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Padrões históricos e processo de hibridação entre duas espécies simpátricas de bromélias da Mata Atlântica

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Padrões históricos e processo de hibridação entre duas espécies simpátricas de bromélias da Mata Atlântica: implicações evolutivas e conservacionistas

Camila Martini Zanella

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SUMÁRIO

RESUMO

A família Bromeliaceae é uma das famílias de plantas com flores mais diversas morfologica e ecologicamente nativas do Novo Mundo, e é bem conhecida por ter sofrido radiação adaptativa recente, evoluindo para habitar inúmeros nichos e ocupando os mais diversos tipos de ambientes. Bromeliaceae surgiu no Escudo das Guianas cerca de 100 milhões de anos atrás (Ma), e linhagens modernas começaram a divergir cerca de 19 Ma, chegando na América tropical e subtropical há 15,4 Ma, aproximadamente. Devido a sua história evolutiva recente, limites genéricos frequentemente sofrem alterações na família Bromeliaceae, com espécies incipientes não completamente definidas. A Floresta Atlântica Brasileira (BAF) é um dos centros de diversidade das bromélias, sendo também um dos centros de diversidade do gênero *Vriesea*. *Vriesea carinata* e *V. incurvata* podem ser consideradas modelos interessantes para o estudo dos padrões históricos da BAF e também para o estudo dos processos de coesão de espécies e barreiras reprodutivas, por serem espécies endêmicas da BAF, com ampla distribuição ao longo dessa ecorregião, sendo encontradas em simpatria e compartilham polinizadores. Assim, este trabalho foi dividido em três manuscritos, buscando uma melhor compreensão da história evolutiva da família Bromeliaceae, bem como da Floresta Atlântica Brasileira. No **Capítulo II**, é apresentado um artigo de revisão, compilando os estudos sobre a família Bromeliaceae, no qual foram abordados aspectos como: diversidade genética, adaptações evolutivas, sistemas de cruzamento e suas consequências sobre a estruturação populacional e conservação *in situ*. Os dados do artigo de revisão demonstraram que Bromeliaceae tem três centros de diversidade, 58 gêneros e cerca de 3.170 espécies. As bromélias são preferencialmente polinizadas por vertebrados e apresentam uma marcante variação nos sistemas de cruzamento, de endogamia predominante à polinização cruzada obrigatória, e uma constância no número de cromossomos (x = 25). Bromélias com sistema de cruzamento autógamo ou misto têm um alto coeficiente de endogamia (F_{IS}), enquanto que as espécies de fecundação cruzada apresentam F_{IS} baixos. O grau de diferenciação entre as populações (F_{ST}) variou de 0,043 a 0,961, e pode ser influenciado pela dispersão do pólen e sementes, crescimento clonal, taxas de fluxo gênico e conectividade entre as populações. Bromeliaceae apresenta algumas adaptações morfológicas e fisiológicas, incluindo o metabolismo ácido das crassuláceas de fotossintese (CAM), formação de rosetas que

acumulam água, tricomas absortivos nas folhas e hábito epífito, que podem ter sido cruciais para a radiação adaptativa desta família. Além disso, muitas espécies são endêmicas com distribuição restrita e/ou são encontradas em ecorregiões ameaçadas de extinção, como a BAF. A preservação deste tipo de ecorregião é vital para a conservação da família Bromeliaceae e das espécies associadas.

No **Capítulo III**, nós estudamos os padrões filogeográficos de *Vriesea carinata* e *V. incurvata* com o objetivo de fornecer informações sobre os processos históricos que influenciaram na diversificação da BAF. Nós testamos a hipótese de que *V. carinata* e *V. incurvata* apresentariam o mesmo padrão filogeográfico, uma vez que podem ter sido submetidas às mesmas alterações climáticas no passado, pois apresentam distribuição geográfica semelhante. Foram amostradas 16 populações de *V. carinata* e 11 de *V. incurvata*, as quais utilizamos para descrever os padrões de variações genéticas do DNA plastidial (cpDNA) e nuclear (microssatélites). *Vriesea carinata* e *V. incurvata* apresentaram padrões filogeográficos semelhantes, com uma forte descontinuidade genética norte/sul entre as populações, sem compartilhamento haplotípico entre essas regiões. A presença de dois grupos genéticos distintos suporta a hipótese de que *V. carinata* e *V. incurvata* sobreviveram em mais de um refúgio durante as oscilações climáticas do Pleistoceno. Um putativo refúgio seria na porção litoranea sul-sudeste (latitude 25°S - PR/SP) e outro no sudeste do Brasil (latitude 20°S - RJ/ES). Os resultados são consistentes com registros encontrados na literatura para a BAF. No entanto, mais estudos são necessários para entender a complexa história da BAF, uma vez que este padrão foi provavelmente moldado ao longo do Pleistoceno, mas eventos anteriores, como soerguimento da costa leste brasileira durante o Terciário, também podem ter influenciado a distribuição e a diversificação dos taxa.

No **Capítulo IV**, nós investigamos a ocorrência de hibridação natural entre *V. carinata* e *V. incurvata* em quatro populações onde elas são encontradas em simpatria. Estas espécies são bem delimitadas taxonomicamente, apresentam morfologia floral similar e compartilham polinizador (beija-flor), apresentando floração sequencial com um curto período de sobreposição do florescimento. O grau de isolamento reprodutivo entre espécies relacionadas é um fator importante que influência a integridade genética das espécies e a formação de híbridos. Foram amostradas e analisadas quatro populações simpátricas, utilizando duas regiões plastidiais e 14 locos de microssatélites, e híbridos

foram encontrados em todas elas, com um total de 19 indivíduos, indicando que ocorre fluxo gênico interespecífico entre *V. carinata* e *V. incurvata.* Análises Bayesianas identificaram híbridos F2 e retrocruzamentos com *V. incurvata* e a rede de haplótipos do cpDNA identificou introgressão bidirecional entre estas duas espécies. Nas populações simpátricas com distribuição mais ao norte foi encontrado um maior número de híbridos, provavelmente por causa de um gradiente latitudinal, que pode influenciar nas estações do ano, temperatura, precipitação e também o período de floração das espécies. A taxa de fluxo gênico interespecífico ($N_em < 0,5$) foi considerada alta, contribuindo para a formação de 10% de híbridos nas populações estudadas, porém a ausência de híbridos F1 indica que a barreira reprodutiva está sendo eficaz. A diferença temporal de floração das duas espécies tem atuado como uma barreira reprodutiva prezigótica forte, sendo a principal força responsável pela coesão das espécies. O conhecimento da hibridação e dos padrões de fluxo gênico interespecífico é importante para a compreensão dos processos de especiação, do movimento de genes entre espécies relacionadas e da manutenção da coesão das espécies. Em suma, os resultados obtidos no presente estudo, utilizando *V. carinata* e *V. incurvata* como modelos, permitiram aumentar a compreensão dos padrões históricos da BAF, dos processos biológicos e ecológicos envolvidos na evolução do isolamento reprodutivo responsável pela manutenção da coesão das espécies de plantas.

ABSTRACT

Bromeliaceae family is one of the morphologically and ecologically most diverse flowering plant families native to the New World and is well known for its recent adaptive radiation, evolving to live in numerous niches and occupying the most diverse types of environments. Bromeliaceae arose in the Guayana Shield roughly 100 million years ago (Ma), and modern lineages began to diverge from each other roughly 19 Ma, arriving in tropical and subtropical America from near 15.4 Ma. Due to its recent evolutionary history, generic boundaries often suffer changes in the Bromeliaceae family, with incipient species not completely defined. Brazilian Atlantic Forest (BAF) is one of the centers of bromeliads diversity, being also one of the diversity centers of *Vriesea* genus. *Vriesea carinata* and *V. incurvata* may be interesting models for the study of BAF historical patterns and also for studying species cohesion processes and reproductive barriers, to be endemic species of BAF, with wide distribution throughout this ecoregion, being found in sympatry and share pollinators. Thus, this thesis was divided in three manuscripts, which seeking a better understanding of the evolutionary history of the Bromeliaceae family, as well as BAF ecoregion. In the **Chapter II**, a review manuscript were presented, compiling the studies on Bromeliaceae family using approaches as: genetic diversity, evolutionary adaptations, mating systems and their consequences on the population structure and *in situ* conservation. The review's results revealed that Bromeliaceae has three diversity centers, 58 genera, and about 3,170 species. Bromeliads are preferentially pollinated by vertebrates and show marked variation in breeding systems, from predominant inbreeding to obligatory outcrossing, as well as constancy in chromosome number (x = 25). Autogamous or mixed mating system bromeliads have a high inbreeding coefficient (F_{IS}), while outcrossing species show low F_{IS}. The degree of differentiation among populations (F_{ST}) of species ranges from 0.043 to 0.961, and can be influenced by pollen and seed dispersal effects, clonal growth, gene flow rates, and connectivity among populations. Bromeliaceae showed some morphological and physiological adaptations, including crassulacean acid metabolism (CAM) photosynthesis, formation of rosettes that accumulate water, leaf absorptive scales and epiphytic habit, which might have been crucial to the adaptive radiation of this family. Also, many species are endemic with restricted distribution and/or are found in endangered ecoregion, as BAF. The preservation of this type of ecoregion is

vital for the conservation of Bromeliaceae and associated species.

In the **Chapter III**, we studied the phylogeographic patterns of *Vriesea carinata* and *V. incurvata* aiming to provide insights into the historical processes that underlined diversification in BAF. We evaluated the hypothesis that *V. carinata* and *V. incurvata* would present the same phylogeographic pattern, since they could be subjected to the same climatic changes in the past because they present similar geographic distribution. We sampled 16 populations of *V. carinata* and 11 of *V. incurvata*, which we use to describe the patterns of genetic variation in plastid (cpDNA) and nuclear DNA (microsatellites). *Vriesea carinata* and *V. incurvata* showed similar phylogeographic patterns, with strong genetic discontinuity among north/south populations and without haplotypic sharing among these regions. The presence of two genetic distinct groups would seem to support the hypothesis that *V. carinata* and *V. incurvata* survived in more than one fragmented refugia during Pleistocene climatic oscillations. One putative refugium was on coastal south-southeastern (latitude 25°S - PR/SP) and another in southeastern Brazil (latitude 20°S - RJ/ES). The results are consistent with records encountered in the literature for the BAF. However, more studies are required for understanding the BAF complex history, since this pattern was probably shaped throughout the Pleistocene, but earlier events, as uplift of Brazilian east coast during Tertiary, may be also influenced the distribution and diversification of taxa.

In the **Chapter IV**, we investigated natural hybridization between *V. carinata* and *V. incurvata* in four populations where they are found in sympatry. These species are well defined taxonomically, show similar floral morphology and share pollinator (hummingbird), presenting sequential flowering and short time of blooming overlap. The degree of reproductive isolation among related species is an important factor influencing species genetic integrity and hybrids formation. We sampled and analyzed four sympatric populations, using two plastid regions and 14 microsatellite loci, and hybrids were found in all of them, with a total of 19 individuals, indicating that interspecif gene flow occurs between *V. carinata* and *V. incurvata*. Bayesian assignment analysis identified F2 hybrids and backcrosses towards *V. incurvata* and cpDNA haplotypic network identified bidirectional introgression between these two species. In sympatric populations with lower latitude we found a greater number of hybrids, probably because of a latitudinal gradient, which may influence the seasons of the year, temperature, precipitation, and also in

flowering period of the species. The rate of interspecific gene flow ($N_em < 0.5$) was considered high, contributing to the formation of 10% hybrids in the studied populations, however the absence of F1 hybrids indicates the reproductive barrier being effective. The temporal difference in the flowering period of the two species has acted as a strong prezygotic reproductive barrier, being the main force responsible for species cohesion. The knowledge of hybridization and patterns of interspecific gene flow are important for understanding the process of speciation, the movement of genes across species boundaries and the maintaining of species cohesion. Finally, the results obtained in this study, using *V. carinata* and *V. incurvata* as a models, allow us to increase the understanding of BAF historical patterns, and of biological and ecological processes involved in the development of reproductive isolation responsible for maintaining the cohesion of plant.

