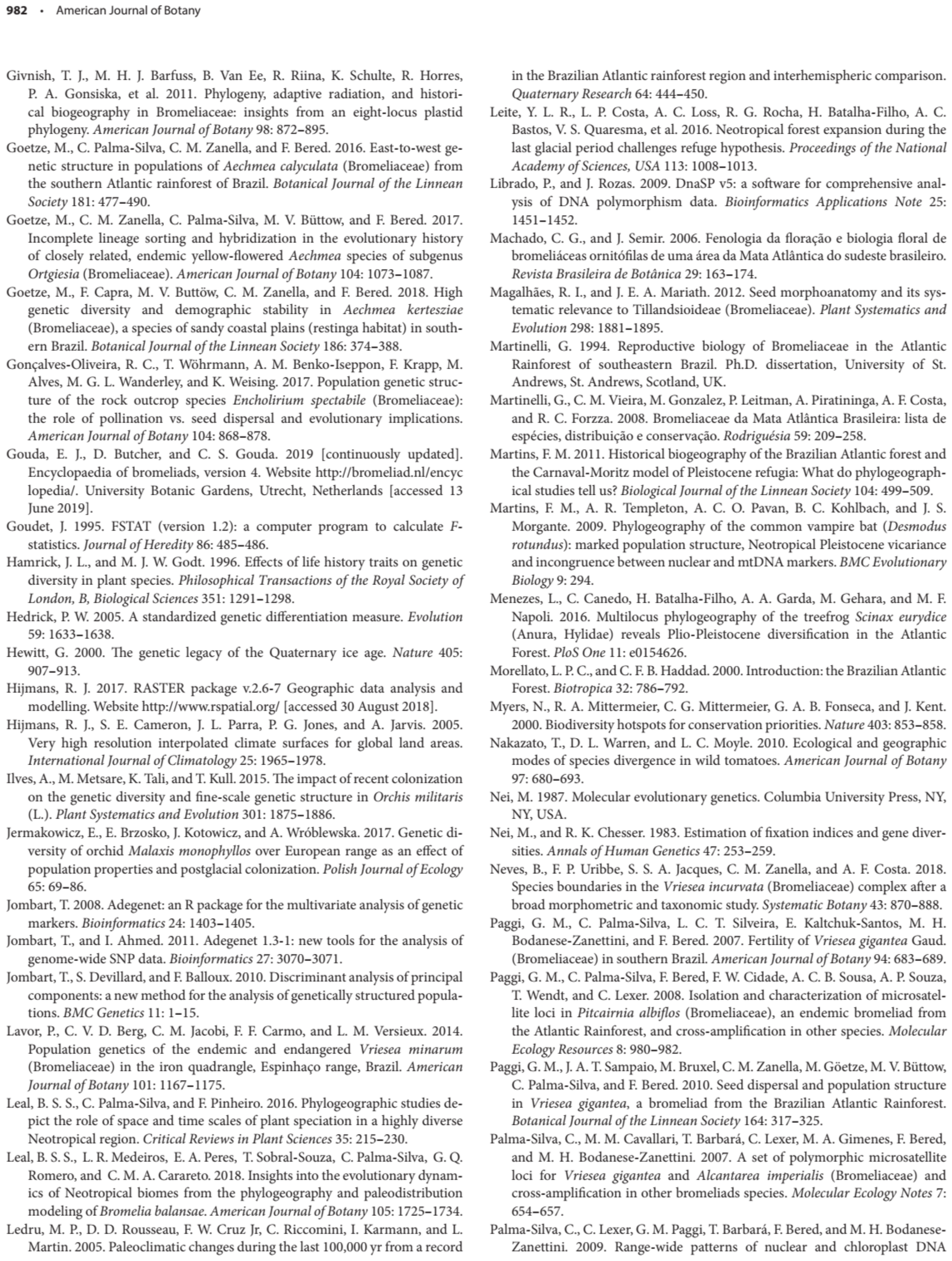

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APPENDIX 1. Vouchers for *Vriesea incurvata* localities analyzed. **(Locality)**, Country, state, city, *collectors*, *collector number*, (herbarium abbreviation), voucher number. There was one voucher per locality to avoid unnecessary extraction, because this is an endemic species from a threatened biome. Localities are described in Table 1; R: Herbário do Museu Nacional; FLOR: Herbário do Departamento de Botânica da Universidade Federal de Santa Catarina; SP: Herbário do Estado "Maria Eneyda P. Kaufmann Fidalgo"-Coleção de Fanerógamas.

Vriesea incurvata Gaudich.: **(MAQ)**, Brazil, Rio Grande do Sul, Maquiné, *B. Neves, R. Moura, S. Kunh and M. L. Gaeta, 175*, (R), 219322; **(FLO)**, Brazil, Santa Catarina, Florianópolis, *B. Neves and F. P. Uribbe*, *124*, (R), 219265; **(ATC)**, Brazil, Santa Catarina, Antônio Carlos, *D. B. Falkenberg and A. Zanin*, *5785*, (FLOR), 22616; **(CRP)**, Brazil, Santa Catarina, Corupá, *B. Neves, F. P. Uribbe and J. Néri,*, *128*, (R), 219324; **(JVL)**, Brazil, Santa Catarina, Joinville, *B. Neves and F. P. Uribbe*, *203*, (R), 219335; **(MTN)**, Brazil, Paraná, Matinhos, *B. Neves and J. Néri*, *206*, (R), 219338; **(MRT)**, Brazil, Paraná, Morretes, *B. Neves, F. P. Uribbe and J. Néri,, 138*, (R), 219267; **(CAN)**, Brazil, São Paulo, Cananéia, *R. Moura, J. Gomes-da-Silva, B. Neves, A. P. Pinto and L. G. Nogueira, 1018*, (R), 219391; **(SPO)**, Brazil, São Paulo, São Paulo, *R. T. Shirasuna, R. Suzuki, and F. Aguiar*, *682*, (SP), 430361.

Appendix S1. Localities and geographical coordinates of the Atlantic Forest used in the ecological niche modelling analyses, based on Neves et al. (2018) data collection.

Municipality/Locality	Brazilian State [a]	Latitude S	Longitude W
Maquiné	RS	-29.504806	-50.239806
Caraá	RS	-29.706944	-50.291056
Joinville	SC	-26.141944	-48.980278
São Bento do Sul	SC	-26.324722	-49.313333
Grão Pará	SC	-28.188333	-49.213889
Blumenau	SC	-26.920833	-49.091667
Corupá	SC	-26.393611	-49.354167
Itapoá	SC	-26.082321	-48.640606
Ilhota-Morro do Baú	SC	-26.799722	-48.930833
Massaranduba	SC	-26.550278	-49.050556
Doutor Pedrinho	SC	-26.7275	-49.585278
Treviso	SC	-28.529167	-49.497222
Imaruí	SC	-28.164722	-48.869722
Siderópolis	SC	-28.549444	-49.603889
Taió	SC	-27.000278	-50.130556
Florianópolis	SC	-27.576667	-48.475556
Antônio Carlos	SC	-27.539444	-48.870278
Paulo Lopes	SC	-27.878889	-48.761944
Benedito Novo	SC	-26.910278	-49.500278
Presidente Nereu	SC	-27.181389	-49.231389
São José	SC	-27.540278	-48.689444
Apiúna	SC	-27.180278	-49.393611
Orleans	SC	-28.259722	-49.411944
Jacinto Machado	SC	-29.045278	-49.945278
Santa Rosa de Lima	SC	-27.989722	-49.230833
Indaial	SC	-27.09	-49.230833
Morro Grande	SC	-28.71	-49.77
São Bonifácio	SC	-27.989167	-49.048889
Praia Grande	SC	-29.249722	-50.129722
Águas Mornas	SC	-27.809722	-48.960833

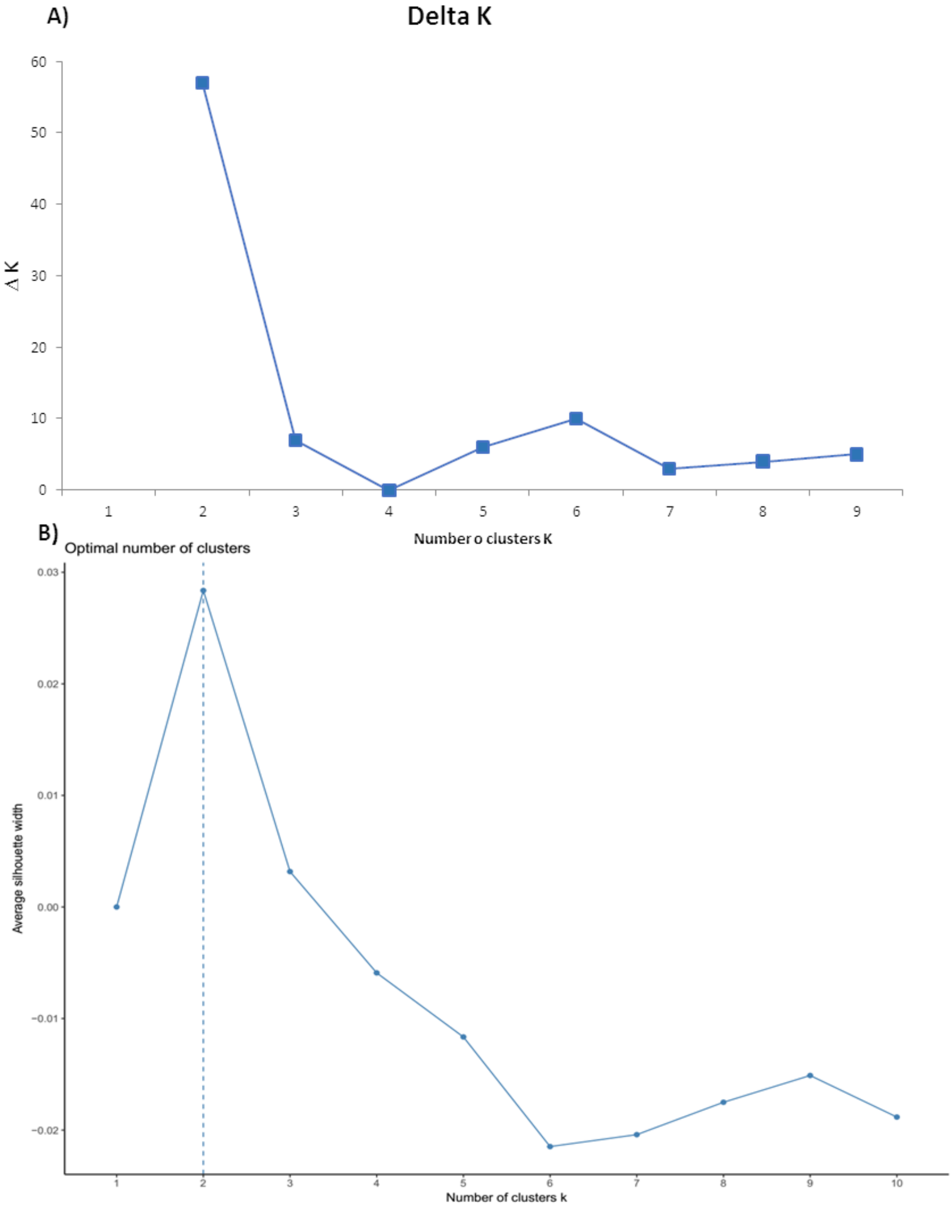
Vitor Meireles	SC	-26.821667	-49.77
Garuva	SC	-26.055025	-48.727225
Santo Amaro da Imperatriz	SC	-27.81	-48.866944
Botuverá	SC	-27.268611	-49.051944
Guabiruba	SC	-27.09	-49.049444
Ituporanga	SC	-27.360833	-49.503056
Nova Veneza	SC	-28.710833	-49.499444
Brusque	SC	-27.005556	-48.869722
São João Batista	SC	-27.359722	-48.8675
Morro da Fumaça	SC	-28.619167	-49.232222
Rio do Sul	SC	-27.090278	-49.590555
Turvo	SC	-28.926667	-49.679444
Guaraqueçaba	PR	-25.180556	-48.385361
Morretes	PR	-25.347389	-48.885889
Matinhos	PR	-25.782323	-48.562387
Guaratuba	PR	-25.868889	-48.966667
Campina Grande do Sul	PR	-25.1377	-48.8215
Tapiraí	SP	-24.035697	-47.382837
Juquiá	SP	-24.16529	-47.55991
Cajati	SP	-24.953056	-48.334167
Ribeirão Grande	SP	-24.271778	-48.40525
Salesópolis	SP	-23.656222	-45.88775
São Miguel Arcanjo	SP	-24.063611	-47.991472
São Lourenço da Serra	SP	-23.824667	-46.939778
Eldorado	SP	-24.643333	-48.400278
Mairiporã	SP	-23.420833	-46.633333
Sete Barras	SP	-24.233333	-48.083333
São Paulo	SP	-23.983333	-46.733333
Caraguatatuba	SP	-23.692222	-45.633333
Iguape	SP	-24.783333	-47.7
Pariquera-Açu/ Cananéia	SP	-24.879444	-47.858333
Miracatu	SP	-24.5	-47.216667
Iporanga	SP	-24.533333	-48.833333
Pilar do Sul	SP	-23.8131	-47.7164

[a] RS, Rio Grande do Sul; SC, Santa Catarina; PR, Paraná; SP, São Paulo.

Appendix S2. Localities pairwise Φ_{ST} based on cpDNA (*mat*K + *trn*L-*trn*F) data set below the diagonal and pairwise F_{ST} based on the 13-nuSSR data set above. For locality abbreviation names, see Table 1.

Locality	MAQ	FLO	ATC	CRP	MTN	MRT	CAN	SPO
MAQ	-	0.071***	0.057***	0.004	0.105***	0.035***	-	0.094***
FLO	-0.079	-	0.016	0.032*	0.065**	0.049**	-	0.112***
ATC	0.065	-0.615	-	0.026*	0.012	0.034*	-	0.066**
CRP	0.008	0.017	0.121	-	0.067***	0.004	-	0.038*
MTN	-0.007	-0.245	-0.355	0.080	-	0.074***	-	0.046
MRT	0.088	-0.186	-0.341	0.115	-0.078	-	-	0.059**
CAN	0.343	-0.200	-0.615	0.366	-0.100	-0.156	-	-
SPO	0.539*	0.205	-0.096	0.556*	0.203*	0.090	-0.167	-

Notes: ***$P < 0.001$; ** $P < 0.01$; *$P < 0.05$



Appendix S3. (A) Magnitude of Δ*K* from structure analysis of *K* (mean ± SD across 10 replicates), calculated using the Δ*K* method of Evanno et al. (2005), and (B) the optimal number of clusters using the silhouette method and PAM algorithm for *Vriesea incurvata* microsatellite data set.