CAPÍTULO I

Introdução Geral

INTRODUÇÃO GERAL

1. Família Bromeliaceae

Bromeliaceae Juss é uma família de angiosperma típica de regiões tropicais e subtropicais do Novo Mundo, apresentando uma ampla diversidade morfológica e ecológica (Benzing, 2000). Sua distribuição geográfica é ampla, sendo encontrada, desde os estados da Virgínia, Texas e Califórnia, nos Estados Unidos (latitude 37° N) até o norte da Patagônia, na Argentina (latitude 44° S). A única exceção é *Pitcairnia feliciana* (A. Chev.) Harms & Mildbr., localizada no Oeste da África, na região da Guiné (Porembsky e Barthlott, 1999); sua ocorrência no continente africano é atribuída a um evento recente de dispersão a longa distância (Givnish *et al*., 2004). Bromeliaceae sofreu um processo de radiação adaptativa recente, sendo que suas espécies são encontradas nos mais variados nichos, com uma grande variação de formas, cores e tamanhos (Benzing, 2000). As plantas podem ocorrer desde o nível do mar até os elevados altiplanos da cordilheira dos Andes (4.000m), em locais úmidos como a Mata Atlântica, ou regiões áridas como a Caatinga, bem como em solos sujeitos a inundações regulares (espécies reófitas) e em locais de baixa ou alta luminosidade (Benzing, 2000). Podem ser terrestres, terrestres ocasionais, rupículas, saxícolas ou epífitas, mas nunca parasitas. Nas espécies epífitas as raízes têm apenas função de fixação, enquanto nas terrestres atuam na fixação e na absorção de água (Coffani-Nunes, 2002).

Paleo-registros oriundos de macro e microfósseis (pólen) indicam a existência de representantes de Bromeliaceae a partir do médio Terciário (Benzing, 2000). Segundo Givnish *et al*. (2011) as bromélias surgiram no Escudo das Guianas há cerca de 100 milhões de anos (Ma) durante o Período Cretáceo, sendo que as subfamílias existentes atualmente começaram a divergir há apenas cerca de 19 Ma. Estes autores também sugeriram que há cerca de 15,4 Ma as bromélias chegaram a regiões da América tropical e subtropical e provavelmente chegaram na região da África tropical há 9,3 Ma (*Pitcainia feliciana*).

A ampla diversidade de hábitats, nos quais as bromélias são encontradas, se deve a algumas adaptações morfológicas e ecológicas adquiridas por essa família ao longo de sua

história evolutiva. Uma importante característica é a formação de um "tanque" que possibilita o armazenamento da água da chuva, esse tanque é formado pela disposição das folhas, de forma helicoidal, formando uma roseta central. A transição de forma de vida terrestre para epífita auxiliou na conquista de novos nichos e territórios, e parece estar associada ao surgimento de tricomas absortivos nas folhas, os quais são responsáveis pela absorção de água e nutrientes presentes no tanque, uma adaptação importante, tendo em vista que espécies epífitas apresentam raízes rudimentares, com função apenas de fixação (Benzing, 2000; Crayn *et al*., 2004). Outra modificação fisiológica que auxiliou no sucesso adaptativo das bromélias foi o surgimento do sistema CAM de fotossíntese (metabolismo ácido das crassuláceas). Todos esses mecanismos auxiliam na resistência à seca, tanto na absorção quanto na conservação de nutrientes em ambientes xéricos e rochosos (Pittendrigh, 1948; McWilliams, 1974; Crayn *et al*., 2004; Givnish *et al*., 2007; Schulte *et al*., 2009).

Smith e Downs (1975; 1977; 1979) publicaram uma Monografia da Flora Neotropica da família Bromeliaceae ("*Flora Neotropical Monograph – Bromeliaceae*"), na qual compilaram todas as espécies descritas da família até as referidas datas de publicação, totalizando três volumes, um para cada subfamília: Bromelioideae, Pitcarnioideae e Tillandsoideae. A família era tradicionalmente dividida nestas três subfamílias, porém, um estudo recente baseado em oito regiões plastidiais demonstrou que Pitcairnioideae é parafilética, separando-a em seis subfamílias e propondo o seguinte relacionamento entre elas: (Brocchinioideae, (Lindmanioideae, (Tillandsioideae, (Hechtioideae, (Navioideae, (Pitcairnioideae, (Puyoideae, Bromelioideae)))))); Givnish *et al*., 2007, 2011). Atualmente são conhecidas cerca de 3170 espécies de bromélias, distribuídas em 58 gêneros (Luther, 2008), sendo que três centros de diversidade são considerados para Bromeliaceae: no norte dos Andes até o México e as Antilhas, no Planalto das Guianas e no leste do Brasil (Smith e Downs, 1974). No Brasil podemos encontrar cerca de 50% das espécies conhecidas, representando um contingente significativo de espécies, tornando o país um importante centro de diversidade desse grupo (Leme e Marigo, 1993).

A família Bromeliaceae também apresenta importância econômica, com espécies sendo utilizadas pelos povos nativos das Américas, estando fortemente presentes em suas culturas. Atualmente, mais de 90 espécies são utilizadas para diversos fins: fibras, forragem, alimentação humana, rituais místicos, combustíveis, ornamentação, medicinais,

cosméticos entre outros (Reitz, 1983; Bennet *et al*., 2001; Zanella *et al*., 2011). Entretanto, o interesse pelo cultivo de bromélias ornamentais para a comercialização é muito recente, datando do início dos anos 1990 (Coffani-Nunes, 2002). A crescente demanda de mercado tem sido responsável pelo aumento na produção e comercialização de bromélias. No entanto, um considerável aumento no extrativismo ilegal, especialmente de espécies com ciclos de vida longos, vem reduzindo muitas populações de espécies oriundas, principalmente, da Mata Atlântica (Coffani-Nunes, 2002). Além disso, a coleta predatória e a perda de habitat devido à ação antrópica vêm contribuindo para o aumento do número de plantas vulneráveis, ameaçadas de extinção ou mesmo em extinção (Bered *et al*. 2008). A espécie com maior importância econômica atualmente é o abacaxi (*Ananas comosus* (L.) Merr.), ocupando a quarta posição entre as frutas tropicais para a produção comercial, atrás da melancia, banana e manga (Chwee e Ahmad, 2008). Ecologicamente, as bromélias também desempenham um papel importante, sendo fonte de frutos carnosos, néctar, água (acumulada nos tanques formados pelas folhas) e abrigo para animais associados (mamíferos, anfíbios, pássaros e insetos; Benzing, 2000). Apesar de um crescente aumento de estudos com espécies desta família e de sua importância ecológica e econômica, a bibliografia científica ainda é consideravelmente restrita (Zanella *et al*., 2012a).

2. Gênero *Vriesea*

O gênero *Vriesea* Lindl. é o segundo maior na subfamília Tillandsioideae e o terceiro maior na família Bromeliaceae (Benzing, 2000), sendo composto por 258 espécies (Luther, 2008) e dividido em duas seções, *Vriesea* e *Xiphion.* O gênero tem dois centros de diversidade, um deles fica no leste do Brasil, onde ocorrem cerca de 84% das espécies, e o outro ocorre mais ao norte, na América do Sul, América Central e Caribe (Costa *et al*., 2009). As espécies desse gênero ocorrem preferencialmente em ambientes mesófilos, mas também ocorrem em campos rupestres, campos de altitude e costões rochosos ("*inselbergs"*). Muitos casos de endemismo são conhecidos no gênero *Vriesea*, mas espécies de ampla distribuição também são reportadas (Smith e Downs, 1977). Como o gênero *Vriesea* é típico de ambientes úmidos, em estudos florísticos na Mata Atlântica avaliando espécies da família Bromeliaceae, o gênero *Vriesea* apresenta alta riqueza de

número de espécies (Martinelli, 1994; Wanderley e Mollo, 1992; Machado e Semir, 2006; Costa e Wendt, 2007).

Vriesea carinata Wawra e *Vriesea incurvata* Gaudichaud, objetos de estudo do presente trabalho, são espécies típicas da Mata Atlântica, bem estabelecidas taxonomicamente, com hábito preferencialmente epifítico, mas também podendo ser terrestres e rupícolas, e ocorrem em lugares úmidos e bem preservados. *Vriesea carinata* é uma espécie com ampla distribuição, ocorrendo desde o norte do Rio Grande do Sul até o sul da Bahia, apresenta roseta infundibuliforme, suas folhas possuem bainha esverdeada podendo apresentar mancha vinosa de tamanho e posição variável (Wanderley e Martins, 2007). A inflorescência é simples, com 4 a 12 flores; brácteas florais com base vermelha e ápice amarelo. Flores dísticas, sépalas amarelas e pétalas amarelas com o ápice verde (Smith e Downs, 1977). É uma espécie diploide com 2n = 50, com cromossomos metacêntricos e submetacêntricos e alta viabilidade de pólen (94,3%; Palma-Silva *et al*., 2004). Seu florescimento ocorre no inverno, de abril a outubro, com pico entre os meses de junho e agosto, as flores normalmente abrem às 07h30min e fecham às 17h, com duração de apenas um dia (Araujo *et al*., 2004; Machado e Semir, 2006).

Vriesea incurvata apresenta uma distribuição um pouco mais restrita, sendo encontrada desde o norte do Rio Grande do Sul até o Rio de Janeiro, podendo apresentar uma altura de até 70 cm, com roseta infundibuliforme e folha com bainha verde. A inflorescência é simples, em racemo, com 10 a 35 flores eretas e oblongas; as brácteas florais são vermelhas, às vezes com margem amarelada. As flores são dísticas, com sépalas e pétalas amarelas (Smith e Downs, 1977). Segundo Palma-Silva *et al.* (2004), *V. incurvata* é diploide, com 2n = 50, com alta viabilidade de pólen (90,0%). As flores de *V. incurvata* tem duração de apenas um dia, abrindo às 06h30min e fechando às 19h, florescendo no verão entre os meses de outubro a maio, com pico de florescimento entre janeiro e março (Machado e Semir, 2006).

Vriesea carinata e *V. incurvata* apresentam morfologia floral semelhante e síndrome de polinização ornitófila, sendo polinizadas por beija-flores (*Phaethornis eurynome* Lesson e *Melanotrochilus fuscus* Vieillot; Machado e Semir, 2006). Estas espécies podem ser encontradas em simpatria e possuem florescimento sequencial, *V. carinata* no inverno e *V. incurvata* no verão, porém uma pequena sobreposição já foi observada entre elas (Araujo *et al*., 2004). A floração sequencial das bromeliáceas em uma região pode ser de extrema

importância para a manutenção dos agentes polinizadores na área, contribuindo para a eficiência no sistema de polinização de espécies ornitófilas da comunidade (Waser e Real, 1979, Feinsinger, 1983, Araujo *et al*., 1994, Fischer e Araujo, 1995). Porém, em espécies simpátricas com polinizadores generalistas, pode ocorrer transferência interespecífica de pólen (Hersch e Roy, 2007).

3. Diversidade genética

Caracterizar os níveis de diversidade genética dentro das populações naturais é de importância primária e está diretamente relacionada com aspectos da história de vida da espécie, podendo fornecer informações importantes com implicações na biologia evolutiva, ecologia e biologia da conservação. A estrutura genética das populações reflete a interação entre diferentes processos, incluindo a sua história evolutiva (distribuição, fragmentação de habitat, isolamento da população), mutações, deriva genética, sistema de cruzamento, fluxo gênico e seleção (Sales *et al*., 2001), as quais podem ajudar a compreender processos de adaptação a circunstâncias ecológicas particulares (Parker *et al*., 1998).

O advento dos marcadores moleculares de DNA, principalmente aqueles baseados na reação em cadeia da polimerase (PCR), oportunizou a caracterização genética de diferentes espécies em fina escala e sem a influência do ambiente (Reif *et al*., 2004). Além disso, análises utilizando marcadores moleculares fornecem informações sobre alguns fatores que determinam a estrutura genética populacional, tais como estimativas de padrões de dispersão do pólen, distância de dispersão e fluxo gênico, que são particularmente importantes para a otimização de programas de conservação *in situ* (Dawson *et al*., 1997; Oubourg *et al*., 1999; He e Smouse, 2002; He *et al*., 2004).

Os marcadores moleculares do tipo microssatélites ou SSR ("*simple sequence repeats*") são marcadores codominantes, normalmente isolados de regiões não codificantes e espécie-específicos. Deste modo, estes marcadores podem ser utilizados para ajudar a resolver problemas que variam desde a taxonomia, questões relacionadas à paternidade, à estrutura genética de populações, padrões de hibridação, sistema de cruzamento, especialização ecológica e capacidade de colonização de populações (McDonald e Potts, 1997; Parker *et al*., 1998; Boneh *et al*., 2003).

Como os locos de microssatélites são espécie-específicos, é necessário isolá-los para cada espécie. Porém a presença de regiões flanqueadoras conservadas permite a amplificação desses locos em espécies próximas. No caso da família Bromeliaceae, a qual sofreu radiação adaptativa recentemente, apresentando baixos níveis de divergência nas sequências de DNA (Maia *et al*., 2012), os marcadores são transferíveis entre espécies da mesma subfamília e até entre as subfamílias (Barbará *et al*., 2007, 2009; Palma-Silva *et al*., 2007; Paggi *et al*., 2008; Wohrmann e Weising, 2011; Wohrmann *et al*., 2012a; 2012b; Zanella *et al*., 2012b, Goetze *et al*., 2013).