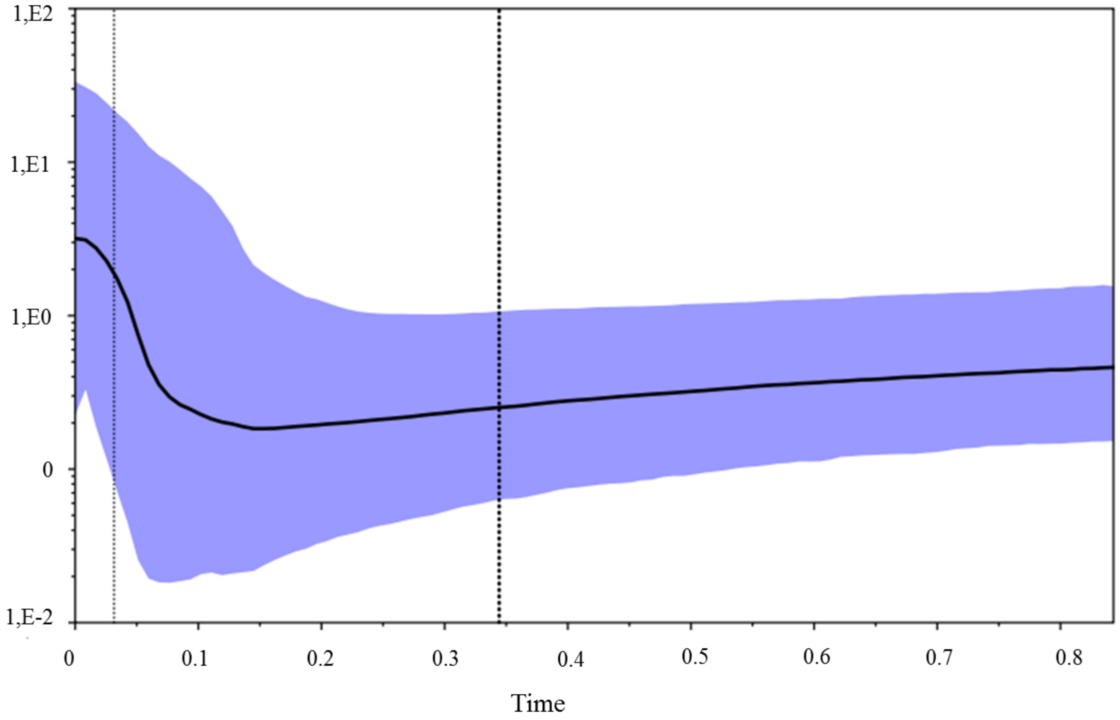
Appendix S4. Bidirectional migration rates (effective number of migrants, $N_e m$) between seven localities of *Vriesea incurvata* for 13-nuSSR data. For locality abbreviation names, see Table 1.

MAQ $\overset{0.738}{\underset{1.201}{\rightleftarrows}}$ FLO	FLO $\overset{0.856}{\underset{1.514}{\rightleftarrows}}$ ATC	ATC $\overset{2.117}{\underset{1.377}{\rightleftarrows}}$ CRP	CRP $\overset{1.232}{\underset{2.202}{\rightleftarrows}}$ MTN	MTN $\overset{2.011}{\underset{3.964}{\rightleftarrows}}$ MRT	MRT $\overset{2.687}{\underset{3.837}{\rightleftarrows}}$ SPO
MAQ $\overset{0.871}{\underset{2.180}{\rightleftarrows}}$ ATC	FLO $\overset{1.012}{\underset{1.351}{\rightleftarrows}}$ CRP	ATC $\overset{2.026}{\underset{1.732}{\rightleftarrows}}$ MTN	CRP $\overset{2.891}{\underset{3.267}{\rightleftarrows}}$ MRT	MTN $\overset{1.701}{\underset{3.157}{\rightleftarrows}}$ SPO	
MAQ $\overset{1.784}{\underset{3.344}{\rightleftarrows}}$ CRP	FLO $\overset{0.811}{\underset{1.738}{\rightleftarrows}}$ MTN	ATC $\overset{2.190}{\underset{3.263}{\rightleftarrows}}$ MRT	CRP $\overset{1.082}{\underset{2.219}{\rightleftarrows}}$ SPO		
MAQ $\overset{1.163}{\underset{1.834}{\rightleftarrows}}$ MTN	FLO $\overset{1.203}{\underset{1.405}{\rightleftarrows}}$ MRT	ATC $\overset{1.022}{\underset{2.087}{\rightleftarrows}}$ SPO			
MAQ $\overset{1.558}{\underset{3.291}{\rightleftarrows}}$ MTN	FLO $\overset{0.778}{\underset{1.618}{\rightleftarrows}}$ SPO				
MAQ $\overset{0.506}{\underset{0.983}{\rightleftarrows}}$ SPO					

* The arrows represent the direction of migration.

Appendix S5. Summary of neutrality tests performed in eight localities of *Vriesea incurvata* and in the total data set based on cpDNA (*matK* + *trnL-trnF*) data. Significant values are in bold ($P < 0.02$). For locality abbreviation names, see Table 1.

Locality Statistic	MAQ	FLO	ANT	CRP	MTN	MRT	CAN	SPO	Total
Tajima's D	-1.401	0.000	0.000	-1.006	-0.447	-0.761	0.000	-1.237	-1.053
Fu's F_s	-1.164	0.201	0.000	-0.922	0.117	-1.139	0.201	-0.922	**-4.681**



Appendix S6. Bayesian skyline plot showing the fluctuation in effective population size over time for *Vriesea incurvata*. The thick solid line represents the median estimate and the shaded area represents the 95% confidence interval. The time scale is in millions of years ago (Ma).

Appendix S7. Most important bioclimatic variable with R ≤ 0.75 and percent contribution of present, and past models (MH and LGM).

Bioclimatic Variable	CODE	Contribution (%)		
		Present	MH	LGM
Min temperature of coldest month	Bio-6	30.9	28.5	19
Mean temperature of warmest quarter	Bio-10	18.4	16.7	27.7
Annual precipitation	Bio-12	28	30.5	0.7
Precipitation of driest month	Bio-14	2.2	4	7.3
Precipitation seasonality (coefficient of variation)	Bio-15	8.6	6.6	1.7
Precipitation of coldest quarter	Bio-19	11.9	13.7	43.6

MH: Mid-Holocene and LGM: Last Glacial Maximum.

CAPÍTULO III

Porous reproductive barriers evidenced by high interspecific gene flow in *Vriesea* (Bromeliaceae) sympatric species from the southern Atlantic Forest

(Artigo a ser submetido à Botanical Journal of the Linnean Society)

Porous reproductive barriers evidenced by high interspecific gene flow in *Vriesea* (Bromeliaceae) sympatric species from the southern Atlantic Forest

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ABSTRACT: Bromeliaceae is a Neotropical family with tremendous morphological and ecological diversity. Due to recent diversification, much of the incipient species may not wholly defined, and permeable reproductive barriers can allow hybridization, which may occur in some species of *Vriesea*. The genus *Vriesea* occurs in preferentially mesophytic environments, such as the ombrophilous and montane areas in the Atlantic Forest, which is considered its diversity center. Thus, in order to understand population structuring, hybridization occurrence, and morphological differences of *Vriesea* species when in sympatry, we analyzed three localities in the Atlantic Forest domain. We used 12 nuclear microsatellites loci and 10 morphological variables (four related to vegetative and six to reproductive structures) to analyze different approaches. Our results revealed a high hybridization rate and low genetic structure between species in sympatric populations from the three localities, suggesting that reproductive barriers are weak in these *Vriesea* species populations. Even more, we observed hybrids with atypical morphology and hybrids that look like purebreds parental. Moreover, we observed anthropogenic impacts in the studied localities, which may create new habitats likely favoring the niche invasion of one species of *Vriesea* to another, facilitating the hybridization events.

Keywords: Bromeliads, hybridization, neotropics, reproductive isolation, sympatry, Tillandsioideae.

INTRODUCTION

In plants, the reproductive isolation is based on pre-pollination barriers, with mechanisms that prevent (ecogeographic isolation) or limit (phenological isolation and pollinator specialization) pollen transfer among species; and post-pollination, which contains pre-zygotic (pollen-stigma interactions, pollen competition) and post-zygotic mechanisms (hybrid inviability, sterility and failure or reduction in successful reproduction in subsequent generations) (Tiffin, Olson & Moyle, 2001; Rieseberg & Willis, 2007; Baack *et al.*, 2015). Studies and reviews on reproductive barriers in plants suggest that pre-pollination barriers were often very strong and have contributed more to total reproductive isolation than post-pollination barriers (Rieseberg & Willis, 2007; Lowry *et al.*, 2008; Widmer, Lexer & Cozzolino, 2009, Baack *et al.*, 2015). However, a strong reproductive isolation usually requires a combination of reproductive barriers in multiple plant-life stages (Cozzolino & Scopece, 2008; Lowry *et al.*, 2008). Indeed, the strength and permeability of the barriers are highly variable among species and strongly depend on the degree of floral specialization and pollination system (Cozzolino & Scopece, 2008; Scopece *et al.*, 2010; Xu *et al.*, 2020). If the reproductive barriers are permeable, the hybridization may occur between species and can lead to distinct consequences in the speciation process, such as new lineages establishment, the maintenance or increase of genetic diversity, the origin and transfer of adaptations and the reinforcement or breakdown of reproductive isolation (Abbott *et al.*, 2013; Todesco *et al.*, 2016). Hybridization usually occurs between related species (Mallet, Besansky & Hahn, 2015) and at different spatial (e.g. hybrid zones) and temporal (e.g. secondary contact after a period of independent evolution) contexts (Abbott *et al.*, 2013). In this way, the anthropogenic impacts, such as disturbance of the habitats, farming, and the introduction of non-native taxa may lead to contact between previously isolated species (Anderson, 1948; Harrison & Larson, 2014; Todesco *et al.*, 2016). In the Brazilian Atlantic Forest (AF), a remarkable biodiversity hotspot (Myers *et al.*, 2000), just 12.4% of the primary vegetation remains due to high anthropogenic impacts (SOSMA/INPE, 2020), being this domain an important area to evaluate the levels of hybridization between species endemic to this forest.

Bromeliaceae is a Neotropical family that suffered recent adaptive radiation driven by some key innovations, thus, providing great morphological and ecological diversification (Givnish *et al*., 2007; 2014; Silvestro, Zizka & Schulte., 2013). It is divided into eight subfamilies and contains 3,652 species in 78 genera (Givnish *et al*., 2007; Gouda, Butcher & Gouda [cont. updated]). Due to the recent family diversification (Givnish *et al*., 2014; Bouchenak-Khelladi, Muasya & Linder, 2014), many incipient species may not be completely defined, and thus, permeable reproductive barriers can allow hybridization (Wendt *et al*., 2008; Matallana *et al*., 2016). Indeed, natural hybridization events have been reported in different genera of bromeliads, such as *Pitcairnia* L'Hér. (Wendt, Paz & Rios, 2000; Wendt *et al*., 2001), *Tillandsia* L. (Gardner, 1984; Gonçalves & Azevêdo-Gonçalves, 2009), *Puya* Molina (Schulte *et al*., 2010), *Dyckia* Schult. & Schult.f. (Hirsch *et al*., 2020), and *Vriesea* Lindl. (Matos *et al*., 2016; Zanella *et al*., 2016; Neri, Wendt & Palma-Silva, 2017a).

Vriesea is one of the species-richest genera in Tillandsioideae subfamily, with 231 species (Gouda, Butcher & Gouda [cont. up.]). The genus is composed mainly by epiphytic plants occurring in mesophilic environments, such as the AF, which is considered its main diversity center with 166 species (Martinelli *et al*., 2008; Costa, Gomes-da-Silva & Wanderley, 2014). The recent diversification of the genus likely caused low infrageneric resolution and unclear species boundaries (Costa, Gomes-da-Silva & Wanderley, 2015; Gomes-da-Silva & Souza-Chies, 2017; Machado *et al*., 2020), leading the relationships in the infrageneric level still under discussion. Groups or complexes of morphologically similar species were recognized and taxonomically revised (Costa, Rodrigues & Wanderley, 2009; Moura, 2011; Versieux, 2011; Gomes-da-Silva & Costa, 2011; Moura & Costa., 2014; Machado, 2017; Neves *et al*., 2018; Uribbe *et al*., 2020). However, some of them were only partially recovered in the molecular-based phylogenetic analyses (Gomes-da-Silva & Souza-Chies, 2017; Machado *et al*., 2020). The species with simple inflorescences, red floral bracts, hummingbird pollinated flowers with tubular and yellow corollas (Costa, Gomes-da-Silva & Wanderley, 2014; 2015) are likely a natural group, identified as “inflated group” which includes *V. carinata* Wawra, *V. incurvata* Gaudich, *V.*

erythrodactylon É.Morren ex Mez, *V. inflata* (Wawra) Wawra, *V. heterostachys* (Baker) L.B.Sm, *V. ensiformis* (Vell.) Beer., *V. simplex* (Vell.) Beer,*V. scalaris* É.Morren and *V. agostiniana* E. Pereira. These species are widely distributed in the AF with cases of narrow distributions (e.g *V. agostiniana*) (Kessous *et al*., 2018a; Flora do Brasil 2020). Floristic inventories and herbarium data along the central and southern AF indicated that several of these *Vriesea* species occurr in sympatry and sometimes form big populations (Kersten & Silva, 2001; Machado & Semir, 2006; Costa & Wendt, 2007; Monteiro & Forzza, 2008; Nunes-Freitas *et al*., 2009; Blum, Roderjan & Galvão, 2011; Vizentin-Bugoni, Maruyama & Sazima, 2014; Machado, Forzza & Stehmann, 2016). However, their morphological limits are not always clear, with overlapping features, especially related to the shape of the leaves and floral bracts (Neves *et al*., 2018; 2020), and length and position of the inflorescence (erect to pendulous; Kessous, 2016). From our field and herbarium experience, several individuals with atypical morphology (other than the most common limits of the cited species) are frequent in localities where they occur in sympatry, leading us to hypothesize their hybrid origin. Moreover, hybridization cases had already been reported between species from this group (*V. carinata* x *V. incurvata*, Matos *et al*., 2016; Zanella *et al*., 2016; *V. simplex* x *V. scalaris*, Neri, Wendt & Palma-Silva, 2017a). In this context, we choose species from this *Vriesea* group as a model to understand the mechanisms involved in the maintenance of these species when in sympatry. For this, we analyzed the population structure, hybridization occurrence and morphological differences in six *Vriesea* species that occur in three localities in the AF. Specifically, we address the following questions: 1) What are the patterns of genetic structure between species into the sympatric populations? 2) How is the genetic diversity of these species in the populations analyzed? 3) What are morphological differences between species within sympatric populations? 4) Do species hybridize as suggested by observations of putative hybrids in the field? 5) If yes, all the hybrids have atypical morphology?