Os trabalhos publicados sobre a diversidade genética de populações de espécies da família Bromeliaceae ainda são escassos, dos 58 gêneros e aproximadamente 3170 espécies conhecidas, apenas 20 delas, de dez gêneros, foram estudadas: *Aechmea*, *Alcantarea*, *Ananas*, *Bromelia*, *Dyckia*, *Encholirium*, *Pitcairnia*, *Puya*, *Tillandsia* e *Vriesea* (Zanella *et al*., 2012a). Marcadores codominantes foram os mais utilizados, com sete trabalhos utilizando microssatélites em dez espécies (Barbará *et al*., 2007; 2009; Palma-Silva *et al*., 2009; 2011; Boisselier-Dubayle *et al*., 2010; Zanella *et al*., 2011; Carlier *et al*., 2012) e sete com aloenzimas (Soltis *et al*., 1987; Murawski e Hamrich, 1990; Izquierdo e Piñero, 2000; Sarthou *et al*., 2001; Alves *et al*., 2004; González-Astorga *et al*., 2004; Hmeljevski *et al*., 2010).

4. Filogeografia e padrões históricos da Mata Atlântica

Filogeografia é a área de estudo que trata dos princípios e processos que governam a distribuição geográfica de linhagens genealógicas, especialmente aquelas em nível intraespecífico. A análise e interpretação da distribuição de linhagens, usualmente, requerem informações da genética molecular, genética de populações, filogenias, demografia e geografia histórica, sendo a filogeografia uma disciplina integrativa (Avise, 1998). Estudos filogeográficos têm sido utilizados para investigar os efeitos de mudanças climáticas do passado, na estrutura genética de espécies animais e vegetais. Estes estudos permitem-nos fazer inferências sobre a evolução de espécies dentro de biomas, e isto pode ser usado para auxiliar a traçar estratégias de conservação das mesmas (Bermingham e Moritz, 1998; Ramos *et al*., 2007). Os estudos filogeográficos têm gerado importantes contribuições para a compreensão da distribuição das espécies no passado e no presente, e

também tem sido uma importante fonte de informação sobre eventos do passado, permitindo a identificação de refúgios do Pleistoceno, rotas pós-glaciais e zonas de contato secundário (Hewitt, 1996; Comes e Kadereit, 1998; Cruzan e Templeton, 2000).

Estudos recentes têm direcionado seu enfoque no entendimento dos efeitos das mudanças climáticas e glaciações durante o Pleistoceno na diversificação de espécies, principalmente no hemisfério norte. A maioria destes estudos investigou a distribuição geográfica de linhagens genéticas e têm demonstrado um papel significativo das mudanças climáticas do passado na formação da história e da estrutura populacional das espécies (Moraes *et al*., 2009). Uma atenção menor tem sido dada para regiões tropicais da América do Sul, principalmente com plantas (Lira *et al*., 2003; Lorenz-Lemke *et al*., 2005; Andrade *et al*., 2007; Palma-Silva *et al*., 2009; Ramos *et al*., 2009; Novaes *et al*., 2010; Pinheiro *et al*., 2011; Ribeiro *et al*., 2011; Turchetto-Zolet *et al*., 2012). Porém, ainda mais escassos são os estudos envolvendo análises de filogeografia para plantas que ocorrem na porção Sul da Mata Atlântica (Rio Grande do Sul, Santa Catarina e Paraná): *Podocarpus* (Ledru *et al*., 2007), *Passiflora actinia* e *P. elegans* (Lorenz-Lemke *et al*., 2005) e *Vriesea gigantea* (Palma-Silva *et al*., 2009).

A Mata Atlântica é um complexo conjunto de ecossistemas de grande importância por abrigar uma parcela significativa da diversidade biológica mundial, sendo também um dos biomas mais ameaçados da América do Sul. Esse bioma cobria originalmente 15% do território nacional, ocupando cerca de 1,3 milhões de Km^2, entre as latitudes 6 e 30° S ao longo da costa leste brasileira e chegando até o Paraguai e a Argentina (SOS Mata Atlântica e INPE, 2009). As características geográficas da Mata Atlântica, combinadas com a grande variação em altitude e o regime de chuvas, favoreceram a ocorrência de uma alta diversidade e endemismo (Oliveira-Filho e Fontes, 2000, Myers *et al*., 2000). Seu grau de endemismo pode atingir 90% para alguns organismos, e sua média geral é de 50%, sendo superado apenas pela Amazônia (Costa *et al*., 2000), incluindo mais de 20.000 espécies de plantas. A maior ameaça à biota da Mata Atlântica é a perda de habitat e o alto grau de fragmentação (Myers *et al.*, 2000). De acordo com a Fundação SOS Mata Atlântica e INPE (2009), restam de 7 a 8% da cobertura original da Mata no país. A maioria dos remanescentes florestais existe em pequenos fragmentos (menores que 100 hectares), isolados uns dos outros, e compostos por florestas secundárias em estágio de sucessão inicial ou médio. Já os poucos fragmentos grandes sobreviveram em locais onde

os terrenos íngremes dificultaram a ocupação humana (Ribeiro *et al*., 2009*).* A atual fragmentação da Mata Atlântica levou à perda da biodiversidade e muitas espécies estão ameaçadas de extinção (Myers *et al*., 2000).

A Mata Atlântica ocupa principalmente a borda leste brasileira, uma área de topografia complexa ao longo de distâncias geográficas curtas que foram moldadas pela atividade das placas tectônicas no Terciário e das transgressões marinhas no Quaternário (Martins e Coutinho, 1981). Esta região é caracterizada por forte sazonalidade, gradientes ambientais (decorrente da topografia) e chuvas orográficas influenciadas pelos ventos vindos do Atlântico. Além disso, bacias hidrográficas e cadeias de montanhas frequentemente delimitam a distribuição das espécies da Mata Atlântica, atuando como barreiras ao fluxo gênico (Thomé *et al*., 2010), entretanto, poucos estudos têm estabelecido eventos geomorfológicos como promotores de diversificação alopátrica neste bioma (Costa, 2003; Pellegrino *et al*., 2005; Grazziotin *et al*., 2006; Cabanne *et al*., 2007; 2008). Essa diversidade de paisagens aliadas a peculiaridades de micro-habitats são responsáveis pela alta taxa de endemismo e biodiversidade da região (Martins, 2011).

Estudos envolvendo a diversificação de espécies da Mata Atlântica demonstram que não há um acordo sobre os mecanismos gerais que explicam a origem de sua diversidade. Uma das hipóteses é a de que espécies neotropicais teriam surgido principalmente durante o Quaternário (nos últimos dois milhões de anos), favorecidas pela alternância do clima glacial/interglacial (Bennett, 2004). Por outro lado, há a teoria de uma origem mais antiga, no Terciário, ligada principalmente a mudanças paleogeográficas (Willis e Niklas, 2004). Estimativas do tempo de diversificação dos Neotrópicos indicam que as linhagens originaram-se continuamente desde o Eoceno tardio, início do Oligoceno até o Pleistoceno (Rull, 2008). O mecanismo comumente invocado para a diversificação na América do Sul é o isolamento de táxons em áreas de habitat estáveis durante as oscilações climáticas do Quaternário (a hipótese de refúgios do Pleistoceno; Haffer, 1969). Refúgios permitiram a persistência em alopatria de populações durante períodos climáticos desfavoráveis e devem mostrar índices de diversidade e endemismos maiores que áreas menos estáveis, as quais não serviram como refúgios (Bennett e Provan, 2008).

Um estudo de paleomodelagem realizado para a Mata Atlântica indica severas contrações florestais ao sul do estado de São Paulo e áreas estáveis de florestas ao norte durante o último máximo glacial, seguido de expansão durante o Holoceno (Carnaval e

Moritz, 2008). Este cenário é compatível com a divergência genética de linhagens observada em vários táxons do norte da Mata Atlântica (Costa, 2003; Pellegrino *et al*., 2005; Moraes-Barros *et al*., 2006; Cabanne *et al*., 2008; Carnaval *et al*., 2009; Fitzpatrick *et al*., 2009; Palma-Silva *et al*., 2009; Ribeiro *et al*., 2011; Silva *et al*., 2012), mas linhagens divergentes também têm sido observadas no sul da Mata Atlântica (Grazziotin *et al*., 2006; Cabanne *et al*., 2007; Fitzpatrick *et al*., 2009), onde não foram previstos refúgios nos paleomodelos. Porém, Thomé *et al*. (2010), no estudo de paleomodelagem para espécies de sapos do gênero *Rhinella*, encontraram a ocorrência de refúgios para a porção sul da Mata Atlântica na região centro-norte do estado do Rio Grande do Sul e oeste de Santa Catarina, quase no centro do Paraná e a região litorânea do sul e sudeste do Brasil, do norte de Santa Catarina até São Paulo. Resultados similares foram encontrados por Amaro *et al*. (2012), com a permanência de populações mais ao sul da Mata Atlântica durante o Pleistoceno. Porém, no estudo realizado por Porto *et al*. (2012) com 14 espécies, nenhum refúgio ao sul da Mata Atlântica foi observado. Os autores discutiram que o poder dos modelos de refúgios quando utilizados para espécies de distribuição muito restrita pode ser mais fraco do que para espécies de distribuição geográfica mais ampla, entretanto, a ocorrência de refúgios ao sul da Mata Atlântica é esperada devido a dados paleoecológicos, de diversidade e endemismo (ver Porto *et al*., 2012). Para a Mata Atlântica é improvável que a hipótese de refúgios ou barreiras explique sozinha os padrões gerais de diversificação de linhagens (Thomé *et al*., 2010), o que demonstra o pobre entendimento dos padrões evolutivos históricos dessa região.

Em Bromeliaceae apenas dois trabalhos de filogeografia foram desenvolvidos até o momento, um deles com *Pitcairnia geyskesii*, uma espécies endêmica da Guiana Francesa, encontrada em afloramentos rochosos (Boisselier-Dubayle *et al*., 2010) e outro com *Vriesea gigantea*, uma espécies endêmica da Mata Atlântica (Palma-Silva *et al*., 2009), no qual foi observado uma divisão filogeográfica entre os estados de São Paulo e Rio de Janeiro (latitude 23°S), provavelmente refletindo um isolamentos das populações no passado, as quais teriam sobrevivido em mais de um refúgio durante as oscilações climáticas do Pleistoceno. Também, foi encontrada uma recente expansão demográfica das populações em direção ao Sul e áreas mais ao norte parecem ter permanecido estáveis durante as oscilações climáticas do Pleistoceno.

5. Hibridação

Hibridação é um fenômeno natural relativamente bem conhecido e documentado, tendo uma função importante na evolução das plantas (Stebbins 1959; Grant 1981; ver Soltis e Soltis, 2009) e sendo responsável pela diversificação de uma grande proporção de angiospermas (50 a 70%; Ellstrand *et al*., 1996; Rieseberg, 1997). O mecanismo mais comumente conhecido de especiação de plantas é através de hibridação alopoliploide (Soltis e Soltis, 1999), porém também há evidências da ocorrência de novas espécies com o mesmo número de ploidia dos parentais (Rieseberg *et al*., 1995; Arnold, 1997; Ungerer *et al*., 1998;Wolfe *et al*., 1998; Buerkle *et al*., 2000). Estes estudos demonstram que a hibridação não é apenas um tipo de "ruido evolutivo" com pouco significado evolutivo, como afirmam alguns autores (Mayr, 1992; Schemske, 2000) e sim uma poderosa força evolutiva que cria oportunidades para a diversificação adaptativa e especiação em populações naturais (Anderson, 1949; Arnold, 1997; Rieseberg e Carney, 1998; Rieseberg *et al*., 2003; Martin *et al*., 2006; Pinheiro *et al*., 2010; Palma-Silva *et al*., 2011).

Os processos de hibridação e suas consequências podem ser estudados pela avaliação da arquitetura genética de zonas híbridas naturais (Rieseberg *et al*., 2000). Uma zona híbrida ocorre quando duas espécies se encontram, cruzam e produzem descendentes viáveis (Harrison, 1990). O estudo da arquitetura genética de zonas híbridas possibilita acessar uma grande variedade de genótipos que podem ter sido produzidos por muitas gerações de recombinação, sendo considerados laboratórios naturais para os estudos de barreiras ao fluxo gênico (Barton e Hewitt 1989; Lexer *et al*., 2005). O estudo do fluxo gênico interespecífico pode fornecer importantes informações quanto ao tipo e poder do isolamento reprodutivo entre as espécies que estão hibridizando (Martinsen *et al*., 2001; Lexer *et al*., 2005; Lorenz-Lemke *et al*., 2006; Palma-Silva *et al*., 2011), também permite avaliar o sucesso reprodutivo do genótipo híbrido adulto em condições naturais, o que é muito difícil de conseguir para espécies de geração longa ou para espécies difíceis de serem manejadas de forma experimental (Rieseberg e Buerkle, 2002; Lexer *et al*., 2003, 2005), como é o caso das bromélias.

Os estudos de zonas híbridas apresentam uma riqueza de informações sobre os fatores que favorecem o fluxo gênico interespecífico, a natureza das barreiras pré- e pószigóticas e a estabilidade de zonas híbridas (Rieseberg e Carney, 1998; Buggs, 2007;

Currat *et al*., 2008). Estes estudos fornecem informações sobre o papel da hibridização em populações naturais contemporâneas, bem como eventos de hibridação no passado. A estrutura e estabilidade das zonas híbridas dependem da extensão em que as espécies parentais são genética e ecologiamente distintas, além do valor adaptativo dos híbridos. O grau de isolamento reprodutivo entre espécies relacionadas é um fator importante que influencia na integridade genética das espécies e na probabilidade de formação de híbridos (Grant, 1981; Harrison, 1993; Avise, 1994; Arnold, 1997; Chari e Wilson, 2001; Mráz *et al*., 2005; Stökl *et al*., 2005). Este grau de isolamento reprodutivo é ainda mais crítico entre espécies simpátricas (Pascarella, 2007).

Marcadores moleculares representam uma ferramenta poderosa no estudo da hibridação, uma vez que são capazes de detectar até mesmo baixos níveis de introgressão. O estudo combinado de marcadores nucleares e plastidiais têm sido utilizados com sucesso na detecção de efeitos combinados de seleção e fluxo gênico (Barton e Hewitt, 1985; Lexer *et al*., 2005). O genoma plastidial não sofre recombinação e é herdado somente de um dos parentais (nas angiospermas, em geral, a herança é materna), indicando a direção do evento de introgressão, sendo a introgressão citoplasmática frequentemente utilizada como evidência de hibridização antiga (Rieseberg e Brunsfeld, 1992).

Bromeliaceae possui grande compatibilidade reprodutiva entre seus gêneros e espécies, produzindo facilmente híbridos artificiais (McWilliams, 1974; Vervaeke *et al*. 2004). No entanto, poucos casos de hibridação natural foram registrados para a família (gêneros *Tillandsia*: Gardner, 1984; Luther, 1985; *Vriesea*: Read, 1984; *Pitcairnia*: Luther, 1984; Wendt *et al*., 2000; Palma-Silva *et al*., 2011). Wendt *et al*. (2008) estudaram 42 espécies de bromélias simpátricas e verificaram um fraco isolamento pré-zigótico entre as espécies estudadas.

O estudo do isolamento reprodutivo tem recebido grande atenção de pesquisadores nos últimos anos, por ser um processo essencial na especiação, principalmente na tentativa de entender como a ecologia e a genética estão atuando sobre as barreiras evolutivas. Muitos são os componentes pré- e pós-zigóticos responsáveis pelo isolamento reprodutivo entre espécies de plantas, como por exemplo, morfologia floral, coloração das pétalas, composição do néctar, fenologia floral, número cromossômico entre outros (Widmer *et al*., 2009). Em plantas, muitas vezes, o isolamento reprodutivo ocorre pela interação entre esses fatores e não pela atuação de uma única barreira ao fluxo gênico interespecífico

(Coyne e Orr, 2004; Rieseberg e Willis, 2007; Widmer *et al*., 2009).

6. Objetivos

A presente tese está inserida em um projeto amplo que visa contribuir para os estudos genéticos e biológicos de plantas neotropicais, com ênfase na família Bromeliaceae. A família Bromeliaceae é um modelo interessante para o estudo dos padrões filogeográficos da Mata Atlântica e de barreiras reprodutivas, por ser uma família que sofreu uma radiação adaptativa recente, tendo a Mata Atlântica como um de seus centros de diversidade. Sendo assim, a presente tese tem como objetivo geral contribuir com informações que auxiliarão na compreensão dos padrões históricos que modelaram a Mata Atlântica, dos mecanismos de especiação, isolamento reprodutivo e coesão de espécies.

Objetivos específicos

- Revisar e compilar os dados de diversidade genética, biologia reprodutiva, citogenética, evolução e conservação sobre a família Bromeliaceae;
- Inferir sobre os padrões de diversidade genética de *V. carinata* e *V. incurvata*, através de marcadores plastidiais e nucleares;
- Compreender os padrões filogeográficos das espécies;
- Comparar os padrões filogeográficos observados para essas espécies com padrões descritos para a Mata Atlântica;
- Identificar se há ocorrência de hibridação natural em populações simpátricas de *V. carinata* e *V. incurvata*,através de marcadores moleculares nucleares e plastidiais;
- Estimar a diversidade e estruturação genética dos indivíduos puros de *V. carinata*, *V. incurvata* e híbridos e avaliar a composição genômica dos híbridos;
- Avaliar os padrões de fluxo gênico interespecífico nuclear e plastidial nas populações simpátricas e definir se a introgressão é uni ou bidirecional;
- Elucidar aspectos evolutivos do sistema reprodutivo e das barreiras reprodutivas pré e pós-zigóticas que estão atuando para a manutenção das espécies.

Capítulo II

Genetics, evolution and conservation of Bromeliaceae

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Research Article

Genetics, evolution and conservation of Bromeliaceae

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Abstract

Bromeliaceae is a morphologically distinctive and ecologically diverse family originating in the New World. Three centers of diversity, 58 genera, and about 3,140 bromeliad species are currently recognized. We compiled all of the studies related to the reproductive biology, genetic diversity, and population structure of the Bromeliaceae, and discuss the evolution and conservation of this family. Bromeliads are preferentially pollinated by vertebrates and show marked variation in breeding systems, from predominant inbreeding to predominant outcrossing, as well as constancy in chromosome number (2n = 2x = 50). Autogamous or mixed mating system bromeliads have a high inbreeding coefficient (F_{IS}), while outcrossing species show low F_{IS}. The degree of differentiation among populations (F_{ST}) of species ranges from 0.043 to 0.961, which can be influenced by pollen and seed dispersal effects, clonal growth, gene flow rates, and connectivity among populations. The evolutionary history of the Bromeliaceae is poorly known, although some studies have indicated that the family arose in the Guayana Shield roughly 100 Mya. We believe that genetic, cytogenetic, and reproductive data will be essential for diagnosing species status and for assisting conservation programs.

Keywords: bromeliads, cytogenetics, genetic diversity, population structure, reproductive biology.

Introduction

The Bromeliaceae is one of the morphologically and ecologically most diverse flowering plant families native to the tropics and subtropics of the New World (Givnish *et al*., 2011). Its geographical distribution ranges from the states of Virginia, Texas, and California in the USA (latitude 37° N) to northern Patagonia in Argentina (latitude 44° S). The family is known for its recent adaptive radiation. Bromeliads have different habits, varying from terrestrial to epiphytical, and are found from sea level to altitudes above 4,000 m, in both desert and humid regions, as well as in soils subject to regular floods and in places with very low or high luminosity. They can thrive on scalding sands and rocks, and withstand temperatures near 0 °C (Benzing, 2000).

Traditionally, the family has been divided into three subfamilies, Bromelioideae (~650 spp.), Pitcairnioideae (~890 spp.), and Tillandsioideae (~1000 spp.), based on Smith and Downs (1979); this classification is adopted in the present study. However, in a recent phylogeny based on eight plastid regions, with representatives from 46 of 58 genera, Givnish *et al.* (2011) confirmed the eight-subfamily classification advanced by Givnish *et al.* (2007). The new classification splits the paraphyletic Pitcarnioideae into six subfamilies and proposes that they are related to each other as follow: (Brocchinioideae, (Lindmanioideae, (Tillandsioideae, (Hechtiooideae, (Navioideae, (Pitcarnioideae, (Puyoideae, Bromelioideae)))))).

Bromeliads are especially appreciated for their ornamental value, but some species have proven medicinal properties (*e.g.*, *Bromelia antiacantha*) or are cultivated as tropical fruits (*e.g.*, pineapple: *Ananas comosus*). Here, we review the main genetic and evolutionary topics concerning Bromeliaceae, from a conservation standpoint.

Pollination and Reproductive Biology

Among the plant families, Bromeliaceae is the one with the highest diversity of pollination modes (ornithophily, chiropterophily, entomophily, mixed/unspecific, and autogamy) throughout its geographic distribution (Kessler

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and Krömer, 2000; Canela and Sazima, 2005; Wendt *et al.*, 2008; Schmid *et al.*, 2010). Bromeliads have evolved floral displays with a great diversity of colors, shapes, and scents, which are related to pollinator attraction, with nectar being the usual reward (Benzing, 2000). The presence of Bromeliaceae in the New World has provided an important resource base, largely absent in the Old World, for small, hovering vertebrate pollinators (Fleming and Muchhala, 2008). A recent study (Krömer *et al*., 2008) strongly supports the hypothesis that the composition of nectar sugars in Bromeliaceae is correlated with the pollinator syndrome (lepidopterophilous, trochilophilous, or chiropterophilous). Although the majority of bromeliads are pollinated by vertebrates, mainly hummingbirds and bats, bees are among the most frequent visitors to some short-corolla species with ornithophilous features. Nevertheless, few studies have identified insects as effective pollinators of these bromeliads (Kamke *et al.*, 2011).

Simultaneously with the divergence of bromeliad subfamilies (see "Evolution" below), the first split of modern hummingbird lineages appears to have occurred in the Andes about 13 Mya, with several other Andean lineages diverging during the Pliocene and Pleistocene (Givnish *et al.*, 2011). This might have contributed to the rapid expansion of the range of bromeliads and pollinators throughout the Neotropics. However, plant-pollinator interactions, seed dispersal, and the mechanisms promoting or constraining species diversification via these interactions are complex and poorly studied in the Neotropics (Antonelli and Sanmartín, 2011).

Bromeliads possess specialized floral features such as herkogamy and dichogamy, which prevent spontaneous self-fertilization and facilitate animal-mediated outcrossing (Benzing, 2000; Martinelli G, 1994, PhD Thesis, University of St. Andrews). Floral morphology, hand-pollination experiments, and population genetics studies have shown that selfing and mixed are the most common mating systems in a large part of the family (Bush and Guilbeau, 2009; Matallana *et al.*, 2010; Table 1), although self-incompatibility systems can be found in all of the subfamilies (Pitcairnioideae: Vosgueritchian and Buzato, 2006; Bromelioideae: Canela and Sazima, 2003, 2005; Schmid *et al.*, 2010; Kamke *et al.*, 2011; Tillandsioideae: Hietz *et al.*, 2006; Ramírez-Morillo *et al.*, 2009). The Tillandsioideae subfamily has a particularly high frequency of selfing and mixed systems in various genera, including *Alcantarea*, *Guzmania*, *Racinea*, *Tillandsia*, *Vriesea*, and *Werauhia* (Benzing, 2000; Lasso and Ackerman, 2004; Paggi *et al*., 2007, 2012; Matallana *et al.*, 2010; Martinelli G, 1994, PhD Thesis, University of St. Andrews; Table 1). Clonality is another reproductive strategy present in the family (Murawski and Hamrick, 1990; Izquierdo and Pinero, 2000; Sarthou *et al*., 2001; Sampaio *et al.*, 2002; Sgorbati *et al*., 2004; Cascante-Marín *et al.*, 2006; Barbará *et al*., 2009), with important ecological and evolutionary consequences (Gonzales *et al*., 2008) such as recruitment and population maintenance (Villegas, 2001).

We studied the mating systems of two bromeliad species. *Vriesea gigantea* presented a high natural production of flowers, fruits, and seeds, with high rates of viable seeds, with an average germination rate of 94% (Paggi *et al*., 2007, 2010). Furthermore, the species showed regular chromosome segregation and high pollen viability (84-98%, Palma-Silva *et al*., 2008), which indicated that the populations analyzed were fertile. Manual hand-pollination indicated that *V. gigantea* is self-compatible (Paggi *et al*., 2007) and showed low to moderate levels of inbreeding depression ($\delta = 0.02$ to 0.39; Sampaio *et al*., 2012). In a study with *Vriesea friburgensis* we highlighted that it is pollinated by hummingbirds and produces high flower, fruit, and seeds together with high seed and pollen viability. We concluded that the wild populations studied were fertile. Self-sterility was observed from spontaneous selfing and manual self-pollination treatments, which may be a consequence of late-acting self-incompatibility. We proposed that this self-sterile species depends on pollinator services to maintain its population fitness and viability through cross-pollination (Paggi *et al*., 2012).