MATERIALS AND METHODS

Study Populations and Species

We selected three localities where two or more *Vriesea* species [populations] occur in sympatry in the southern portion of the Brazilian Atlantic Forest domain (Table 1; Figure 1). In Santa Virgínia there are four *Vriesea* species (*Vriesea carinata* Wawra

(VCA), *V. taritubensis* var. *patens* B.Neves & A.F.Costa (VTP), *V. simplex* and (VSI), and *V. agostiniana* (VAG) (Figure 1). We also found four individuals that share the same atypical morphological features hypothesized as a *Vriesea* putative hybrid (VPH). (Figure 1G; Table 1). Santa Virgínia belongs to Parque Estadual da Serra do Mar – São Paulo Brazilian State (SP). The vegetation is composed of dense montane rainforest, small stretches of altitude fields, and fog forest. Also, it presents secondary forest in different successional stages and anthropogenic impacts, as field pastures and discarded *Eucalyptus* spp. farming (São Paulo, 2005; Liu, 2014). In Boraceia (SP), we collected *V. inflata* (VIF) and *V. incurvata* (VIC) (Figure 1; Table 1). Boraceia is a Biological Station composed of dense montane rainforest within the Casa Grande Reserve, part of the Parque Estadual da Serra do Mar (Custodio-Filho, 1989; Cavarzere, Moraes & Silveira, 2010). The Station has more than 60 years, and the urbanization in its surroundings in the last decades may have influenced its conservation status (Machado, 2014). Joinville (Santa Catarina Brazilian State - SC) is the southernmost locality studied (Figure 1) and is also covered by dense montane rainforest (Vibrans *et al*., 2013). The species *V. carinata* and *V. incurvata* (Figure 1; Table 1) were sampled in trails around Serra Dona Francisca Road, a touristic highway opened between the years of 1853 to 1895 (Goulart-Filho, 2014).

As the species studied here occur almost always in sympatry, it is difficult to sample allopatric populations for each one. For this reason, we used as allopatric reference those individuals that presented a pure molecular profile of each species and preferably not occurring with the species tested in each situation (Table 1). *Vriesea simplex* was collected in Ubatuba (SP), in a low montane forest at Parque Estadual da Serra do Mar, Núcleo Picinguaba, to be used as allopatric population in the genetic analyses of Santa Virgínia locality (Table 1).

All the individuals sampled were assigned as a particular species, during the collection in the field by the taxonomist AFC (including VPH that was hypothesized to be a hybrid taxa) based on her vast experience in identification of these plants, and literature descriptions as follows: *V. agostiniana* (Kessous *et al*., 2018a), *V. carinata* and *V. inflata* (Costa, Wanderley & Moura, 2007), *V. incurvata* and *V. taritubensis* var. *patens* (Neves *et al*., 2018), *V. simplex* (Kessous, Salgueiro & Costa, 2018b). All the genetic analyses were performed and the results were discussed under this taxonomic approach.

Sampling, Storage and DNA Isolation

A total of 222 individuals from six species (plus VPH) was sampled in four localities (Table 1). Of these, 160 individuals were evaluated by genetic and 168 by morphology approaches. For DNA isolation, fresh leaves were collected and stored in silica gel for drying. The total genomic DNA was isolated using the cetyl trimethyl ammonium bromide (CTAB) method (Doyle & Doyle, 1990). For morphological analyses, we collected one or two leaves and the whole inflorescence of each individual, which were dried in the forced-air plant drier and stocked appropriately until the moment of being measured. Representative vouchers of each taxa from each locality studied were deposited at the Herbaria of the Universidade Federal do Rio Grande do Sul (ICN) and Museu Nacional do Rio de Janeiro (R).

Molecular Markers and Genotyping

We used 12 nuclear microsatellites loci (nuSSR) isolated from *Vriesea carinata* (*Vcar143*, *Vcar153*, *Vcar91*, *Vcar95.1*, *Vcar115*, *Vcar258*, *Vcar280.1*, *Vcar293*; Todeschini *et al*., 2018), *Vriesea gigantea* Gaudich. (*VgB12*; Palma-Silva *et al*., 2007), *Alcantarea imperialis* (Carrière) Harms (*Ai4.03*; Palma-Silva *et al*., 2007) and *Tillandsia fasciculata* Sw. (*e6* and *e6b*; Boneh, Kuperus & Van Tienderen, 2003). For each nuSSR, the forward primers were synthesized with a 19-bp M13 tail (5′-CACGACGTTGTAAAACGAC-3′) at the 5′ end for labeling with a tailed fluorescent dye M13 primer during genotyping procedures, following the method of Schuelke (2000). All PCR amplifications were performed in the Veriti 96- Well Thermal Cycler following the protocol of Palma-Silva *et al*. (2007). The microsatellite alleles were resolved on an ABI 3100 DNA Analyzer Sequencer (Applied Biosystems) and sized against the GS500 LIZ molecular size standard (Applied Biosystems) using GeneMarker Demo version 1.97 (SoftGenetics, State College, PA, USA). *Vriesea taritubensis* var. *patens* and *V. incurvata* revealed to be recalcitrant taxa concerning DNA isolation and PCR amplifications, probably due to secondary compounds (personal observation). For this reason, due to high lost data for *V. incurvata* in Boraceia and Joinville, we used seven and 10 nuSSR in these populations, respectively (Table S1).

Data Analyses

Patterns of genomic admixture to hybrid detection

To investigate the occurrence of structure in each sympatric population, we performed a Bayesian clustering algorithm implemented in STRUCTURE version 2.3.4 (Pritchard, Stephens & Donnelly, 2000). For each *K* from 1 to 10 (Santa Virgínia) and 1 to 6 (Boraceia and Joinville), we performed 10 replicates, using the admixture model, assuming correlated allele frequencies and using a burn-in period of 500,000 and a run length of 1,000,000 to confirm the stabilization of summary statistics (Pritchard, Stephens & Donnelly, 2000). Based on the ΔK method described by Evanno, Regnaut & Goudet (2005), implemented in STRUCTURE HARVESTER 0.6.94 (Earl & vonHoldt, 2012), we determined the highest number of *K* for each sympatric population. Besides that, the run with the best *K* and 10 replicates was performed for each sympatric population using pure genetic profiles obtained from populations used as allopatric (Table 1). The threshold to classify pure individuals was $q \geq 0.90$ and $0.10 < q < 0.90$ to hybrid individuals (Vähä & Primmer, 2006). In addition, we performed the clustering method (Anderson & Thompson, 2002) implemented in the NEWHYBRIDS version 1.1 to assign the individuals into different genotypic classes (pure parental species (P) 1 or 2, F1, F2 and backcross 1 or 2), using a threshold value of $q \geq 0.90$, individuals with $q < 0.90$ remained unassigned. The two possible runs were performed in the program: one informing the parents and hybrids (according to STRUCTURE results) and the other with no information. The Santa Virgínia population has more than one parental combination (VCA+VSI; VCA+VTP and VSI+VTP), so independent runs with each parental possibility plus specific allopatric population and hybrids were performed.

Genetic diversity, structure, and gene flow

The analyzes were performed for each sympatric population assuming pure and hybrid individuals according to STRUCTURE results (Table 2). The diversity indices were characterized using the number of alleles (*A*), private alleles (PA), allelic richness (AR), observed and expected heterozygosities (*H*O and *H*E respectively), and inbreeding coefficient (F_{IS}) (Weir & Cockerham, 1984). All these indices were calculated using ARLEQUIN 3.1 (Excoffier, Laval & Schneider, 2005), FSTAT version 2.9.3.2 (Goudet, 1995), and MSA 4.05 (Dieringer & Schlötterer, 2003). Departures from the Hardy–Weinberg equilibrium (HWE) for species in each population were identified using exact tests in GENEPOP 4.7 (Raymond & Rousset, 1995; Rousset, 2008) with 10,000 permutations. Also, AMOVA was carried out in ARLEQUIN, with 10,000 permutations, to examine the partition of nuclear genetic diversity within and among species in sympatric populations. Pairwise effective migration rates (N_em) were estimated among species in sympatric populations following a coalescent theory and maximum likelihood-based approach using MIGRATE 3.0.3 (Beerli & Felsenstein, 1999). The computations were carried out under a Brownian motion model, and mutation rates (μ) among the loci were estimated from the data.

Pollination experiments

To test genetic compatibility between species, we performed interspecific artificial crosses with intraspecific cross-pollination as a control. Manual crossing experiments were conducted at the greenhouse of the Botanical Department (UFRGS, RS, Brazil) following Cafasso, Widmer & Cozzolino (2005). Most of the crosses, including intraspecific ones, were conducted in both directions (each plant provided and received pollen) and were performed between different individuals. Not all possible combinations could be made due to technical difficulties as differential blooming (Table S2).

Morphological Analyses

We analyzed 10 morphological variables in each sympatric population, four related to vegetative, and six to reproductive structures (Table 5). The data was standardized, and analyses were conducted in PAST v.4.03 (Hammer, Harper & Ryan, 2001). The normality Shapiro-Wilk test (Shapiro & Wilk, 1965) was performed, and the variances did not show the normal distribution for most of the variables. Therefore, the non-parametric Kruskall Wallis (KW, H test) for two or more populations (Callegari-Jacques, 2003) was chosen. For Santa Virgínia population (four species plus VPH), we performed a multiple comparison test (Mann-Whitney pairwise) after KW. Moreover, a Principal Component Analyses (PCA) was performed to investigate each species' position within sympatric populations. Besides that, a hierarchical clustering analysis was performed with Ward’s method distance (Ward, 1963) exclusively with individuals used in STRUCTURE analyses. The individuals with admixture molecular profile or with another species' genetic profile were indicated in the morphological analyses (special symbols).

RESULTS

Genetic composition of sympatric populations

STRUCTURE analysis revealed high hybridization between species in sympatric populations from the three localities (Figure 2). The Evanno method indicated three most likely clusters in Santa Virgínia (Figure S1A) related to VCA, VTP, and VSI species (Figure 2A). The plants identified as *V. agostiniana* (VAG) showed admixture molecular profile or the molecular profile characteristic of another species, and all plants identified morphologically as *Vriesea* putative hybrid (VPH) revealed an admixture molecular profile (Table 2; Figure 2A). In this way, of the 80 plants sampled in Santa Virgínia based on morphological identification (four species – VCA, VTP, VSI, VAG plus the putative hybrid, VPH), 36 (45%) showed hybrid profile (Table 2; Figure 2A). In Boraceia, the Evanno method indicated two most likely clusters (Figure S1B), corresponding mostly to VIF and VIC species (Figure 2B). However, of the 26 sampled plants, 11 (~42%) showed a hybrid profile (nine sampled as VIF and two sampled as VIC; Table 2). As expected, for Joinville population, the Evanno method indicated two groups (Figure S1C) corresponding to the two species collected in that locality (Figure 2C). Of the 35 plants collected in Joinville, 27 had the molecular profile according to each species' taxonomic identification. Two VCA samples showed a profile of VIC, and two others showed a hybrid profile. Also, four samples identified as VIC revealed a hybrid profile, totaling eight plants (~23%) detected with the intermediate molecular profile (Table 2; Figure 2C). Herein the individuals that showed admixture molecular profile and molecular profile characteristic of another species from STRUCTURE results (Table 2), including VPH and VAG, will be called just as HYBRIDS in the tables, figures and results of the genetic analyses. The exceptions are VPH and VAG individuals in pollination experiments and morphological analyses, and also in some points of the discussion.

Considering NEWHYBRIDS analysis, many hybrid individuals identified by STRUCTURE could not be assigned into any hybrid class. The program was able to classify better when we did not inform the parents and hybrids. Of the 36 hybrid individuals analyzed into the three possible parents set in Santa Virgínia locality, 11 (VCA+VSI), 17 (VCA+VTP) and 16 (VSI+VTP) fitted in F2 category (Table S3). For Boraceia population, two individuals of the 11 hybrids were classified as parental and F2 class (data not shown). Joinville also presented just two of the eight hybrid individuals classified, both as parental types (data not shown).

Genetic Diversity

The nuSSR were polymorphic in all populations studied (Table 3). The number of alleles (*A*) in Santa Virgínia ranged from 54 (VCA) to 87 (VTP), and the hybrid individuals showed 99 alleles. Private alleles (*PA*) varied from 3 (VSI) to 15 (VTP), and the hybrids presented 10. The VTP was the taxon with the highest observed (H_O= 0.65720) and expected (H_E =0.82464) heterozygosities, and the hybrids presented H_O =0.55240 and H_E =0.78752 (Table 3). In Boraceia, the hybrid individuals also have the highest number of alleles (46) when compared to the two other species (30 for both) (Table 3). The private alleles varied from 2 (VIF) to 6 (hybrids). Allelic richness was 3.095 for hybrid individuals and 2.758 and 2.581 for VIC and VIF, respectively. In Joinville, the species with the highest diversity indices was VIC (Table 3). The hybrids presented 48 alleles and VCA 39 with 5 *PA* for both. The allelic richness was 4.367 and 3.127 for the hybrids and VCA , respectively. The indices obtained for populations used as allopatric for each sympatric population are summarized in Table 3. Inbreeding coefficients (F_{IS}) departed significantly from the HWE for all species and hybrids, except for VIF in sympatric and allopatric populations (Table 3).

Structure and Gene Flow

AMOVA revealed more variation within than among species in all sympatric populations, with moderate fixation indices: F_{ST} = 0.101 (Santa Virgínia), 0.099 (Boraceia) and 0.152 (Joinville); all significant (P < 0.001) (Table 4). The maximum-likelihood-based estimates of effective migration rates (N_em) were high in the three sympatric populations (Figure 3). In Santa Virgínia, it ranges from 0.325 (VSI to VCA) to 3.176 (VTP to hybrids) (Figure 3A). For Boraceia, the N_em varied from 0.380 (VIF to VIC) to 4.499 (hybrids to VIF) (Figure 3B). In Joinville it ranges from 0.261 (VCA to VIC) to 1.875 (VIC to hybrids) (Figure 3C). Concerning the artificial crosses performed (n=225), only 26 fruits were generated: four from controls (VCA x VCA); 18 from interspecific crosses (VCA x VIC; VSI x VAG; VAG x VIC) and four from VCA x VPH (Table S2).