Diversity and Genetic Structure

The genetic diversity of only a few species of Bromeliaceae has been studied. We compiled data from all diversity and genetic structure studies published before June 2011 (Table 1). Of the 58 genera and about 3,140 bromeliad species (Givnish *et al*., 2011), only 20 species of the following nine genera have been previously evaluated: *Aechmea*, *Alcantarea*, *Bromelia*, *Dyckia*, *Encholirium*, *Pitcairnia*, *Puya*, *Tillandsia*, and *Vriesea*. Most of the studied species are endemic to the Atlantic rainforest in southeastern Brazil.

The use of co-dominant markers has been the preferred method for studying bromeliad population genetics, with nuclear microsatellite markers being the most frequently used molecular markers (nine species), followed by allozymes (eight species). Dominant markers such as amplified fragment length polymorphisms have been used in only one study of one species, and random amplified polymorphic DNA was applied in another study of three species (Table 1). A comparison of genetic diversity parameters among such studies is difficult, as the highly polymorphic SSRs usually show higher observed and expected heterozygosity (H_O and H_E, respectively) compared with other markers. For example, populations of *Pitcairnia geyskesii* have been evaluated using allozymes (Sarthou *et al.*, 2001) and SSRs (Boisselier-Dubayle *et al*., 2010). With allozymes, H_O and H_E were 0.188 and 0.246, respectively; with SSRs, H_O and H_E were 0.293 and 0.324, respectively.

We found low inbreeding coefficient indices (F_{IS}) in almost all species with outcrossing mating systems. The ex-

Table 1 - Bromeliads studied: Mating system, genetic diversity and population structure descriptors, molecular markers used and geographical distribution.

Species	Mating system	Marker	H_O mean/all	H_E mean/all	F_{IS} mean	F_{ST} mean	Geographical distribution	Reference
Aechmea magdalenae	ND	Allozyme	0.099/-	0.084/-	-	0.356[a]	Mexico to Ecuador	Murawski and Hamrich, 1990
Aechmea tuitensis	ND	Allozyme	0.061/-	0.12/-	0.631	0.196	Endemic to Mexico	Izquierdo and Piñero, 2000
Alcantarea geniculata	Out	SSR	0.356/0.357	0.380/0.429	0.094	0.111	Rio de Janeiro, Brazil	Barbará *et al*., 2007
Alcantarea glaziouana	Out	SSR	0.259/0.299	0.334/0.472	0.156	0.217	Rio de Janeiro, Brazil	Barbará *et al*., 2009
Alcantarea imperialis	Out	SSR	0.357/0.362	0.398/0.615	0.099	0.434	Rio de Janeiro, Brazil	Barbará *et al*., 2007
Alcantarea Regina	Out	SSR	0.479/0.484	0.458/0.523	-0.051	0.195	Rio de Janeiro, Brazil	Barbará *et al*., 2009
Bromelia antiacantha	Out	SSR	0.326/ -	0.559/ -	0.431	0.224	southeastern Brazil	Zanella *et al*., 2011
Dyckia ibiramensis	Mix	Allozyme	0.055/0.064	0.098/0.219	0.436	0.674[b]	Endemic to southern Brazil	Hmeljevski *et al*., 2010
Encholirium biflorum	ND	RAPD	-	-	-	0.160[c]	Cadeia do Espinhaço, Brazil	Cavallari *et al*., 2006
Encholirium pedicellatum	ND	RAPD	-	-	-	0.084[c]	Cadeia do Espinhaço, Brazil	Cavallari *et al*., 2006
Encholirium subsecundum	ND	RAPD	-	-	-	0.012[c]	Cadeia do Espinhaço, Brazil	Cavallari *et al*., 2006
Pitcairnia albiflos	Out	SSR	0.383/ -	0.429/ -	0.109	0.336	Rio de Janeiro, Brazil	Palma-Silva *et al*., 2011
Pitcainia geyskesii	ND	SSR	0.293/-	0.325/-	0.125	0.156	French Guyana and Suriname	Boisselier-Dubayle *et al*., 2010
Pitcairnia geyskesii	ND	Allozyme	0.185/0.188	0.183/0.246	-0.037	0.266	French Guyana and Suriname	Sarthou *et al*., 2001
Pitcairnia staminea	Aut	SSR	0.347/-	0.452/-	0.240	0.336	Rio de Janeiro, Brazil	Palma-Silva *et al*., 2011
Puya raimondii	Aut	AFLP	-	-	-	0.961[a]	Peru	Sgorbati *et al*., 2004
Tillandsia achyrostachys[d]	ND	Allozyme	0.127/ -	0.210/ -	0.433	0.391	Mexico	González-Astorga *et al*., 2004
Tillandsia ionantha	ND	Allozyme	0.064/ -	0.069	0.056	0.043	Central Mexico to Nicaragua	Soltis *et al*., 1987
Tillandsia recurvata	ND	Allozyme	0/-	0.01/-	1.000	0.906	USA to Argentina	Soltis *et al*., 1987
Vriesea friburgensis	Mix	Allozyme	-/0.234	-/0.226	-0.035	-	Rio Grande do Sul to Pernambuco, Brazil	Alves *et al*., 2004
Vriesea gigantea	Mix	SSR	0.431/-	0.579/-	0.273	0.211	Brazil (south and southeast)	Palma-Silva *et al.,* 2009

ND = Not determined; Out = Outcrossing; Mix = Mixed; Aut = Autogamous; AFLP = Amplified Fragment Length Polymorphism; RAPD = Random Amplified Polymorphic DNA; SSR = Microsatellite.
[a]G_{ST} (Nei, 1973, 1977).
[b]G'_{ST} (Hedrick, 2005).
[c]ϕ_{ST} (Excoffier *et al.*, 1992).
[d]*Tillandsia achyrostachys* var *achyrostachys*.

ceptions were *B. antiacantha* (F_{IS} = 0.431), possibly due to the Wahlund effect and/or null alleles, and *Alcantarea glaziouana* (F_{IS} = 0.156), owing to biparental inbreeding. *Pitcairnia staminea*, which is autogamous, had a high inbreeding coefficient (F_{IS} = 0.240; Table 1). *V. gigantea* and *Dyckia ibiramensis*, which have a mixed mating system, also showed high inbreeding coefficients (F_{IS} = 0.273 and 0.436, respectively; Table 1). The degree of differentiation among populations (F_{ST}) of species evaluated ranged from 0.043 to 0.961. These differences in plant population structure can be influenced by pollen and seed dispersal effects, clonal growth (Gliddon *et al*., 1987), gene flow rates, and connectivity among populations. Compared with species from continuous forest habitats, species restricted to inselberg habitats (Barbará *et al*., 2007, 2009; Palma-Silva *et al*., 2011; Table 1) showed more highly structured populations, with extremely high population differentiation and isolation based on the distance among inselbergs. Thus, rock outcrops could be highly useful venues for studies regarding the molecular ecology and genetics of continental radiations.

Cytogenetics

Few cytogenetic studies of Bromeliaceae are available. Chromosome numbers have been determined for nearly 12% of the known species (Cotias-de-Oliveira *et al.*, 2004), most of which are horticulturally important as ornamentals or fruit producers. Owing to the scarcity of cytogenetic data, the chromosomal evolution of the family has not been completely elucidated. The major hindrances to cytogenetic studies are probably the very small size and poor staining ability of the chromosomes, together with a marked cytoplasmic content (Sharma and Ghosh, 1971; Brown and Gilmartin, 1986).

Billings (1904) was the first to determine the chromosome number of a bromeliad, using *Tillandsia usneoides*, after which several studies were carried out. The first reports revealed a great variety of diploid numbers (2n = 16, 34, 36, 46, 48, 50, 52, 54, 56, 64, 96, and 100) and basic numbers (x = 5, 8, 9, 16, 17, and 25; Brown and Gilmartin, 1986; Bellintani *et al*, 2005). In contrast, most of the 72 bromeliad species studied by Marchant (1967) showed a basic number of x = 25 (except *Cryptanthus*: x = 17). Since then, studies in several different species have generally found the basic chromosome number to be a multiple of x = 25, corroborating Marchant's finding (Brown and Gilmartin, 1989; Cotias-de-Oliveira *et al.*, 2000, 2004; Palma-Silva *et al*., 2004; Gitaí *et al*., 2005; Ceita *et al*., 2008; Louzada *et al.*, 2010). Polyploidy of this base number (2n = 4x = 100 and 2n = 6x = 150) has been observed in all subfamilies, but with low frequency (Brown and Gilmartin, 1989; Gitaí *et al*., 2005; Louzada *et al*., 2010).

Brown and Gilmartin (1989) have proposed a model to explain the evolution of the chromosome base number. In their model, two paleodiploids (x = 8 and x = 9) hybridized, resulting in a paleotetraploid lineage (x = 17), which in turn hybridized with the x = 8 paleodiploid, and the poliploidization stabilized at the hexaploid level of x = 25. Eletrophoretic data (Soltis *et al.*, 1987) suggest that a "diploidization" of the dibasic paleohexaploid occurred. The dibasic model could explain the origin of the distinctive chromosome number in *Cryptanthus*, which may represent a paleotetraploid with 2n = 34. One alternative hypothesis is that *Cryptanthus* evolved from x = 25 via aneuploidy (Brown and Gilmartin, 1989). Flow cytometric results obtained by Ramírez-Morillo and Brown (2001) indicated that the *Cryptanthus* chromosome number originated by descending aneuploidy.

Bromeliaceae chromosomes are usually exceedingly small (0.21-2.72 μm), although the size varies widely among species. According to Gitaí *et al.* (2005), larger chromosomes are usually found at lower ploidy levels, with diploids exhibiting a higher contrast between maximal and minimal chromosome sizes compared with polyploids. Chromosome banding and triple staining with CMA_3/Actinomycin/DAPI has revealed that bromeliads have relatively little heterochromatin, with only one or two CMA^+/$DAPI^-$ terminal bands corresponding to nucleolus organizing regions. B chromosomes have been reported in three Bromelioideae species (Cotias-de-Oliveira *et al.*, 2000, 2004; Bellintani *et al.*, 2005).

Evolution

Recently, Givnish *et al*. (2011) reinforced the *i.e.* of Smith (1934) that bromeliads arose in the Guayana Shield roughly 100 Mya during the Cretaceous Period, with the extant subfamilies beginning to diverge only about 19 Mya. Givnish *et al*. (2011) also suggested that about 15.4 Mya, bromeliads began to spread from that hyper-humid, extremely infertile center to other parts of tropical and subtropical America, and probably arrived in tropical Africa about 9.3 Mya, in a recent long-distance dispersal event. During the evolution of this family, events such as climatic oscillations throughout the Pleistocene have resulted in the dispersion of some clades, including Bromelioidae (Givnish *et al*., 2011). As of the current time, *V. gigantea* has survived glaciation periods in two fragmented refugia in southeastern Brazil (Palma-Silva *et al*., 2009).

The "bromeliad revolution" probably occurred after the uplift of the northern Andes and shift of the Amazon to its present course (Givnish *et al*., 2007). Some morphological and physiological adaptations, including crassulacean acid metabolism (CAM) photosynthesis and the formation of rosettes and leaf absorptive scales, might have been crucial to the adaptive radiation of bromeliads (Benzing, 2000; Crayn *et al*., 2004).

An ecological peculiarity of Bromeliaceae, compared with other families of the order Poales, is their epiphytic habit (Linder and Rudall, 2005). Based on plastid loci,

Crayn *et al*. (2004) proposed that the epiphytic habit of bromeliads evolved a minimum of three times, most likely in response to geological and climatic changes in the late Tertiary.

The more than 3,000 bromeliad species that currently occupy the Neotropical region have evolved to fill numerous niches, with an incredible diversity of adaptations. Some aspects of the complex evolutionary history of this family are still unclear, indicating the need for further molecular studies, in combination with paleontological data, to explain the evolutionary gaps in the wide diversity of bromeliad forms and adaptations.

Conservation

Bromeliads are widely distributed in the Neotropics, with three centers of diversity: the Brazilian Atlantic rainforest; the Andean slopes of Peru, Colombia, and Ecuador; and Mexico and adjacent Central America (Zizka *et al*., 2009). Many species are presently distributed in endangered biomes, are endemic, or have a relict distribution, threatening the survival of many members of this family. For example, the Brazilian Atlantic rainforest is a diverse biome with multiple extremely endangered vegetation types occupying only 7.91% of the extent of their original distribution (Fundação SOS Mata Atlântica and Instituto Nacional de Pesquisas Espaciais, 2009; Carnaval and Moritz, 2008). As the Atlantic rainforest contains at least 803 bromeliad species, 653 of which are endemic and 40% of which are endangered, the preservation of the Atlantic rainforest is vital for the conservation of Bromeliaceae (Martinelli *et al.*, 2008).

Few studies of Bromeliaceae connect genetic data and conservation planning. All of the works cited in the above section "Diversity and genetic structure" contain data that could be used in making conservation decisions. Considerations of the clonal and sexual reproduction, demography, genetic structure within and among populations, gene flow, and mating systems of Bromeliaceae are of primary importance in developing successful conservation strategies (Bizoux and Mahy, 2007).

Our group has studied mainly Brazilian bromeliads, and our field records show a significant reduction in the current distribution of species, compared with the first records in the literature. We believe that genetic, cytogenetic, and reproductive data will be essential for diagnosing species status and for assisting conservation programs and will help to elucidate aspects of evolution and environmental and climate change for Bromeliaceae and the Brazilian Atlantic rainforest.

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Capítulo III

Phylogeography of two sympatric species, *Vriesea carinata* and *V. incurvata* (Bromeliaceae), as a contribution to unravel the evolutionary history of the southern portion of Brazilian Atlantic Forest

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Original Article

Phylogeography of two sympatric species, *Vriesea carinata* and *V. incurvata* (Bromeliaceae), as a contribution to unravel the evolutionary history of the southern portion of Brazilian Atlantic Forest.

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Short running head: Phylogeography of two BAF sympatric bromeliads.

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ABSTRACT

Aim Phylogeographic and paleomodeling studies in Brazilian Atlantic Forest (BAF) may provide valueable insights into the historical processes underlying diversification in this region. Here, we compared the phylogeographic patterns of *Vriesea carinata* and *V. incurvata*, evaluating if these species could share some similar phylogeographic patterns,

since they share some life-history traits and similar geographic distribution, being subjected to the same climatic changes in the past.

Location Southeastern and southern Brazilian forest fragments, along of the BAF.

Methods Fourteen nuclear microsatellites and two plastial DNA regions were used to genotype and sequence individuals from 16 populations of *V. carinata* and from 11 populations of *V. incurvata*. For both sets of markers, we estimated genetic diversity and population differentiation. Bayesian structure analysis of nuclear markers and plastid haplotype network were used to infer population structure. Neutrality tests were used to infer demographic expansion.

Results *V. carinata* and *V. incurvata* showed moderate levels of nuclear and plastial genetic diversity. Both species showed isolation by distance and present expansion towards southern margins. They showed similar phylogeographic patterns and we proposed that they survived in more than one refugium during climatic oscillation of Pleistocene, one putative refugium was on coastal south-southeastern (25°S - PR/SP) and another in southeastern Brazil (20°S- RJ/ES).

Main Conclusions The results are consistent with few records encountered in the literature, proposing the multiple refugia hypothesis to BAF with genetic discontinuity among southern and northern, influenced mainly by climatic oscillations of the Pleistocene. These studies are essential for a better understanding of the biome's evolutionary history as a whole, and particularly for the southern portion.

Keywords: Brazilian Atlantic Forest, Phylogeography, Pleistocene, Bromeliaceae, microsatellite, cpDNA, *Vriesea*.

INTRODUCTION

The Brazilian Atlantic Forest (BAF) is the third largest hotspot of the world, with approximately 20 000 plant species of which 8 000 are endemic. This is a considerable proportion of the South America biodiversity but, unfortunately, the BAF currently retains only 7.5% of its primary vegetation (Myers et al., 2000). The BAF includes mainly ombrophilous and semi-deciduous forest, having large geographic extension and floristic diversity (Oliveira-Filho & Fontes, 2000). Much effort has been put into understanding the

complex and high levels of biodiversity in BAF through phylogeographical and paleomodeling studies, which may provide valuable insights into the historical processes underlying diversification in this region (Martins, 2011).

Modern phylogeographical methods, studying and reconstructing evolutionary relationships of lineages, make possible to infer the role of past events in shaping the current patterns of biodiversity (Excoffier, 2004). Added to it, the combination of multiple types of markers with variable mutation rates and modes of inheritance (organellar and nuclear markers) provides a mean of separating the contribution of different events spanning broad time-scales (Petit et al., 2005). Comparative phylogeography focus on the comparison of geographical patterns and genetic variation among multiple co-distributed taxa, and has strong parallels with historical biogeography (Cracraft, 1989; Zink, 1996). Phylogeographical approach would help to reconstruct the evolutionary history of BAF, improving conservation and management measures such as the identification of priority populations/areas for conservation. There are some studies on BAF species diversification, but they show limited agreement on general mechanisms used to explain the origin of its diversity.

Recent paleoclimatic modelling of predicted habitat stability in the BAF corroborates the hypothesis that the distribution of forested habitat was spatially and temporally variable during Late Pleistocene glaciations (Carnaval & Moritz, 2008; Carnaval et al., 2009; Thomé et al., 2010; Silva et al., 2012). The Pleistocene glacial cycles were responsible by vicariant events and the isolation of populations in refuges along the coast (Haffer 1969; Grazziotin et al. 2006; Martins, 2011). Those climatic changes in combination with the geomorphologic complexity of coastline could result in fine-scale habitat heterogeneity (Fitzpatrick et al., 2009). BAF diversification was influenced by uplift of Brazilian east coast during Tertiary, resulting in geographical and climatic modifications, leading to forest fragmentation, isolation of regional faunas and flora, and correlated speciation events (Simpson, 1979). Phylogeographical studies have recently identified a north–south division in the BAF and most of these studies have given forest fragmentation as the most likely scenario for the geographical structure described (Harris et al., 2005; Pellegrino et al., 2005; Grazziotin et al., 2006; Cabanne et al., 2007; Martins et al., 2007; Fitzpatrick et al., 2009; Palma-Silva et al., 2009; Novaes et al., 2010; Pinheiro et al., 2011; Ribeiro et al., 2011). Paleomodeling of the BAF ecoregion predicts severe forest contraction in south

97 portion, nearly of São Paulo state and large stable forested areas in northern regions during
98 the last glacial maximum (LGM), followed by Holocene expansion (Carnaval & Moritz,
99 2008). However, the historical biogeography of the BAF is complex and many processes
100 might have to be invoked (Martins, 2011; Silva et al., 2012). Many factors, including
101 Pleistocene refugia, marine transgression, and tectonic activity, might be responsible for
102 shaping the current distribution of lineages (Martins, 2011).

103 The Bromeliaceae family is one of the morphologically and ecologically most
104 diverse flowering families native to the tropics and subtropics of the New World, with
105 more than 3 000 species that currently occupy numerous niches, with an incredible
106 diversity of adaptations (Zanella et al., 2012). BAF contains at least 800 bromeliad species,
107 ~600 of which are endemic and 40% are endangered, being the preservation of the BAF
108 vital for its conservation (Martinelli et al., 2008). *Vriesea* Lindl. is the second largest genus
109 in subfamily Tillandsioideae and the third largest in Bromeliaceae (Benzing, 2000). The
110 genus is presently composed of 258 species and has two centers of diversity, one of them
111 lies in eastern Brazil (BAF), where approximately 84% of the species occurs (Costa et al.,
112 2009). *Vriesea carinata* Wawra is an epiphytic or terrestrial bromeliad which is endemic to
113 BAF, with distribution from 19° to 29°S. *Vriesea incurvata* Gaudichaud is also epiphytic
114 and endemic to BAF, with a more restrict distribution, from 22° to 29°S (Smith & Downs,
115 1977). These species can be found in sympatry, occur preferentially in mesophilic
116 environments, showing sequential flowering along the year and similar floral morphology.
117 Also, they share pollinator (hummingbirds; Machado & Semir, 2006) and show seed
118 dispersion mediated by wind (Smith & Downs, 1977). The synchrony of sequencial
119 flowering is common in Bromeliaceae, being important mainly for maintenance of pollen
120 vectors to allow great diversification of food supply, (Araujo et al., 1994; Machado &
121 Semir, 2006). *Vriesea carinata* and *V. incurvata* were chosen for this study, since they are
122 typically BAF species, with wide distribution across this ecoregion and for having a
123 distribution further south (29°S), where the history of BAF is poorly known.

124 Here we used these two species of BAF to test the hypothesis that they would be
125 subject to the same climate changes of the past, using a comparative phylogeography
126 approach. Specifically, we addressed the following questions: I) Do *V. carinata* and *V.*
127 *incurvata*, having sympatric populations, similar distribution and sharing some life-history
128 traits, show related phylogeographic pattern? II) What is the influence of the historical

events on the genetic diversity and population's structure of *V. carinata* and *V. incurvata*? III) The phylogeographic patterns observed are compatible with previous hypotheses of the origins and patterns of diversification of BAF? We discuss the phylogeographical and genetic structure of *V. carinata* and *V. incurvata* in the light of palaeoclimate, vegetation reconstructions and the biogeographical history of the BAF.

MATERIALS AND METHODS

Plant material and sampling strategy

Sixteen populations of *V. carinata* and 11 of *V. incurvata* were sampled across the BAF from 2009 to 2011 (Table 1; Figure 1). The populations were located in six Brazilian Federative States: Rio Grande do Sul (RS), Santa Catarina (SC), Paraná (PR), São Paulo (SP), Rio de Janeiro (RJ) and Espírito Santo (ES). The distance among populations ranged from approximately 23.8 km (MQvc and CAvc) to 1459 km (CAvc and STvc; Figure 1). In six sites the species were found in sympatry (MQ, CO, JV, MT, MO, and IC) and all of them were sampled (Table1). These species are epiphytic, occurring in habitat with high humidity and well preserved, being most of samples from biological reserves. Leaves were collected from up to 44 individuals in each population, totaling 279 individuals for *V. carinata* and 186 for *V. incurvata*. Fresh leaves were stored in silica gel for drying. Total genomic DNA was isolated using CTAB method (Doyle & Doyle, 1990).

Nuclear microsatellite markers and genotyping assays

A total of 14 nuclear microsatellite markers (nuSSR) were used in this study, seven isolated from *Vriesea gigantea* (*loci*: VgA04, VgA06, VgB10, VgB12, VgC01, VgG02, VgG03; Palma-Silva et al., 2007), three from *Alcantarea imperialis* (*loci*: Ai5.18, Ai4.10, Ai4.03; Palma-Silva et al., 2007), three from *Tillandsia fasciculata* (*loci*: e6, p2p19, e6b; Boneh et al., 2003) and one from *Pitcairnia albiflos* (*locus*: PaA10; Paggi et al., 2008). For each SSR, the forward primers were synthesized with a 19-bp M13 tail (5′-CACGACGTTGTAAAACGAC-3′) at the 5′ end to allow labeling with a tailed fluorescent dye M13 primer during genotyping procedures, following the method of Schuelke (2000).

All polymerase chain reaction (PCR) amplifications were performed in a Veriti 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA) following the protocol described by Palma-Silva et al (2007). The microsatellite alleles were resolved on a *ABI 3100* DNA Analyzer Sequencer (Applied Biosystems, Foster City, CA, USA) and sized against the *GS500 LIZ* molecular size standard (Applied Biosystems, Foster City, CA, USA) using GENEMARKER Demo version 1.97 (*SoftGenetics*, State College, PA, USA).

Plastidial non-coding region: amplification and sequencing

Nine chloroplast genome regions (cpDNA) were analyzed by amplification and sequencing: *3'rps16-5'trnK*, *rpl32-trnL* (Shaw et al., 2007), *trnH-psbA* (Shaw et al., 2005), *trnL-trnF*, *trnTa-trnLb* (Taberlet et al., 1991), *trnD-trnT* (Demesure et al., 1995), *petG-trnP* (Hwang et al., 2000), *trnL intron* (Taberlet et al., 2007), *matK* (Schulte et al., 2005). Two cpDNA regions, *trnL-trnF* spacer and *matK* gene, revealed polymorphisms in the analyzed individuals and were therefore selected for a large-scale survey of haplotype variation in *V. carinata* and *V. incurvata.* PCR reactions for *trnL-trnF* were run using the following parameters: denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 54 °C for 1min, and 72 °C for 1 min, and a final extension for 10 min at 72 °C, using primers *trnL5'*^{UAA}F (TabC) and *trnF*^{GAA} (TabF) for PCR and sequencing as described by Taberlet et al. (1991). *matK* gene amplification and sequencing were carried out as described in Schulte et al. (2005). All PCR was carried out in a total volume of 20 µl containing 10 ng DNA template, 1x *GoTaq* buffer, 2mM $MgCl_2$, 250 µM dNTP mix, 5 ρmol forward and reverse primers and 1U of *GoTaq* DNApolymerase (Promega, Madison, WI, USA). PCR amplifications were performed in a Veriti 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA). Plastid PCR products were sequenced from both ends using BigDye Kit (Applied Biosystems) at the Macrogen Inc. (South Korea). Sequences were aligned to obtain the consensus using the software MUSCLE (Edgar, 2004) implemented in MEGA5 version 5.0 (Tamura et al., 2011) and were edited manually.