Morphological Analyses

The Kruskall-Wallis test revealed significant differences in all variables analyzed in Santa Virgínia and Joinville populations. In Boraceia, seven variables exhibited significant differences between *V. inflata* (VIF) and *V. incurvata* (VIC) (Table 5). In Santa Virgínia, six of ten variables did not show differences between *V. carinata* (VCA) and *Vriesea* putative hybrid (VPH) (Table 5). Moreover, the variable that presented the most differentiation between species was the second internode's length of inflorescence (SIL).

In the PCA analysis, the first two components explained 79.24% (Santa Virgínia), 56.84% (Boraceia), and 79.78% (Joinville) of the variance in these populations (Figure 4; Table S4). The variables that most contributed were the width of inflorescence (IW) and the length of the second internode of inflorescence (SIL) in Santa Virgínia, length of the leaf sheath and length of floral bract (SHL; FBL) in Boraceia, and width of leaf sheath (SHW) and SHL in Joinville (Figure 4; Table S4). Three groups were highlighted in Santa Virgínia, corresponding mainly to VCA+VPH, VTP, and VSI (Figure 4A). The VAG individuals (except one) were located between VTP and VSI groups (Figure 4A). In Boraceia, we found two groups with an overlap of some individuals (Figures 4B). The Joinville populations also formed two groups, with a clear differentiation between species and no morphological overlapping (Figure 4C).

The hierarchical clustering of Santa Virgínia population presented two main groups (0.8251 of cophenetic correlation): I (VCA+VPH) and II (VTP+VSI+VAG) (Figure 5A). The Group II split into III (VSI+VAG) and IV (VTP). The Boraceia populations also showed two main groups (Cophen. Corr.: 0.7749): I (VIC) and II (VIF+VIC) (Figure 5B). Group II was constituted mainly by VIF with only three individuals of VIC. Joinville presented 0.9281 of cophenetic correlation and formed two well-defined groups, one with VCA species (Group I) and the other with VIC (Group II) (Figure 5C).

The morphological analyses corroborate with clusters assigned by STRUCTURE (Figures 2, 4, and 5), which allow us to classify the individuals in three different ways (Table 2): (1) Own Species Molecular Profile-OSMP; the hybrids presented (2) admixture molecular profile (ADXP) and (3) in few cases they presented the molecular profile characteristic of another species (ASMP).

DISCUSSION

The subtribe Vrieseinae harbors the genera *Vriesea*, *Alcantarea*, *Stigmatodon,* and *Waltillia* (Barfuss *et al*., 2016; Leme, Halbritter & Barfuss, 2017; Kessous *et al*., 2020), and its origin was estimated in 5.4–10.2 Mya (Million years ago) in the Miocene, with the crown ages in the Pliocene (5.3–2.6 Mya) (Kessous *et al*., 2020). *Vriesea* is paraphyletic in its current circumscription (Barfuss *et al*., 2016; Gomes-da-Silva & Souza-Chies, 2017; Machado *et al*., 2020), and the Brazilian lineage diverged from *Stigmatodon* in Late Miocene and started to diversify in Early Pliocene in the AF as ancestral area. The infrageneric lineages started to diversify in the Late Pliocene and Early Pleistocene (Kessous *et al*., 2020; Neves *et al*., 2020). The species complexes encountered within *Vriesea* are probably associated with its recent diversification, being responsible for the difficulty in the species' taxonomic delimitation (Costa, Gomes-da-Silva & Wanderley, 2015; Gomes-da-Silva & Souza-Chies, 2017; Machado *et al*., 2020). In addition, incomplete reproductive isolation between species within the genus has been reported (Matos *et al*., 2016; Zanella *et al*., 2016; Neri, Wendt & Palma-Silva., 2017a). It is widely known that related species, which recently diverged and occur in sympatry, have an increased hybridization chance, a phenomenon well described in plants (Soltis & Soltis, 2009; Harrison & Larson, 2014; Turchetto, Schnitzler & Freitas, 2019; Turchetto *et al*., 2020). One of our motivations for this study was the observation, by our team, of atypical and intermediate phenotypes in sympatric populations of *Vriesea*, suggesting the occurrence of hybridization. In this context, we used molecular and morphological approaches to identify the occurrence of hybridization and verify the morphological identity of sympatric species of *Vriesea*. Our results show high rates of hybridization but maintaining the morphological identity of the species.

Factors that may favor hybridization

Although certain taxa are more prone to hybridization than others (Mitchell *et al*., 2019), species are usually isolated by a number of barriers to gene flow (Charlesworth & Charlesworth, 2000). However, several factors facilitate and promote the overlap of these obstacles in sympatric populations, which will be discussed in this topic. The most obvious factor is the occurrence of weak reproductive barriers between species. In the present study, the molecular profile of the sympatric species revealed a high admixture rate (Figure 2). However, we were not able to robustly identify probable hybrid classes. The NEWHYBRIDS analysis (Table S3), despite having identified many profiles corresponding to F2 plants, was not effective in identifying several samples. The capability to classify hybrids through genetic analysis partially depends on the number of diagnostic loci detected and that the markers used are completely fixed in the two parental species (Moccia, Windmer & Cozzolino, 2007; Neri, Wendt & Palma-Silva, 2017a). In this study, most loci were not diagnostic, probably due to the sharing of ancestral polymorphism between these related species, which is common in Bromeliaceae (Palma-Silva *et al.*, 2011; Zanella *et al.*, 2016; Mota *et al.*, 2019). This difficulty in distinguishing hybrid classes was also found in other studies with some *Vriesea* species evaluated here (*V. carinata* and *V. incurvata* - Zanella *et al.*, 2016; *V. simplex* - Neri, Wendt & Palma-Silva., 2017a). One of the issues that could help to identify the type of barrier ongoing in the populations sampled in this study is the correct identification of the classes of hybrids. The rarity of F1s in hybrid populations, for example, may be associated with strong prezygotic reproductive isolation (Arnold *et al.*, 2010). This strong prezygotic isolation would minimize the formation of F1 hybrids, but once an F1 is formed, all of its descendants will be hybrid derivatives, and these can thus quickly multiply in number, producing later-generation hybrids and becoming possible the occurrence of introgression (Yan *et al.*, 2017). In this sense, despite the high admixture rate observed in our study and the hybridization reported for other *Vriesea* studied (*V. carinata* x *V. incurvata*: Matos *et al.*, 2016; Zanella *et al.*, 2016; *V. simplex* x *V. scalaris*: Neri, Wendt & Palma-Silva, 2017a) we cannot be categorical in saying which of the barrier type is weak.

The pollinator specialization and phenological isolation are related to pre-pollination (pre-zygotic) barriers and may reduce or prevent hybridization events. The pollinator specialization occurs when plants with different floral syndromes favor the decrease of pollinator visits (Tiffin, Olson & Moyle, 2001; Baack *et al.*, 2015). The phenological isolation is associated with differences in the reproductive period of species (Baack *et al.*, 2015). Wendt *et al.* (2008) studied the phenology and flower visitors of 42 sympatric bromeliads (13 *Vriesea* species, including *V. simplex*) and revealed a lack of pre-pollination barriers (pre-zygotic mechanisms) to avoid interspecific breeding, with high flowering overlapping and flower visitors sharing between these species. In the bromeliads the hummingbirds are among the most frequent pollinators (Benzing, 2000; Zanella *et al*., 2012). The hummingbirds *Phaethornis eurynome* Lesson and *Ramphodon naevious* Dumont were observed visiting *V. carinata*, *V. incurvata*, and *V. simplex* (Araujo, Fischer & Sazima, 1994; Machado & Semir, 2006; Wendt *et al*., 2008; Silva & Piratelli, 2014). Moreover, in a study of plant–pollinator network at Santa Virgínia Station, Vizentin-Bugoni, Maruyama & Sazima (2014) showed that the hummingbird *P. eurynome* was the only pollinator of the six species of *Vriesea* occurring in that area, including *V. carinata*, *V. simplex* and *V. taritubensis* var. *patens* (treated at that time as *V. incurvata*). We did not make methodological observations of pollinators in the present study; however, the floral features shared between the species studied here (see Introduction), and based on the aforementioned studies, it is likely that these species share pollinators, which would facilitate the hybridization.

The blooming period of these *Vriesea* species varies greatly, with significant overlap between most species (*V. carinata*: April to September; *V. taritubensis* var. *patens*: December to February; *V. incurvata*: September to May; *V. inflata*: February to November; *V. simplex*: all year with small breaking (Araujo, Fischer & Sazima, 1994; Machado & Semir, 2006; Wendt *et al*., 2008; Silva & Piratelli, 2014; Martínez, 2016; Neves *et al*., 2018). We did not find, in the literature, the flowering period of *V. agostiniana*, but, according to our observations in the field, this species blooms in March in the observed population (data not shown). We confirmed the sequential and overlapping flowering reported in the literature in the three sympatric populations analyzed in our field expeditions. The occurrence of two or more species with overlapping blooming is common in sympatric bromeliads populations (Wendt *et al*., 2008). The overlap of flowering time among species causes a high overall flower abundance, making this period an effective food source for pollinators, leading to increased pollinators and, consequently, interspecific pollen transfer (Wendt *et al*., 2008, Zanella *et al*., 2016, Ambroise *et al*., 2020).

Considering the aspects of reproductive biology, it seems evident that reproductive systems that require crossbreeding (i.e. higher outcrossing rates) may be genetically predisposed to hybridize (Mitchell *et al*., 2019). The bromeliads have different reproductive strategies, such as specialized floral features (herkogamy and dichogamy), which prevent spontaneous self-fertilization and favoring outcrossing (Martinelli, 1994; Benzing, 2000; Zanella *et al*., 2012). Concerning the target species of this study, *V. carinata*, *V. incurvata* and *V. simplex* were described as self-compatible (Martinelli, 1994; Wendt *et al*., 2008; Matallana *et al*., 2010; Wolowski *et al*., 2013), being *V. simplex* herkogamous and dependent on a pollinator visit to reproduce (Neri *et al*., 2017b; Souza *et al*., 2017). Neri *et al*. (2017b) detected variations in reproductive systems and floral traits in sympatric populations of *V. simplex* and *V. scalaris*. In sympatric populations, *V. scalaris* tends to have higher selfing rates than in allopatric ones, suggesting a potential pre-pollination (pre-zygotic) barrier that maintain species boundaries even when hybridization occurs (Neri, Wendt & Palma-Silva, 2017a).

In order to increase the discussion on reproductive barriers between the target species in this study, we carried out controlled pollination experiments between them, including putative hybrids (VPH). Despite the effort to obtain crossbreeding between all species, only five out of the 15 possible combinations were made (plus three control pollinations; Table S2). Of these five crosses, only those between *V. carinata* and *V. incurvata*, *V. simplex* and *V. agostiniana*, and *V. agostiniana* and *V. incurvata* produced fruits (Table S2). Although the experiment should be repeated to increase the number of crosses, these results indicate that the species of this group may cross and produce seeds, as already reported by other authors that studied *Vriesea* species (Matallana *et al*., 2016; Neri *et al*., 2017b; Souza *et al*., 2017). Matallana *et al*. (2016) evaluated the pollen tube growth in cross experiments with 13 sympatric bromeliad species (three *Vriesea* species) and observed 73.5% of heterospecific pollen inhibition, indicating a strong pre-zygotic mechanism (post-pollination). However, in some cases, it was incomplete, with 26.5% of crosses resulting in complete pollen tube development, allowing hybridization.

Another factor that may be involved in increasing hybridization rates is environmental disturbance. Over fifty years ago, Anderson & Stebbins-Jr. (1954) predicted that human disturbance would create novel and suitable habitats for newly formed hybrids, allowing them to establish and persist (Guo, 2014). Different anthropogenic disturbance forms have been hypothesized to increase taxa's hybridization rates (Guo, 2014; Ortego, Gugger & Sork, 2017). The disturbance hypothesis postulates that habitat alterations increase hybridization opportunities via the breakdown of premating reproductive isolating mechanisms and/or creating environmental gradients with novel or intermediate niches where hybrids outperform parental species (Ortego, Gugger & Sork, 2017). Examples of anthropogenic disturbances associated with increased hybridization rates include land-use alterations and climate change, among other factors (Gilman & Behm, 2011; Ortego, Gugger & Sork, 2017). Although natural hybridization processes have been described for different species of bromeliads (Gardner, 1984; Gonçalvez & Azevêdo-Gonçalves, 2009; Wendt, Paz & Rios, 2000; Wendt *et al*., 2001; Schulte *et al*., 2010; Palma-Silva *et al*., 2011, 2015; Matos *et al*., 2016; Zanella *et al*. 2016; Neri, Wendt & Palma-Silva, 2017a; Hirsch *et al*., 2020), the high rates of hybridization observed in the present study deserves a closer look to identify the main factors involved in this finding. Although two of the three locations sampled here are located in protected areas (Table 1), many anthropogenic impacts were identified and may have contributed to increase the hybridization rate (see Materials and Methods section). In the same sense of this hypothesis, the hybrids of *Vriesea* described by Matos *et al*. (2016) were located in disturbed areas. Moreover, in the family Rosaceae several genera seem to hybridize readily in areas that had been disturbed by frequent fires, clearing, or grazing (Wiegand, 1935). Even more, hybrids between *Banksia hookeriana* and *B. prionotes* (Proteaceae) were observed in 12 disturbed populations but not in undisturbed vegetation (Lamont *et al*., 2003). Therefore, the occurrence of hybridization among the studied species in undisturbed areas is a hypothesis that remains to be tested.

Diversity and genetic structure of populations

In general, both sympatric and allopatric populations showed genetic diversity indices similar to those reported in other, or the same species, of *Vriesea* from different populations (Palma-Silva *et al*., 2009; Zanella *et al*., 2016; Soares *et al*., 2018). Besides, there is a clear trend of increasing levels in hybrids (Table 3). High indices of diversity (i.e. number of alleles, alleles richness and observed and expected heterozygosities) have been observed in hybrid individuals in different species (Pinheiro *et al*., 2010; Mota *et al*., 2019). The higher genetic diversity in hybrids may result from allelic combinations from the parental species (Pinheiro *et al*., 2010). On the other hand, all species (except *V. inflata*) showed significant departure from the HWE (Table 3) because of the high proportion of homozygotes. This pattern is common in bromeliads when used nuSSR markers (Palma-Silva *et al*., 2009; Zanella *et al*., 2011, 2012, 2016; Soares *et al*., 2018; Aguiar-Melo *et al*., 2019). Factors such as genetic drift, inbreeding, and the proper plant's mating system may be related to the heterozygote deficit (Hamrick & Godt, 1996). Most of the species analyzed here are self-compatible (Martinelli, 1994; Wendt *et al*., 2008; Matallana *et al*., 2010; Wolowski *et al*., 2013). In this way, the excess of homozygotes may occur due to selfing or biparental inbreeding as observed in *Vriesea gigantea*, which has a mixed mating system and high biparental inbreeding (Paggi *et al*., 2015). No significant departure from HWE for *V. inflata* was observed in both sympatric and allopatric populations, despite the low number of individuals analyzed (Table 3). The excess or deficit of heterozygotes observed within a population varies markedly with mating systems, and the high rates of heterozygotes may suggest a self-incompatibility system (Stoeckel *et al*., 2006). We do not have information on the *V. inflata* mating system, but our results indicate that this is an interesting subject for future studies.