Data analysis

Nuclear SSR: Descriptive statistics were performed for each population of both species. The nuclear microsatellite diversity was characterized using the number of alleles (*A*), observed (H_O) and expected (H_E) heterozygosities, and the inbreeding coefficient (F_{IS}) (Weir & Cockerham, 1984), calculated using the programs FSTAT version 2.9.3.2 (Goudet, 1995) and MSA 4.00 (Dieringer & Schlötterer, 2003). Departures from the Hardy–Weinberg equilibrium (HWE) for each population were identified using exact tests in GENEPOP 4.0 (Raymond & Rousset, 1995). We also evaluated the presence of null alleles using the program MICRO-CHECKER version 2.2.3 (Van Oosterhout et al., 2004).

The genetic structure of *V. carinata* and *V. incurvata* populations was investigated with a Bayesian clustering algorithm implemented in STRUCTURE version 2.3.3 (Pritchard et al., 2000), considering each species data set separately (n = 279 for *V. carinata* and n = 186 for *V. incurvata*). We performed 20 runs and calculated the mean posterior probability of the data ['log probability of data', L(K)]. We determined the most probable number of populations, K, by using the method described by Evanno et al. (2005) that examines ΔK, an *ad hoc* quantity related to the change in posterior probability between runs of different K. Analyses were carried out under the admixture model assuming independent allele frequencies and using a burn-in period of 100 000, run length of 500 000, to confirm stabilization of summary statistics and K ranging from 1 to 17 for *V. carinata* and 1 to 13 for *V. incurvata* (Pritchard et al., 2000).

We assessed nuclear genetic differentiation using estimates of F_{ST} (Weir & Cockerham, 1984); the unbiased estimator of relative differentiation G_{ST} (Nei & Chesses, 1983) and the standardized genetic differentiation measure G'_{ST} (Hedrick, 2005) calculated in the software FSTAT version 2.9.3.2 (Goudet, 1995). Pairwise comparisons of F_{ST} between populations were estimated with 10 000 permutations for each of the 26 populations using the software ARLEQUIN 3.1 (Excoffier et al., 2005). Partitioning of genetic diversity within and among species were examined by analysis of molecular variance (AMOVA) implemented in the software ARLEQUIN 3.1 (Excoffier et al., 2005).

The hypothesis that populations are differentiated because of isolation-by-distance (Wright, 1965) was tested by calculating the correlation between geographic and genetic distance matrices (F_{ST}) with a standardized Mantel test using GENEPOP (Raymond & Rousset, 1995). The significance was assessed through a randomization test using 10 000 Monte Carlo simulations.

Recent population size reductions (i.e. genetic bottlenecks) were tested based *M*-statistic values calculated using the software ARLEQUIN 3.1 (Excoffier et al., 2005), for each population according to Garza & Williamson (2001), to detect reductions in effective population size. The threshold value of $M = 0.680$ was used in comparison between the mean value *M* across all loci, following the procedure described by Garza & Williamson (2001). Populations with $M < 0.680$ suggested bottlenecks evidence. To estimate the relative contribution of pollen versus seed flow, G_{ST} values from nuSSR (biparentally) and cpDNA (maternally) inherited markers were compared, following the formula # 1 presented by Petit et al (2005).

Plastidial sequences: Sequences were aligned and polymorphisms at mononucleotide microsatellites were excluded due to ambiguous alignment and higher mutation rates. Long indels (usually with more than 5 bp) were coded as one evolutionary event (one character), and each base pair was equally weighted before analysis. For statistical analyses, the sequences of the two chloroplast regions were concatenated. Haplotype (*h*) and nucleotide (π) diversity (Nei, 1987) were estimated for each population and overall populations and species using the software ARLEQUIN 3.1 (Excoffier et al., 2005). Genealogical relationships among haplotypes for chloroplast data set were inferred using median-joining method (Bandelt et al., 1999), implemented in the software NETWORK 4.6.1.1 (http://www.fluxus-engineering.com) for total cpDNA data set sequences (n = 212).

Estimates of differentiation (G_{ST} and F_{ST} statistics) were calculated in the software DNASP v5.10 (Librado & Rozas, 2009), taking into account the pairwise distance between cpDNA haplotypes, excluding the only sample of JVvi population. Population pairwise F_{ST} comparisons were calculated using ARLEQUIN software ($P < 0.05$) considering each species data set. The program spatial analysis of molecular variation (SAMOVA, Dupanloup et al., 2002) was used to analyze the population structure. This method defines groups of populations that are geographically homogenous and maximally differentiated from each other, through a priori definition of the number of groups (K) of populations, and generates *F* statistics (F_{SC}, F_{ST}, and F_{CT}). The optimal number of groups was selected according to the highest F_{CT} value (differentiation among groups). For each value of K, 100 simulated annealing processes were used, ranging K from two to 12. We performance two SAMOVA analysis, one for each species. To test the hypothesis of population differentiation, the

genetic structure was further examined by an AMOVA, using SAMOVA results in the software ARLEQUIN 3.1 (Excoffier et al., 2005).

We employed Monmonier's maximum difference algorithm to highlight geographical features that are corresponding to pronounced genetic discontinuity using the program BARRIER 2.2 (Manni et al., 2004). Geographical coordinates were used for each sample and connected by Delauney triangulation using a cpDNA pairwise F_{ST} genetic matrix. Putative genetic boundaries were identified across the geographical landscapes. The data derived from each species matrix were analyzed separately to detect if putative barriers of gene flow are similar for two species.

Neutrality tests were performed using Fu's Fs (Fu, 1997), based on the haplotype distribution, and Tajima's D (Tajima, 1989), Fu & Li's (1993) F* and D*, considering the segregating sites. Tests were carried out with 10 000 simulation steps using ARLEQUIN 3.1 (Excoffier et al., 2005) and DNASP v5.10 softwares (Librado & Rozas, 2009), considering four groups based in SAMOVA (cpDNA) and STRUCTURE (nuSSR) results: *V. carinata* north distribution – VcN; *V. carinata* southern – VcS, *V. incurvata* north distribution – ViN and *V. incurvata* southern – ViS.

RESULTS

Nuclear genetic diversity

For the 15 populations of *V. carinata* and nine of *V. incurvata* genotyped with 14 nuSSR, moderate levels of genetic diversity were observed for all genetic parameters (Table 1). The results obtained from genetic diversity and population structure were similar for both species. The number of alleles ranged from 43 to 132, with a total of 208 alleles in *V. carinata* and ranged from 64 to 124, with a total of 192 alleles in *V. incurvata*. The observed and expected heterozygosities per population ranged from 0.133 to 0.647 and from 0.373 to 0.756 for *V. carinata* and from 0.267 to 0.588 and from 0.554 to 0.787 in *V. incurvata*, respectively. The inbreeding coefficient (F_{IS}) was high and significant in all populations ($P < 0.001$); null alleles were detected in some populations (data not shown).

Nuclear population structure

Vriesea carinata and *V. incurvata* showed low levels of genetic differentiation among populations, F_{ST} = 0.090, G_{ST} = 0.095 and G'_{ST} = 0.100; and F_{ST} = 0.080, G_{ST} = 0.080 and G'_{ST} = 0.089, respectively. Individual F_{ST} estimates between pairs of *V. carinata* and *V. incurvata* populations ranged from 0.012 to 0.291 and from 0.002 to 0.185, respectively, and most values observed were significant ($P < 0.05$; Appendices S1 and S2 in Supporting Information). For both species Bayesian analysis confirmed structure, with a model of K = 2 populations being able to best capture the variation in the data, clearly separating the northern populations (SMvc and STvc in *V. carinata* and CMvi and GPvi in *V. incurvata*; Figures 2A e 2B, respectively). AMOVA results, for each species revealed that a higher proportion of the genetic variance resided 'whitin populations' in *V. carinata* (93.3%; $P < 0.001$) and in *V. incurvata* (92.4%; $P < 0.001$), with fewer variation among populations (Table 2).

Mantel tests in each species indicated significant correlation between geographical and genetic distance, thus suggesting the presence of isolation by distance in populations of *V. carinata* and *V. incurvata* ($R^2 = 0.412$, $P < 0.001$; $R^2 = 0.716$, $P < 0.001$; respectively). *M*-ratios test for recent population bottlenecks, which ranged from 0.565 to 0.768 across all sites, suggested that bottlenecks have occurred in some populations for both species (Table 1).

Using the values of genetic differentiation G_{ST} among populations for nuSSR markers (0.095) and for cpDNA markers (0.251), the ratio of pollen flow to seed flow (Ennos, 1994; Petit et al., 2005) was estimated at 2.12, indicating that gene flow through pollen in *V. carinata* is twice more efficient than through seeds. For *V. incurvata*, the ratio of pollen flow to seed flow was estimated at 4.43, suggesting that gene flow via pollen is fourfold greater than that via seeds.

Chloroplast genetic diversity

A total of 2719 bp from two plastidial DNA regions (*matK* gene - 1844 bp, and *trnL-trnF* intergenic spacer - 875 bp) of 139 individuals of *V. carinata* and 73 of *V. incurvata* were sequenced. The molecular diversity indexes are shown in Table 1. These cpDNA sequences represented 25 haplotypes with 26 polymorphic sites, including 16 transitions,

eight transversions and four indels. The plastid haplotype network, performed with cpDNA sequences, revealed low haplotypic sharing among species (H2, H3 and H12) and a not clear separation of them, with few mutational steps (Figure 3A, 3B).

Vriesea carinata showed 15 haplotypes and in *V. incurvata* 13 were found (Table 3). The haplotype diversity (*h*) for *V. carinata* population ranged from 0 to 1.000 and the nucleotide diversity (π) from 0 to 0.00111 (Table 1). The total haplotype and nucleotide diversities were 0.785 and 0.00787, respectively. Two of the 15 analyzed *V. carinata* populations were monomorphic (MQvc and SMvc), the highest haplotype number was observed in population MOvc (seven haplotypes), whereas the remaining populations showed two to four haplotypes with ten unique haplotypes (Table 3; Figure 3C). In the 11 populations of *V. incurvata*, the haplotype diversity (*h*) ranged from 0 to 1.000, and the nucleotide diversity (π) from 0 to 0.00246. The total haplotype and nucleotide diversities were 0.731 and 0.00107, respectively (Table 1). Only the JVvi population was monomorphic due to single sample. The populations MOvi, and GPvi exhibited four haplotypes, whereas the others showed two or three haplotypes with seven unique haplotypes (Table 3; Figure 3D). Haplotypes H1 and H16 are the most frequent and widespread for *V. carinata* and *V. incurvata*, respectively. We found that 79.14% of the individuals in 12 out of 15 *V. carinata* sampled populations showed the three most common haplotypes (H1 – 33.81%, H3 – 28.06% and H2 – 17.27%). For *V. incurvata*, we found that H16 (43.24%) and H17 (22.97%) were the most frequent haplotypes, being found in nine out of 11 sampled populations (Table 3). Populations from northern distribution in each species do not share haplotypes with south populations; in contrast, in the southern populations several haplotypes were shared among them in both species (H2; H3 and H12; Table 3 and Figure 3).

Chloroplast population structure

Pairwise F_{ST} values among *V. carinata* populations ranged from -0.177 to 1.000 (Appendix S1). In general, lower F_{ST} values were observed between adjacent populations. Differentiation measures across all populations were moderate, with F_{ST} = 0.355 and G_{ST} = 0.251. For *V. incurvata* we estimated a F_{ST} = 0.678 and G_{ST} = 0.298, showing moderate to

high structure among populations. Pairwise F_{ST} values among populations ranged from -0.615 to 0.967 (Appendix S2).

The SAMOVA analyses using cpDNA allowed the identity of three groups with F_{CT} = 0.524 (P < 0.001) for *V. carinata* and two groups for *V. incurvata* (F_{CT} = 0.832; P < 0.001), separating the southern and northern populations for both species (Figure 4). AMOVA results, for each species, considering cpDNA data, revealed that a higher proportion of the genetic variance resided 'whitin populations' in *V. carinata* (66.2%; P < 0.001) and 'among populations' in *V. incurvata* (70.3%; P < 0.001 (Table 2). Neutrality tests revealed no significant values (P > 0.05) for most groups and analyses, except VcS and ViS that showed negative significant values, indicating past population expansion and/or purifying selection (Table 4). The Barrier prediction analysis using Monmonier's maximum difference algorithm identified one putative barrier when all sites were included for each species analysis (Figure 1). The barrier separated RJ and ES Brazilian States populations (northern) from southern populations.