AMOVA results showed low genetic differentiation among species within each locality (Table 4), reflecting the interspecific gene flow recorded in the present study. Even more, the effective number of migrants (N_em) between the species and hybrids was high (Figure 3), corroborating the permeability of the reproductive barriers of the studied sympatric species. Variable levels of interspecific gene exchange have been identified in sympatric populations of other bromeliads. Neri, Wendt & Palma-Silva (2017a) found low levels of interspecific gene flow (N_em = 0.05–0.24) in sympatric populations of *Vriesea* and suggest that the studied species are indeed independent evolutionary units. On the other hand, Mota *et al.* (2019) found different interspecific gene flow levels according to the population of *Pitcairnia* studied, N_em varying from 0.06 to 0.33. In general, the rates found in the present study were much higher (0.261 – 4.499).

Morphological identity, difficulty in identifying hybrids by morphology and "hybrid swarm"

The morphological analysis differentiated well the species, forming three main groups in Santa Virgínia (*V. carinata*; *V. simplex* and *V. taritubensis* var. *patens* - Figure 4A; 5A), and two in Boraceia (Figure 4B; 5B) and Joinville (Figure 4C; 5C), corroborating with *K* assigned by STRUCTURE (Figure 2; S1) in all localities. In Santa Virgínia, *V.*

agostiniana differs from other species only by the length of inflorescence (IL) (Table 5), and grouped with *V. simplex* and *V. taritubensis* var. *patens* (Figure 4A) or together with *V. simplex* (Figure 5A), corroborating its hybrid molecular profile (Figure 2A). Moreover, of the individuals collected and assigned by morphology as parental purebreds, 45% showed a hybrid profile by STRUCTURE in Santa Virgínia (including VPH), 42.32% in Boraceia and 22.86% in Joinville (Table 2). In some of these individuals (except VPH), we observed features slightly different from usual, but they can still be classified as parental species by morphology. Zanella *et al*. (2016), studying the hybridization between *V. carinata* and *V. incurvata*, also observed discrepancies between morphological and molecular assignment. In that study, of the 16 putative morphological hybrids observed in the field, only six were confirmed by STRUCTURE. In addition, 11 individuals identified in the field as parental purebreds showed intermediated admixture proportion, indicating a hybrid ancestry. In the same sense, Neri, Wendt & Palma-Silva (2017a) did not observe a clear morphological distinction between parental and hybrids between *V. simplex* and *V. scalaris*. In addition to these inconsistencies, four individuals from Santa Virgínia identified with atypical morphology (*Vriesea* putative hybrid, VPH) presented an intermediate admixture molecular profile by STRUCTURE analysis (Table 2; Figure 2A). Among them, one has a mixture between *V. carinata* and *V. taritubensis* var. *patens* molecular profile, whereas the others exhibit an admixture of the profiles assigned as *V. carinata*, *V. taritubensis* var. *patens* and *V. simplex* (Figure 2A). Besides, *Vriesea* putative hybrid grouped with *V. carinata* in morphological analyses (Figure 4A; 5A), sharing the dimensions of leaf sheath, inflorescence, peduncle bract and the length of the leaf blade (Table 5). Moreover, it is also morphologically similar in the width of inflorescence, length of peduncle and floral bracts to the *V. simplex* and *V. taritubensis* var. *patens* species (Table 5). Indeed, *Vriesea* putative hybrid differs morphologically from other species in just three variables (width of leaf blade and flower bract, and length of second internode) (Table 5). Matos *et al*. (2016) described the nothospecies *V.* × *brueggemannii* with intermediate morphological traits between *V. carinata* and *V. incurvata* parents in Atlantic Forest populations from Santa Catarina Brazilian State. The main morphological characteristics that differ the nothospecies from the parents are: width of the inflorescence and the rachis, length and width of the stigma, and the length of anthers, being this taxa very genetic similar to *V. carinata*. Our *Vriesea* putative hybrid individuals are not similar to *V.* × *brueggemannii*, showing bracts with less width and internodes more spaced (Figure 1G). Therefore, the analyses suggested that three species (*V. carinata*, *V. taritubensis* var. *patens* and *V. simplex*) are putative parental of the *Vriesea* putative hybrid individuals. This hypothesis needs further analysis to be confirmed or rejected, such as crosses experiments and analyses using plastid genome.

The incongruity between the molecular profile and the morphological identification, as well as the high number of samples with intermediate (hybrid) genetic profile, suggests the occurrence of introgression in the studied sympatric locations. Introgression is the transfer of genetic material from one species to another through hybridization and backcrossing (Soltis & Soltis, 2009). Although NEWHYBRIDS was unable to classify backcrossing individuals from our data set, the STRUCTURE analysis revealed some species with a molecular profile from another (Figure 2A; 2C; Table 2). *Vriesea agostiniana* was the only species that did not present a distinctive genetic profile, maybe due to the few individuals analyzed by STRUCTURE (Figure 2A). However, the low number of individuals present in the population and compatibility with other species (e.g. *V. simplex* - Table S2), may have led to the collapse of this species in this population, with possible introgression from *V. simplex*, a fact that was corroborated by morphological analyses (Figure 4A; 5A). Introgressive hybridization can lead to the production of recombinant genotypes that have properties different from the parents (Harrison, 1993). Anderson (1953) argued that introgression is an important source of new variation and that these variants are most likely to succeed in disturbed or changing environments. The occurrence of high rates of hybridization or introgression may suggest that we are facing hybrid swarms. The hybrid swarms can be defined as complex mixtures of parental forms, F1 hybrids, backcross types and segregation products, comprising a wide range of early and later generation hybrids (Abbott, 2017; Yan *et al.*, 2017). Within a hybrid swarm, the pure individuals or the parental species grow adjacent to F1 and advanced generation (backcross) hybrids. These extraordinary populations effectively connect the morphological, genetic and spatial gaps that otherwise distinguish species. Indeed, whole species complexes have arisen through hybridization in diverse plant groups (Floate *et al.*, 2016). The locations studied here (mainly Santa Virginia), considering the results of this study and other field observations by our team, have all the characteristics to be considered a hybrid swarm. However, this hypothesis should be tested more robustly in future studies.

MAIN CONCLUSIONS AND PROSPECTS

We showed a high contemporary gene flow between *Vriesea* species in the three localities analyzed (Santa Virgínia, Boraceia and Joinville). Moreover, we observed hybrids with atypical morphology and hybrids that looks like purebreds parental. The results suggested permeable reproductive barriers in these species. However, other approaches (e.g. cytology, genome size, more artificial crosses and seed viability) are needed to better understand which mechanisms are involved in the high interspecific gene flow encountered. The use of cytoplasmatic markers (i.e. chloroplast genome) is an interesting approach to be evaluated in these localities, aiming to understand the structure and sharing of haplotypes of these species in the past. It is also desirable to analyze other *V. agostiniana* populations (Kessous *et al*., 2018a) to test the species' genetic profile. Moreover, the anthropogenic impacts, such as farming, illegal plant extractions, and exotic plants introduction may be creating new habitats and favoring the niche invasion of one species of *Vriesea* to another, increasing the hybridization events. Therefore, the conservation of habitats is fundamental to the maintenance of species boundaries.

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Table 1. Localities studied where two ou more *Vriesea* species occur in sympatry, allopatric populations sampled, their sites and coordinates, as well species names and codes, N sampled and N evaluated in genetic and morphological analyses. SP: São Paulo Brazilian State; SC: Santa Catarina Brazilian State.

Locality	Site	Coordinates		Species	Species codes	N sampled	N evaluated	
		Lat.S	Long.W				Genet.	Morph.
SANTA VIRGÍNIA	Parque Estadual Serra do Mar, Núcleo Santa Virgínia, São Luís do Paraitinga, SP	23º20’	45º09’	*Vriesea carinata*[1]	VCA	34	30	24
				Vriesea putative hybrid	VPH	4	4	4
				Vriesea taritubensis var. *patens*	VTP	27	23	21
				Vriesea simplex	VSI	17	17	14
				Vriesea agostiniana	VAG	6	6	5
				Vriesea inflata[2]	VIF	4	4	-
UBATUBA	Parque Estadual Serra do Mar, Núcleo Picinguaba, Ubatuba, SP	23º21’	44º46’	*Vriesea simplex*[3]	VSI	15	15	-
BORACEIA	Estação Biológica da Boracéia, Salesópolis, SP	23º39’	45º53’	*Vriesea inflata*	VIF	43	16	41
				Vriesea incurvata[4]	VIC	22	10	19
JOINVILLE	Serra Dona Francisca Road, Joinville, SC	26º13’	49º03’	*Vriesea carinata*[5]	VCA	18	18	12
				Vriesea incurvata[6]	VIC	32	17	28
					TOTAL	222	160	168

[1] Used as allopatric population in comparison with Joinville
[2] Used as allopatric population in comparison with Boraceia
[3] Used as allopatric population in comparison with Santa Virgínia
[4] Used as allopatric population in comparison with Joinville
[5] Used as allopatric population in comparison with Santa Virgínia
[6] Used as allopatric population in comparison with Boraceia

Table 2. Morphological and molecular identification of *Vriesea* species in the three localities studied. The molecular profile was based on STRUCTURE results, and each sample was classified as: Own Species Molecular Profile (OSMP) and Hybrid Profiles, which is divided in Admixture Molecular Profile (ADXP) and Molecular Profile Characteristic of Another Species (ASMP).

Localities/Species	MorphoID	MolecularID		
			Hybrid Profiles	
		OSMP	ADXP	ASMP
SANTA VIRGÍNIA				
VCA	30	16	14	-
VPH	4	-	4	-
VTP	23	17	6	-
VSI	17	11	6	-
VAG	6	-	4	2
TOTAL	**80**	**44**	**36**	
BORACEIA				
VIF	16	**7**	9	-
VIC	10	8	2	-
TOTAL	**26**	**15**	**11**	
JOINVILLE				
VCA	18	14	2	2
VIC	17	13	4	-
TOTAL	**35**	**27**	**8**	

MorphoID = number of plants sampled as each taxon by morphological traits.

MolecularID = number of plants that match to each molecular profile.

Table 3. Diversity indexes estimated using nuclear microsatellites (nuSSR) in sympatric and respective allopatric populations for *Vriesea* species. Sample size (N) were based on molecular identification from STRUCTURE results, number of alleles (A), private alleles (PA), allelic richness (AR), observed (H_O) and expected (H_E) heterozygosities and inbreeding coefficient (F_{IS}). For species code and molecular identification, see Tables 1 and 2, respectively.

Localities/Species	N	A	PA	AR	H_O	H_E	F_{IS}[a]
SANTA VIRGÍNIA							
VCA	16	54	5	4.020	0.39962	0.57650	0.314**
VTP	17	87	15	6.448	0.65720	0.82464	0.209**
VSI	11	57	3	4.662	0.51944	0.73063	0.300**
HYBRIDS[b]	36	99	10	6.056	0.55240	0.78752	0.302**
Allopatric							
VCA	14	49	4	3.826	0.47024	0.57407	0.187**
VSI	15	65	6	4.641	0.55195	0.63140	0.130*
BORACEIA							
VIF	7	30	2	2.581	0.65306	0.69074	0.059
VIC	8	30	3	2.758	0.51190	0.73074	0.335**
HYBRIDS[b]	11	46	6	3.095	0.67201	0.82499	0.194**
Allopatric							
VIF	4	18	0	2.160	0.34524	0.58061	0.442
VIC	6	32	2	2.899	0.58571	0.78600	0.276*
JOINVILLE							
VCA	14	39	5	3.127	0.45000	0.54312	0.177**
VIC	13	61	13	5.000	0.53945	0.81862	0.352**
HYBRIDS[b]	8	48	5	4.367	0.42012	0.75116	0.463**
Allopatric							
VCA	15	43	7	3.205	0.33000	0.54633	0.405**
VIC	8	34	6	2.590	0.39815	0.71650	0.515**

[a] Inbreeding coefficient (F_{IS}) departed significantly from Hardy–Weinberg equilibrium (HWE) at **$P < 0.001$; * $P < 0.05$.

[b] Hybrids = individuals that showed admixture molecular profile or molecular profile characteristic of another species from STRUCTURE results, including VPH and VAG in Santa Virgínia location.

Table 4. Analyses of Molecular Variance (AMOVA) for sympatric populations based on nuclear microssatellities (nuSSR). The Species property were based on STRUCTURE results (see Table 2).

	Localities		
	SANTA VIRGÍNIA	BORACEIA	JOINVILLE
Source of variation	Percentage of variation		
Among Species	10.08	9.98	15.24
Within Species	89.92	90.02	84.76
F_{ST}	0.101*	0.099*	0.152*

F_{ST}, fixation index; $^*P < 0.001$.

Table 5. Description matrix reporting the median, minimum and maximum values of each variable per species in each locality of *Vriesea* studied. Differences among species were accessed using Kruskal-Wallis test (H* = $P < 0.05$) followed by multiple comparison test (Mann Whitney pairwise). Values with no significant differences ($P > 0.05$) are represented by the same superscript letters. Species codes and N evaluated, see Table 1.

	Localities and Species		
Variables	SANTA VIRGÍNIA	BORACEIA	JOINVILLE
Length of the leaf sheath (SHL)	*H* = 43.38*	*H*= 0.2919	*H*=19.21*
	VCA 6.35 (4.0-8.7)^{a}	VIF 9.4 (5.6-12.2)	VCA 7.0 (5.0-8.2)
	VPH 5.85 (3.8-7.2)^{a}	VIC 9.3 (7.2- 12.9)	VIC 8.8 (7.1-13.8)
	VTP 10.3 (7.1-14.6)^{b}		
	VSI 9.15 (6.6-12.7)^{b}		
	VAG 10.0 (8.4-11.1)^{b}		
Width of leaf sheath (SHW)	*H* = 42.98*	*H*= 6.917*	*H*= 15.52*
	VCA 3.3 (2.2–4.8)^{a}	VIF 4.5 (3.0–5.4)	VCA 3.75 (2.6–4.8)
	VPH 4.0 (2.7–5.0) $^{a, b}$	VIC 5.1 (3.7–5.9)	VIC 4.95 (3.5–6.3)
	VTP 5.3 (3.6–6.1)^{c}		
	VSI 5.2 (4.3–5.8)^{c}		
	VAG 5.5 (4.8–5.7)^{b,c}		
Length of leaf blade (BL)	*H* = 33.25*	*H*= 0.6698	*H*=22.58*
	VCA 12.75 (8.9–22.2)^{a}	VIF 18.9 (11.2–38.3)	VCA 13.25 (10.1–17.3)
	VPH 13.4 (9.2–17.2)^{a}	VIC 20.3 (12.0–29.3)	VIC 20.9 (14.9–34.5)
	VTP 22.0 (10.2–34.2)^{b}		
	VSI 19.35 (12.0–32.2)^{b}		
	VAG 21.7 (17.2–32.2)^{b}		
Width of leaf blade (BW)	*H* = 47.71*	*H*= 13.19*	*H*=23.86*
	VCA 1.35 (1.0–2.0)^{a}	VIF 2.0 (1.5–2.6)	VCA 1.4 (0.9–2.0)
	VPH 1.8 (1.6–2.0)^{b}	VIC 2.3 (2.0–3.0)	VIC 2.5 (1.9–2.9)
	VTP 2.4 (1.7–3.1)^{c}		
	VSI 2.55 (2.0–3.3)^{c}		
	VAG 2.4 (2.2–2.9)^{c}		
Length of inflorescence (IL)	*H* = 40.19*	*H*= 3.728	*H*=19.21*
	VCA 29.55 (24.4–37.5)^{a}	VIF 26.5 (13.9–34.0)	VCA 24.55 (17.6–30.8)
	VPH 37.15 (28.5–40.1)^{a}	VIC 31.0 (14.3– 55.1)	VIC 36.5 (24.1–48.7)
	VTP 57.0 (21.2–88.4)^{b}		
	VSI 49.3 (32.0–76.1)^{b}		
	VAG 75.8 (57.7–82.6)^{c}		
Width of inflorescence (IW)	*H* = 46.77*	*H*= 5.72*	*H*=11.92*
	VCA 4.5 (3.1–5.2)^{a}	VIF 4.1 (2.6–6.0)	VCA 3.0 (2.3–4.7)
	VPH 3.55 (3.0–5.0)$^{a, b}$	VIC 3.7 (2.3–5.4)	VIC 4.2 (2.8–5.5)
	VTP 3.1 (1.7–4.5)^{b}		
	VSI 8.6 (7.0–9.3)^{c}		
	VAG 5.7 (3.5–9.5)$^{a, c}$		
Length of second internode of inflorescence (SIL)	*H*= 56.12*	*H*= 16.74*	*H*=24.44*
	VCA 0.5 (0.4–0.9)^{a}	VIF 0.5 (0.4–0.8)	VCA 0.5 (0.4–0.7)
	VPH 1.3 (1.1–1.6)^{b}	VIC 1.0 (0.4–1.9)	VIC 1.3 (0.7–1.7)
	VTP 1.9 (1.1–3.1)^{c}		
	VSI 2.75 (1.5–4.0)^{d}		
	VAG 3.4 (2.1–4.1)^{d}		

Length of peduncle bract (PBL)	*H*= 41.07*	*H*=24.03*	*H*=21.61*
	VCA 2.5 (1.8–3.7)^{a}	VIF 2.6 (1.7–3.4)	VCA 2.4 (2.0–3.0)
	VPH 2.7 (2.1–3.7)$^{a, b}$	VIC 3.6 (2.5–4.4)	VIC 3.9 (2.2–4.7)
	VTP 3.8 (2.7–4.5)^{c}		
	VSI 3.25 (2.9–4.0)^{b}		
	VAG 2.9 (2.9–4.1)$^{b, c}$		
Lenght of flower bract (FBL)	*H*= 52.9*	*H*=31.56*	*H*=24.59*
	VCA 3.0 (2.4–3.6)^{a}	VIF 4.1 (3.6–4.9)	VCA 2.8 (2.3–3.2)
	VPH 4.0 (3.4–4.8)^{b,d}	VIC 5.0 (4.0–5.6)	VIC 5.35 (4.3–6.0)
	VTP 5.0 (3.8–5.8)^{c}		
	VSI 4.25 (3.5–4.7)^{b,d}		
	VAG 4.8 (4.4–5.5)^{b,c}		
Width of flower bract (FBW)	*H*= 52.37*	*H*=19.38*	*H*=24.59*
	VCA 0.65 (0.5–0.8)^{a}	VIF 1.6 (1.3–2.3)	VCA 0.5 (0.4–0.7)
	VPH 0.8 (0.7–1.1)^{b}	VIC 1.9 (1.2–2.3)	VIC 1.9 (1.6–2.2)
	VTP 1.4 (0.9–1.6)^{c}		
	VSI 1.2(1.0–1.4)^{d}		
	VAG 1.3(1.2–1.5)$^{c, d}$		

Table S1. Nuclear microssatellities markers (nuSSR) used in genetic analyses with total N of species in populations and how much work in each locus. The N of species and hybrids are based on STRUCTURE results. For species code and molecular identification, see Tables 1 and 2, respectively.

	N	Vcar 91	Vcar 95.1	Vcar 115	Vcar 143	Vcar 153	Vcar 258	Vcar 280.1	Vcar 293	VgB12	Ai4.03	e6	e6b
Localities/Species													
SANTA VIRGÍNIA													
VCA	**16**	12	16	11	16	16	16	16	16	16	16	16	16
VTP	**17**	9	13	16	9	17	11	17	11	12	16	16	11
VSI	**11**	9	11	11	10	11	11	11	11	11	11	11	11
HYBRIDS[a]	**36**	27	35	29	33	32	32	36	32	34	36	35	33
Allopatric													
VCA	**14**	13	14	14	14	14	14	14	14	14	14	14	14
VSI	**15**	15	13	15	15	15	14	15	15	15	15	15	15
BORACEIA													
VIF	**7**	7	X	X	7	7	X	7	X	X	7	7	7
VIC	**8**	3	X	X	8	8	X	8	X	X	6	8	2
HYBRIDS[a]	**11**	10	X	X	11	11	X	11	X	X	9	9	10
Allopatric													
VIF	**4**	4	X	X	4	3	X	4	X	X	4	4	4
VIC	**6**	5	X	X	5	6	X	6	X	X	6	6	6
JOINVILLE													
VCA	**14**	13	14	14	14	14	X	14	X	14	14	14	14
VIC	**13**	11	13	13	11	13	X	12	X	9	11	11	11
HYBRIDS[a]	**8**	7	7	8	5	5	X	7	X	7	8	6	7
Allopatric													
VCA	**15**	11	15	10	15	15	X	15	X	15	15	15	15
VIC	**8**	3	0	2	8	8	X	8	X	2	6	8	2

[a] Hybrids = individuals that showed admixture molecular profile or molecular profile characteristic of another species from STRUCTURE results, including VPH and VAG in Santa Virgínia location.

Table S2. Artificial crosses between *Vriesea* species. For species codes, see Table 1.

Artificial Crosses	Plants	Crosses	Fruits
VCA x VTP	-	-	-
VCA x VSI	-	-	-
VCA x VAG	3	5	0
VCA x VIF	-	-	-
VCA x VIC	16	70	7
VTP x VSI	-	-	-
VTP x VAG	-	-	-
VTP x VIF	-	-	-
VTP x VIC	-	-	-
VSI x VAG	3	14	7
VSI x VIF	-	-	-
VSI x VIC	3	9	0
VAG x VIF	-	-	-
VAG x VIC	3	16	4
VIF x VIC	-	-	-
Controls:			
VCA x VCA	11	27	4
VTP x VTP	-	-	-
VSI x VSI	2	3	0
VAG x VAG	-	-	-
VIF x VIF	-	-	-
VIC x VIC	6	5	0
Hybrids:			
VPH 60 x VPH 58	2	23	0
VPH 60 x VPH 80	2	12	0
VPH 58 x VPH 80	2	1	0
VPH 60 x VCA	9	27	1
VPH 58 x VCA	5	11	3
VPH 80 x VCA	2	2	0
Total		225	26

Table S3. Bayesian admixture proportions (*Q*) of hybrid individuals (assigned by STRUCTURE) estimated in NEWHYBRIDS for Santa Virgínia population. The three possible combinations of parents were present (VCA+VSI; VCA+VTP; VSI+VTP). NA: no assigned in any class; P1, P2 and F2: class assigned. The threshold was: $q \geq 0.90$. For species code, see Table 1.

Hybrid Numbers	VCA+VSI	VCA+VTP	VSI+VTP
34	NA	F2	F2
35	NA	P1	NA
48	NA	NA	F2
59	NA	P1	F2
70	NA	F2	F2
78	P1	NA	F2
82	F2	F2	P1
103	NA	NA	NA
143	F2	F2	NA
155	F2	F2	F2
156	F2	F2	F2
193	NA	P1	NA
197	F2	NA	NA
200	NA	NA	F2
63	P2	P2	P1
87	NA	NA	F2
91	NA	F2	NA
98	NA	F2	P1
142	P2	P2	NA
151	P2	P2	P1
58	NA	F2	NA
60	P1	NA	NA
75	F2	F2	F2
80	NA	F2	NA
137	F2	F2	NA
140	F2	F2	F2
145	NA	F2	NA
153	F2	F2	F2
138	P2	NA	P1
159	P2	P2	P1
39	NA	NA	F2
47	F2	F2	F2
52	P2	P2	NA
53	NA	P2	F2
64	P2	P2	P1
186	F2	F2	F2

Table S4. The most important principal components (PC) of localities with loadings per variable and percentage of variance. Variables code, see Table 5.

Variables	Localities					
	SANTA VIRGÍNIA		BORACEIA		JOINVILLE	
	PC1	PC2	PC1	PC2	PC1	PC2
SHL	0.33285	-0.091706	0.25834	0.52504	0.26782	0.46027
SHW	0.35235	0.034018	0.34282	0.41859	0.24967	0.59163
BL	0.29704	-0.091974	0.069726	0.40018	0.282	-0.35568
BW	0.34078	0.069294	0.39362	0.077556	0.33021	0.20672
IL	0.31902	0.047069	0.29736	-0.17103	0.29755	-0.33891
IW	0.085737	0.86019	0.041653	0.2829	0.27171	-0.29543
SIL	0.31561	0.36099	0.3452	-0.40538	0.3525	0.037675
PBL	0.32171	-0.23202	0.39498	-0.2781	0.34141	-0.23477
FBL	0.34386	-0.20168	0.4234	-0.16652	0.37545	-0.057024
FBW	0.36086	-0.10104	0.33367	0.065299	0.36501	0.083426
Variance (%)	67.496	11.744	41.592	15.249	66.145	13.638
Total Variance (%)	79.24		56.84		79.78	

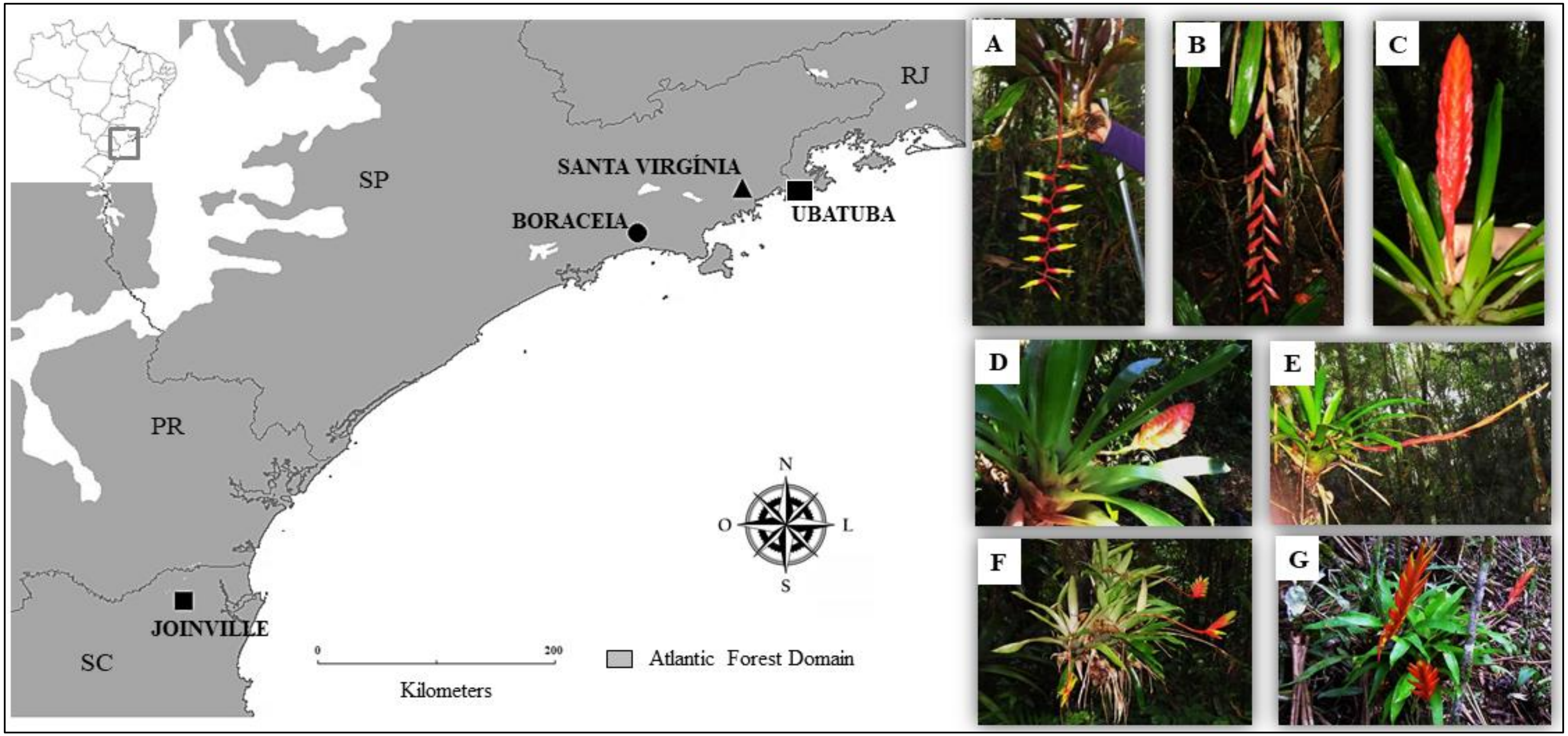

Figure 1. Map with studied localities (Santa Virgínia, Boraceia, Joinville and Ubatuba) in southeast and south Brazil in the Atlantic Forest domain and exemplaries of species collected in these localities: A: *Vriesea simplex*; B: *V. agostiniana*; C: *V. incurvata*; D: *V. inflata*; E: *V. taritubensis* var. *patens*; F: *V. carinata*; G: *Vriesea* putative hybrid. SC: Santa Catarina, PR: Paraná, SP: São Paulo, RJ: Rio de Janeiro Brazilian States. For specific species collected in each population, see Table 1.

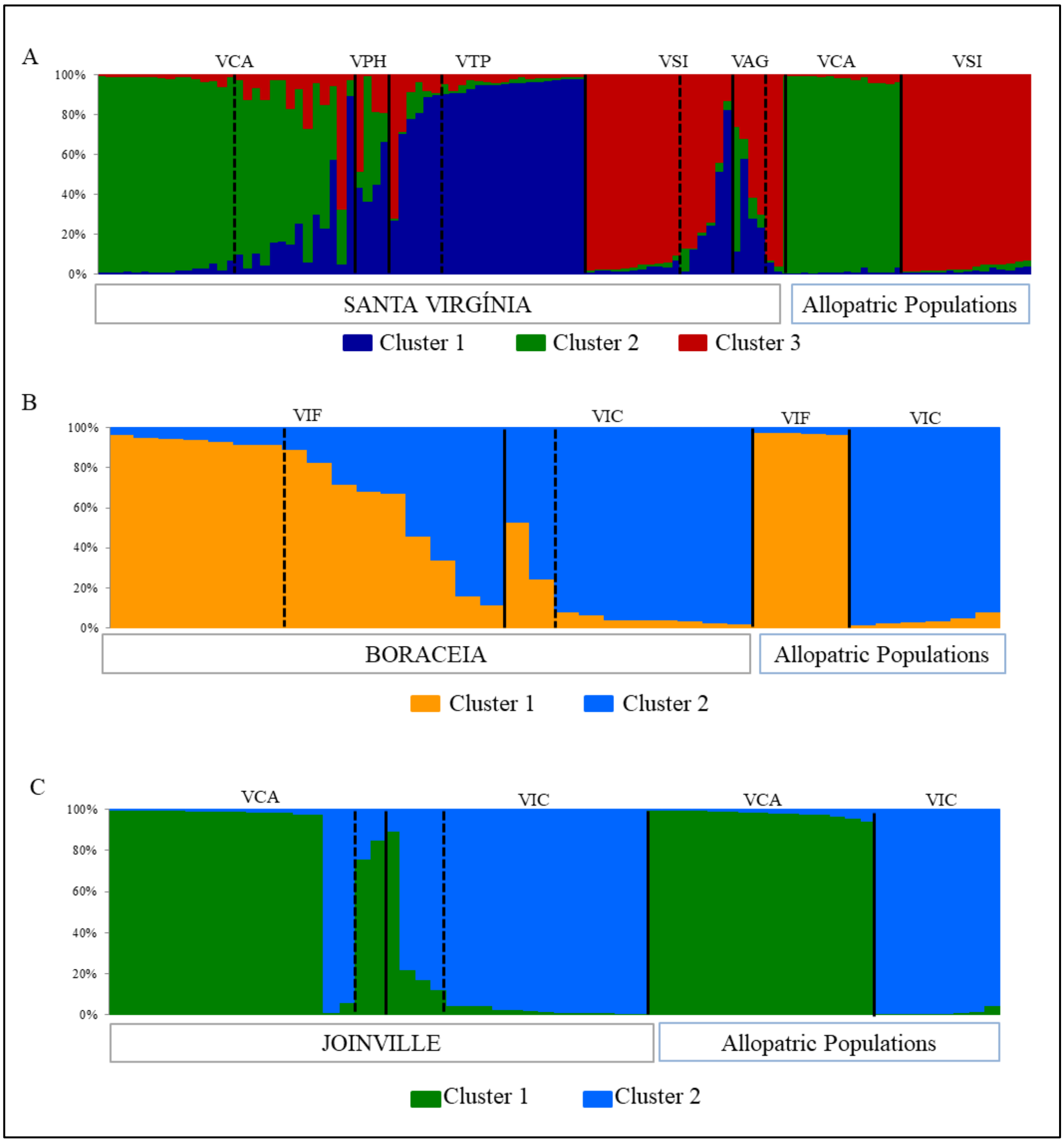

Figure 2. Population structure using Bayesian assignment analysis for A) Santa Virgínia, $K = 3$ based on 12 nuSSR loci; B) Boraceia, K=2 based on 7 nuSSR and C) Joinville, K=2 based on 10 nuSSR. The individuals between dashed lines correspond to the admixture molecular profiles, classified by $0.10 < q < 0.90$ threshold. The species, in each locality and populations, are separated by solid lines. For species code, see Table 1**.**

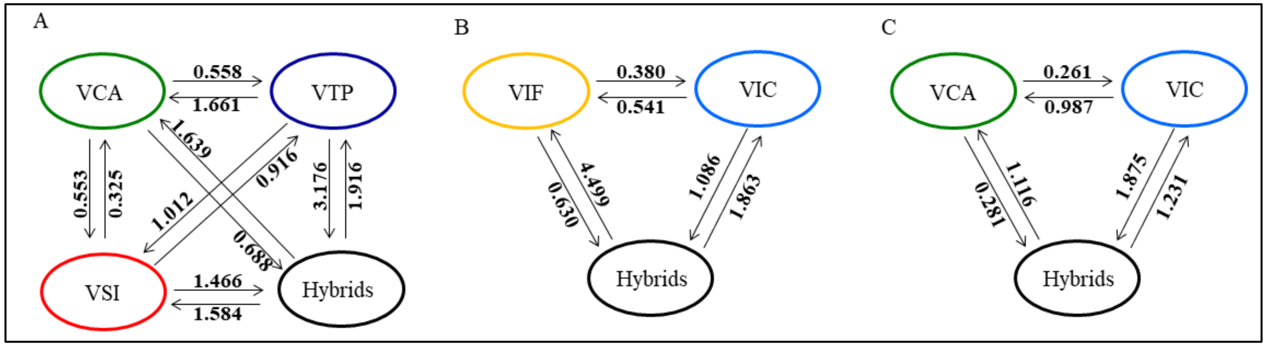

Figure 3. Bidirectional migration rates (effective number of migrants, N_em) for nuSSR markers between *Vriesea* species in sympatric populations: A) Santa Virgínia, B) Boraceia, C) Joinville. Species code, see Table 1. The arrow represents the direction of migration.

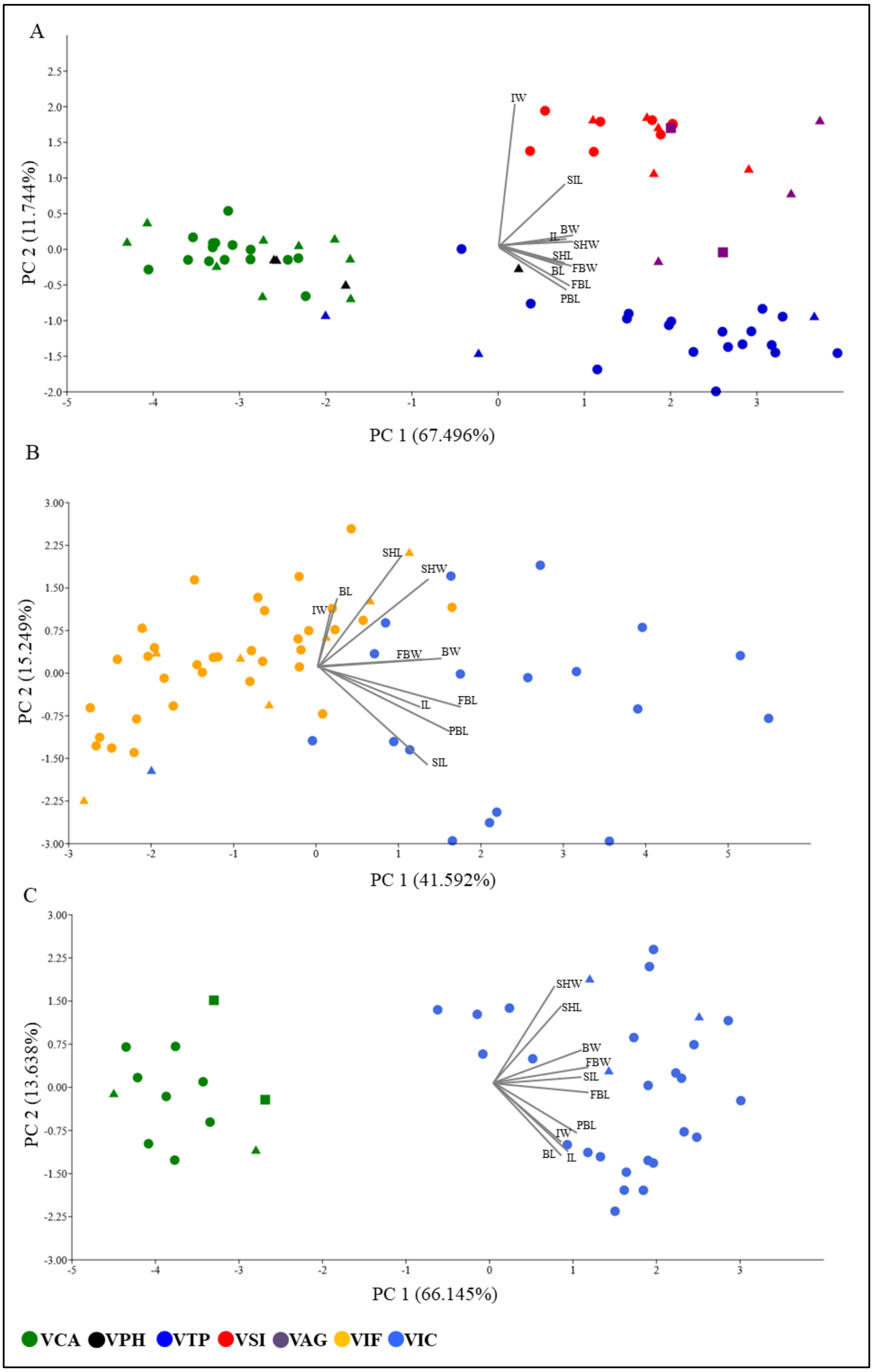

Figure 4. Principal components analysis using morphological variables from Santa Virgínia (A), Boraceia (B), and Joinville (C). The individuals also analyzed for their genetic profile by Structure are represented by triangles and squares. The triangle represents individuals with a mixed molecular profile, and the squares represent those individuals that have a molecular profile characteristic of another species.

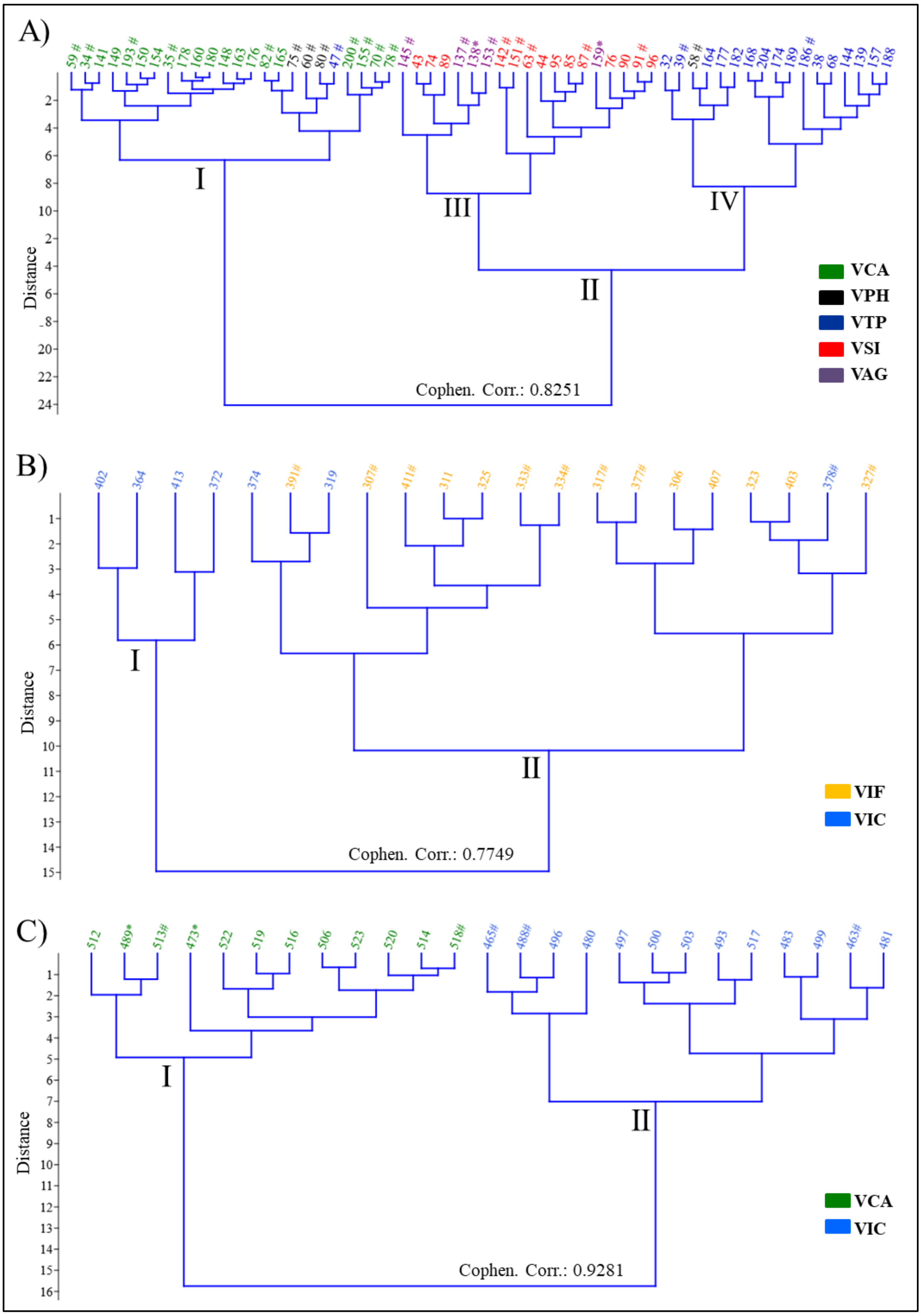

Figure 5. Hierarchical Clustering morphological analysis based on Ward's method for: A) Santa Virgínia, B) Boraceia and C) Joinville. The symbols represent individuals with admixture molecular profile (#) and another species molecular profile (*) according STRUCTURE results. For species code, see Table 1.

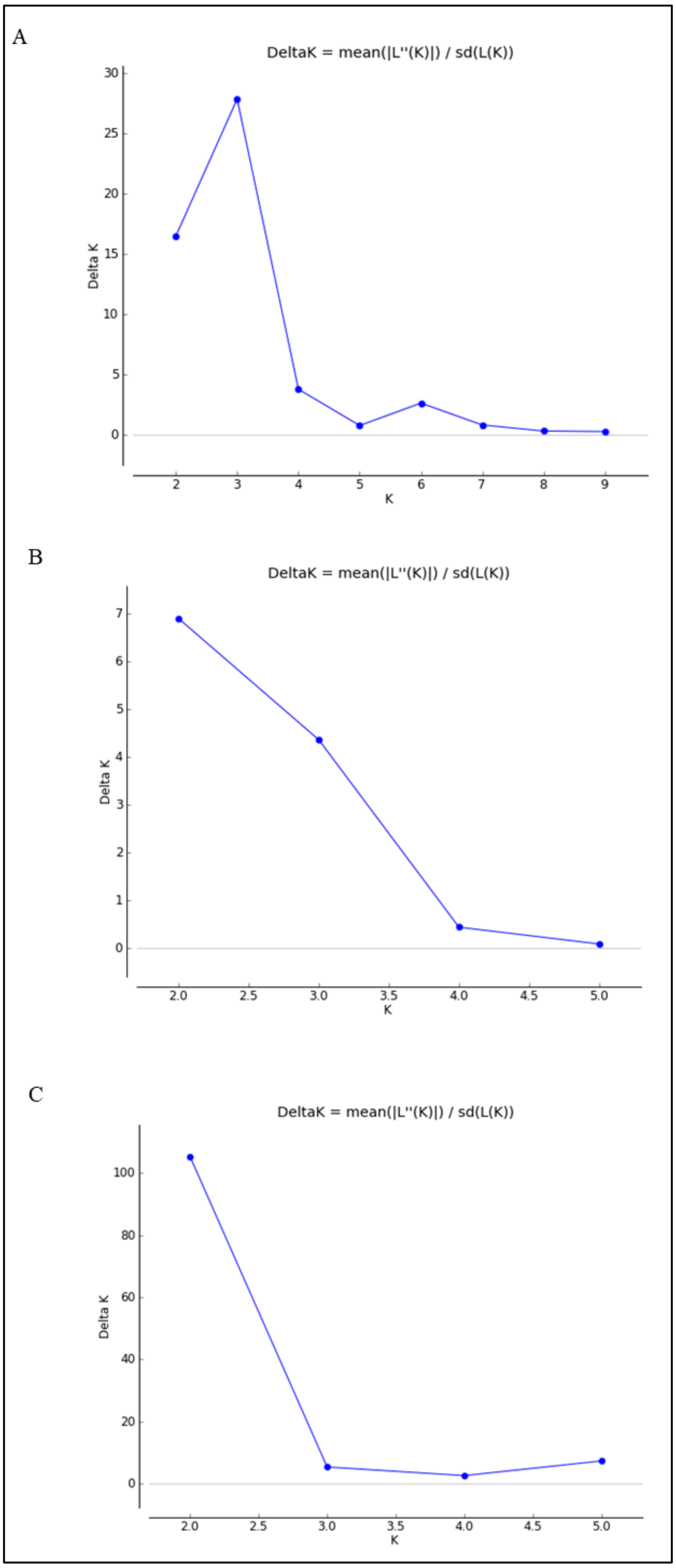

Figure S1. Mostly likely number of clusters (*K*) by Structure Harvester, calculated by following the Δ*K* method proposed by Evanno, Regnaut & Goudet (2005), for the A) Santa Virgínia; B) Boraceia and C) Joinville.

CAPÍTULO IV

CONSIDERAÇÕES FINAIS

CONSIDERAÇÕES FINAIS

A Mata Atlântica é reconhecida como o principal centro de diversidade do gênero *Vriesea* (Martinelli *et al*., 2008; Costa *et al*., 2014), além de ser considerada, juntamente com o Chaco, o berço das linhagens ancestrais da subtribo Vrieseinae, ao qual *Vriesea* pertence (Barfuss *et al*., 2016; Leme *et al*., 2017; Kessous *et al*., 2020). Sendo assim, esse bioma pode ser considerado um interessante laboratório para estudos evolutivos do gênero. Na presente tese, foram estudadas espécies do "grupo das infladas", um dos grupos monofiléticos presentes na Mata Atlântica (Costa *et al*., 2015; Gomes-da-Silva e Souza-Chies, 2017), a fim de compreender os padrões históricos (Capítulo II) e o relacionamento interespecífico destas espécies (Capítulo III), vindo à somar junto a outros trabalhos (Matos *et al*., 2016; Zanella *et al*., 2016; Neri *et al*., 2017b; Neves *et al.*, 2018; 2019; 2020) na compreensão dos processos evolutivos destas espécies e do gênero *Vriesea* como um todo.

A espécie *Vriesea incurvata* originalmente tinha sua distribuição geográfica descrita como sendo do estado do Rio Grande do Sul ao Rio de Janeiro (Smith e Downs, 1977; Martinelli, 1994; Martinelli *et al*., 2008). No entanto, em um trabalho de morfometria, com espécies que formam o chamado "complexo *V. incurvata*", os indivíduos do norte de São Paulo e Rio de Janeiro foram recircunscritos como *V. taritubensis* e suas variedades, e a distribuição de *V. incurvata* ficou delimitada do centro-sul de SP ao RS (Neves *et al*., 2018). Além do mais, a partir de sequencias plastidiais observou-se que não há compartilhamento de haplótipos entre *V. incurvata* e as variedades de *V. taritubensis*, e também a ocorrência de uma quebra filogeogeográfica entre estas espécies na região da Serra da Bocaína em SP, por volta de 5 Ma, no Plioceno (Neves *et al*., 2019). Tais evidências históricas, somadas ao fato de *V. incurvata* apresentar uma das maiores distribuições geográficas do "grupo das infladas" (Neves *et al*., 2018) motivou nossa equipe a investigar os padrões históricos, de estrutura e diversidade genética desta espécie em populações da porção sul da Mata Atlântica, região à qual ela é endêmica. Essa investigação culminou no segundo capítulo desta tese, no artigo intitulado "Ecological niche modeling and a lack of phylogeographic structure in *Vriesea incurvata* suggest historically stable areas in the southern Atlantic Forest", publicado no ano de 2019 na revista American Journal of Botany. Em resumo, os resultados dos marcadores plastidiais indicaram uma falta de estruturação filogeográfica com um alto compartilhamento de haplótipos entre as populações e uma diversidade genética que pode ser considerada moderada/alta nas populações estudadas. Na modelagem de nicho, observamos que as áreas mais adequadas para a ocorrência da espécie nos períodos do Último Máximo Glacial e Holoceno Médio não variaram muito em relação a distribuição atual. Assim, juntamente com os resultados demográficos, concluímos que após a colonização da porção sul da Mata Atlântica, *V. incurvata* se manteve estável, não tendo sua estrutura populacional afetada pelas mudanças climáticas do Quaternário, indicando que a porção sul da Mata Atlântica foi estável durante este período. Já os marcadores microssatélites nucleares também indicaram uma moderada/alta diversidade genética e um elevado número de migrantes entre as populações; apesar disso foi observada uma ligeira estruturação populacional. Embora a maioria das populações amostradas estejam situadas em áreas de proteção ambiental, e de algum grau de conectividade existir entre elas (indicada pelos níveis de diversidade e número de migrantes), estas estão presentes em fragmentos da floresta, com diferentes graus de impacto antrópico. Desta forma, a recente estruturação genética, mesmo que baixa, deve ser considerada um sinal de alerta, para que haja a manutenção dos fragmentos florestais que ainda restam, ou mesmo possíveis corredores ecológicos entre estes.

A fim de investigar a possível hibridação e, por conseguinte inferir sobre a eficácia das barreiras reprodutivas em espécies do "grupo das infladas", foram selecionadas três localidades na Mata Atlântica onde estas espécies ocorrem em simpatria, dando origem ao Capítulo III desta tese (dados ainda não publicados). Uma das localidades é Santa Virgínia onde foram analisadas as espécies *V. carinata, V. taritubensis* var. *patens, V. simplex* e *V. agostiniana*. As outras duas localidades são Boraceia e Joinville onde foram estudadas as espécies *V. incurvata* e *V. inflata*, e *V. carinata* e *V. incurvata*, respectivamente. Em Santa Virgínia, além das espécies mencionadas, foi possível encontrar quatro indivíduos com uma morfologia atípica, os quais denominamos de *Vriesea* híbridos putativos (VHP). Para avaliar o fluxo gênico interespecífico entre as espécies em cada localidade, foram utilizados até 12 marcadores nucleares do tipo microssatélite. Além disso, para avaliar as diferenças morfológicas entre as espécies nas localidades foram analisadas 10 variáveis, quatro relacionadas à parte vegetativa e seis a parte reprodutiva. Os resultados indicaram uma alta taxa de hibridação e uma baixa estruturação entre as espécies em cada localidade. Dos indivíduos identificados morfologicamente como uma das espécies, 45% apresentaram perfil molecular híbrido em Santa Virgínia (incluindo VHP), em Boraceia e Joinville essa porcentagem foi de 42,32% e 22,86%, respectivamente. Afora os indivíduos VHP, os demais indivíduos que apresentaram perfil molecular híbrido, apesar de ser possível observar algumas características morfológicas ligeiramente diferentes do comum em alguns deles, nas análises morfológicas, de modo geral, agruparam junto aos indivíduos puros, ou seja, fenotipicamente eles se parecem com os parentais. Dos quatro indivíduos identificados com características morfológicas atípicas (VHP) e corroborados pelos resultados genéticos, um apresentou perfil molecular entre *V. carinata* e *V. taritubensis* var. *patens*, os outros três apresentaram perfil molecular de três espécies (*V. carinata*, *V. taritubensis* var. *patens* e *V. simplex*). Apesar dos cruzamentos entre *V. carinata* e VHP terem gerado frutos e nas análises morfológicas estes indivíduos se agruparem com *V. carinata*, não foi possível realizar o cruzamento destes híbridos com *V. taritubensis* var. *patens* e *V. simplex*, e também não foi possível classificar com precisão a qual classe híbrida estes indivíduos pertencem, dificultando afirmar quem seriam seus parentais. *Vriesea agostiniana*, de Santa Virgínia, foi a única espécie dentre as demais (*V. carinata*, *V. taritubensis* var. *patens* e *V. simplex*), que não apresentou um perfil molecular próprio; dos seis indivíduos coletados, quatro apresentaram perfil molecular híbrido e dois apresentaram perfil da espécie *V. simplex*. O fato de poucos indivíduos terem sido encontrados na população é algo que deve ser levado em consideração na discussão sobre o motivo de *V. agostiniana* não ter apresentado um perfil próprio.Também, devido a este fato pode-se especular que esta espécie possa estar à beira de um colapso nesta população, podendo ser extinta em poucas gerações. Outro aspecto que pode ser levado em consideração é a possível origem híbrida desta espécie. Para testar essa hipótese, as outras duas populações reconhecidas (Kessous *et al.*, 2018) devem ser analisadas, avaliando as espécies que coocorrem com ela, e também analisando o genoma plastidial destas espécies. As altas taxas de hibridação observadas nas localidades estudadas indicam que a barreiras reprodutivas são permeáveis nestas espécies. Apesar de não termos realizado observação de polinizadores, é relatado na literatura que muitas destas espécies apresentam como visitantes florais os beija-flores *Phaethornis eurynome* Lesson e *Ramphodon naevious* Dumont (Araujo *et al*., 1994; Machado e Semir, 2006; Wendt *et al*., 2008; Silva e Piratelli, 2014). Além disso, um estudo de rede de polinizadores na localidade de Santa Virgínia, identificou *P. eurynome* como o único polinizador das espécies de *Vriesea* da região

(Vizentin-Bugoni *et al*., 2014). Ademais, nas saídas de campo foi possível observar que havia floração sequencial com alguma sobreposição. Tal observação pode ser corroborada pelos períodos de floração descritos para as espécies na literatura, além do fato de que a sobreposição de floração é comum em populações simpátricas de bromélias (Wendt *et al*., 2008). O compartilhamento de polinizadores e sobreposição de floração estão relacionados às barreiras reprodutivas pré-polinização, contudo não podemos afirmar categoricamente quais das barreiras reprodutivas (pré-polinização (mecanismo pré-zigóticos) ou pós-polinização (mecanismos pré e pós-zigóticos)) estão sendo mais permeáveis nestas espécies. Para isso seriam necessários mais experimentos, como cruzamentos, viabilidade das sementes e crescimento do tubo polínico. Outro fator a ser levado em consideração é que as localidades analisadas, apesar de duas estarem em áreas de proteção ambiental, apresentam um impacto antrópico evidente. De fato, os distúrbios antrópicos como o desmatamento, agricultura, introdução de espécies exóticas, podem levar a formação de novos nichos e ao contato entre populações que estavam isoladas, podendo ocasionar a hibridação entre as espécies (Lamont *et al*., 2003; Harrison e Larson, 2014).

Desta forma, os resultados obtidos na presente tese, contribuíram para o melhor entendimento dos padrões filogeográficos do gênero *Vriesea* na Mata Atlântica (Palma-Silva *et al*. 2009; Neves *et al*. 2020) e dos padrões históricos desta floresta, revelando estabilidade da porção sul durante o Quaternário, ao contrário do relatado em trabalhos anteriores. Além disso, o Capítulo III da tese mostrou uma alta taxa de hibridação entre as espécies do "grupo das infladas", sendo que a partir deste trabalho muitos outros estudos poderão ser desenvolvidos, buscando o entendimento de quais barreiras estão sendo menos eficientes ou quais ainda estão ajudando a manter o limite entre estas espécies nas localidades estudadas. Também, sugerimos a investigação da possível origem híbrida de *V. agostiniana*, e mesmo a descrição dos quatro indivíduos (VHP) como uma provável notoespécie, a partir de uma investigação mais aprofundada no que diz respeito a determinação dos possíveis parentais. Considerando as diferentes abordagens dessa tese, ficou evidente a necessidade de conservação do bioma Mata Atlântica, para que o fluxo gênico entre as populações possa ocorrer e que a manutenção do limite entre as espécies fique em equilíbrio.

CAPÍTULO V

REFERÊNCIAS BIBLIOGRÁFICAS

(CAPÍTULOS I E IV)

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