DISCUSSION

Genetic structure and diversity

Vriesea carinata and *V. incurvata* showed moderate levels of genetic diversity for all parameters (Table 1). All populations evaluated exhibited an excess of homozygote genotypes, similar to previous reports for other bromeliads using nuclear microsatellite markers (Barbará et al., 2007; Palma-Silva et al., 2009; Hmeljevski et al., 2010; Zanella et al., 2011). *Vriesea incurvata* is self-compatible (Martinelli, 1994), characteristic predominant in *Vriesea* genus (Matallana et al., 2010). As *V. carinata* and *V. incurvata* are related species (Maia et al., 2012) and possibly present the same breeding system, the excess of homozygotes encountered in these species probably occur due to selfing or biparental inbreeding, as observed in *V. gigantea*, which is characterized by a mixed mating system and high biparental inbreeding (Paggi, 2012).

Our analyses of cpDNA sequences from *V. carinata* and *V. incurvata* populations exhibited high haplotype and nucleotide diversities in 2719 bp sequenced of *matK*/*trnL-trnF* concatenated regions (Table 1). Qualitative inspection of the data indicated that the

383 center of genetic diversity for *V. carinata* may be represented by populations MTvc,
384 MOvc, PAvc and IPvc (southern species distribution – 24° and 25°S latitude), and
385 diversity decreased steadily towards northern and southern range margins (Table 1). For *V.*
386 *incurvata*, the genetic diversity center may be represented by MTvi, MOvi and GPvi
387 populations, in the same region of *V. carinata*. Moreover, the geographical and genetic
388 distances are significantly correlated, suggesting isolating by distance as observed for *V.*
389 *gigantea* (Palma-Silva et al 2009. *Vrisea carinata* and *V. incurvata* sharing three
390 haplotypes, indicanting a recent separation among then, as verify by Maia et al (2012).
391 The genetic divergence among *V. carinata* (F_{ST} = 0.090) and *V. incurvata* (F_{ST} =
392 0.080) populations, considering nuSSR, was low and similar to values observed for some
393 outcrossing Bromeliaceae species (*Alcantarea geniculata* F_{ST} = 0.111 and *Alcantarea*
394 *regina* F_{ST} = 0.195, Barbará et al., 2007, 2009, respectivaly). Considering cpDNA
395 haplotypes, F_{ST} values revealed stronger genetic structure compared with nuSSR (see
396 results), and this difference reflects the relative role of pollen and seed dispersal in
397 generating the observed genetic structure, where pollen was more efficient than seeds in
398 both species. In species which seed flow is lower than pollen flow, it is predicted that the
399 plastid genome will be highly structured when compared with nuclear genes (Petit et al.,
400 2005), if the plastid DNA is inherited maternally (Ennos, 1994; Petit et al., 2005) as
401 observed in most angiosperms. *Vriesea carinata* and *V. incurvata* have pollen gene flow
402 mediated by hummingbirds (Machado & Semir, 2006) and seed dispersion mediated by
403 wind (Smith & Downs, 1977). The presence of strong population structure using cpDNA
404 markers was also reported in *V. gigantea*, were seeds are thought to be wind-dispersed and
405 pollen grains are thought to be dispersed by bats (Palma-Silva et al., 2009). The results
406 indicated an important role for pollinators in maintaining population connectivity, once
407 seed gene flow is lower, and the seeds probably reach short distance as reported for *V.*
408 *gigantea* (Paggi et al., 2010).
409
410 **Phylogeography, demographic patterns and putative refugia on BAF**
411
412 The comparative analysis of phylogeographic and demographic pattern between *V.*
413 *carinata* and *V. incurvata* identified similarities between then, suggesting that these species
414 being subjected to the same climatic changes in the past. We identified the existence of two

distinct groups for both species, one located in the southern distribution, and other in the northern portion of the sampled area, comprising populations of RJ and ES Brazilian States, where a genetic discontinuity were identified (Figures 1 and 3). This genetic discontinuity agrees with patterns previously described for another *Vriesea* species (*V. gigantea*) that showed deep phylogeographic structure divided into two major groups, one in northern and other in southern species' distribution (Palma-Silva et al., 2009). The presence of two distinct groups would seem to support the hypothesis that *V. carinata* and *V. incurvata* survived in more than one fragmented refugia during Pleistocene climatic oscillations. One probably located between 23°S and 25°S, comprising populations of Matinhos (MT), Morretes (MO), Pariquera-açu (PA) and São Paulo (SP). These populations hold the greatest amount of genetic diversity for both nuSSR and cpDNA, corresponding to the boundary between the southern and southeastern Brazilian regions, named here PR/SP refugium (Table 1 and Figure 1). The second putative refugia, was located in the northern region of species distribution, comprising populations of RJ and ES states, named here RJ/ES refugium (Figure 1). Patterns of haplotype distribution in the northern populations (RJ and ES States), without haplotypic sharing in three sampled localities for *V. carinata* (Figure 3C), combined with SAMOVA results (Figure 4), reveal a complex history in this region. On the other hand, the southern populations showed high haplotypic sharing and signal of expansion towards southern. Vegas-Villarubia et al. (2011) argued that palaeoecological information shows that spatial reorganizations and persistence in suitable microrefugia have been more common than extinction during the Quaternary. The persistence of some species in multiple refugia localized throughout their distribution indicates that these species might have persisted in heterogeneous environments, demonstrated the importance of dynamic evolutionary processes and the mosaic of habitats in heterogeneous landscapes (Turchetto-Zolet et al., 2013). These microrefugia are difficult to identify with common palaeoecological methods because of their assumed small size and unknown distribution (Rull, 2010), but intra-specific genetic patterns of the involved species provide evidence of their existence and suggestions for their geographical distribution (Vegas-Villarubia et al., 2011).

Neutrality test values for southern populations of *V. carinata* and *V. incurvata* were significantly negative (Table 4), providing evidence of expansion of these populations. This pattern may result from the localization of southern BAF, where higher latitudes

probably suffered more intensely the effect of the Pleistocene climatic oscillations leading to smaller patches of continuous forest and population expansion after the end of the last glacial cycle (Carnaval et al., 2009). Thereby, we would predict relatively high stability in northern populations and signatures of more recent population expansion in southern regions after the Last Glacial Maximum.

Concerning the evidences discussed here, *V. carinata* and *V. incurvata* probably survived in more than one refugium (PR/SP and RJ/ES) during the Pleistocene climatic oscillations. Carnaval et al. (2009) and Thomé et al (2010), in their studies on ecological niche models under paleoclimates, modeled the spatial range of the BAF for three climatic scenarios using frogs and toads as models. The results support a picture of habitat fragmentation associated with glacial cycling and the identification of putative stable areas, of which two correspond to those found here to *V. carinata* and *V. incurvata*. One in southeastern Brazil, ranging from RJ to ES States and eastern Minas Gerais, which corresponds to northern populations distribution of *V. carinata* and *V. incurvata* (RJ/ES refugium) and other in coastal south-southeastern Brazil, ranging from north Paraná to São Paulo States, which coincides with PR/SP refugium (Figure 1). These north-south division in the BAF was also observed in some animals phylogeographical studies (Harris et al., 2005; Cabanne et al., 2007; Martins et al., 2007; Fitzpatrick et al., 2009), with a contact zone after the forest expansion for the monkey *Alouatta guariba* (Harris et al., 2005) and the bird *Xiphorynchus fuscus* (Cabanne et al., 2007), being this contact zone well described near the coastal area of São Paulo state where the tropic of Capricorn lies. This is an indication of a Pleistocene separation between the Serra do Mar in São Paulo state and the other forested areas along the coast to the north (Martins, 2011). There is a great discussion regarding the importance of Pleistocene vs. Pliocene/Miocene events in promoting the diversification of Neotropical species (Hewitt, 1996, 2001; Moritz et al., 2000; Bennett, 2004; Rull, 2008, 2011; Brunes et al., 2010; Hoorn et al., 2010; Werneck et al., 2011; Hughes et al., 2013). Both Pleistocene climatic oscillations and Pliocene/Miocene orogenic events have contributed to shaping the current diversity and distribution of modern species in the Neotropical regions (Turchetto-Zolet et al., 2013). Under this hypothesis, many species must have begun diversifying by Neogene tectonic events and palaeogeographical reorganizations, and maintained by the action of Pleistocene climatic oscillations (Rull, 2011). The results obtained here may contribute to unravel this question.

CONCLUSIONS

Vriesea carinata and *V. incurvata* showed moderate nuclear and plastidial genetic diversity and both had a negative correlation between genetic and population distances, indicating isolation by distance. Bottlenecks were detected in marginal populations, and expansions events were observed in southern populations, consistent with historical south forest expansion after the Last Glacial Maximum (Carnaval et al., 2009). The species presented similar phylogeographic patterns, with a north-south division in the BAF, corroborating with the few records encountered in the literature. In this way, we proposed the multiple refugia hypothesis to BAF, with genetic discontinuity between southern and northern *V. carinata* and *V. incurvata* distribution. It was identified a putative coastal south-southeastern refugium (PR/SP) and other in southeastern Brazil (RJ/ES), influenced mainly by climatic oscillations of the Pleistocene and by Pliocene/Miocene orogenic events.

More studies are required for understanding the BAF complex history, since this pattern was probably shaped throughout the Pleistocene, but earlier events, as uplift of Brazilian east coast during Tertiary, may be also influenced the distribution and diversification of taxa (Silva et al., 2012; Turchetto-Zolet et al., 2013). Moreover, a discordant predictive performance of paleomodeling in the south-southeastern regions of BAF (Carnaval et al., 2009; Thomé et al., 2010; Martins, 2011; Silva et al., 2012), aggregated to a few phylogeographic studies in this region, reinforce the need of studies that focus on taxa with broader distributions in the southernmost BAF region for a better coverage of this region.

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775

776 **BIOSKETCH** This study is part of Camila M. Zanella's doctoral research on the
777 phylogeography and hybridization of Bromeliaceae, carried out at the Federal University
778 of Rio Grande do Sul. The authors of this paper share interests in the genetics and
779 conservation of the Brazilian Atlantic Forest plant species, with main emphasis on the
780 family Bromeliaceae. We use different approaches and tools to resolve issues related to
781 biology, ecology, genetics and evolution of species of this taxonomic group. (http://
782 www.ufrgs.br/ngcp/).

783 **TABLES**

784 **Table 1** Population sampled with their identification code (ID) and geographical coordinates of *Vriesea carinata* and *V. incurvata* in the
785 Brazilian Atlantic Forest and the estimated diversity indexes for 14 nuclear microsatellite and chloroplast DNA sequences (*matk* + *trnL-trnF*).
786 Sample size analyzed for both genomes (*N*), number of alleles (*A*), observed (H_O) and expected (H_E) heterozygosities, inbreeding coefficient
787 (F_{IS}), mean Garza-Williamson index for bottleneck test probabilities (*M*), haplotype diversity (*h*), nucleotide diversity (π) and number of
788 haplotypes (*NH*).

				Nuclear microsatellite						cpDNA			
Population	ID‡	Lat S	Long W	N	*A*	H_O	H_E	F_{IS}†	*M**	N	*h*	π	*NH*
Vriesea carinata													
Maquiné – RS	MQvc	29°30'	50°14'	15	74	0.407	0.595	0.324	0.659**	10	0	0	1
Caraá – RS	CAvc	29°43'	50°17'	8	62	0.437	0.687	0.383	0.619**	5	0.400	0.00015	2
Corupá – SC	COvc	26°24'	49°20'	19	96	0.534	0.680	0.219	0.700	12	0.682	0.00048	4
Joinville – SC	JVvc	26°10'	48°59'	18	99	0.412	0.708	0.427	0.641**	7	0.762	0.00059	3
Garuva – SC	GAvc	25°56’	48°48’	19	83	0.408	0.600	0.326	0.659**	9	0.639	0.00059	3
Matinhos – PR	MTvc	25°47'	48°31'	24	109	0.513	0.696	0.268	0.753	13	0.564	0.00028	3
Morretes – PR	MOvc	25°20'	48°52'	44	132	0.524	0.709	0.264	0.765	19	0.819	0.00105	7
Ilha do Mel – PR	IMvc	25°33'	48°18'	-	-	-	-	-		2	1.000	0.00111	2
Pariquera-açu – SP	PAvc	24°38'	47°48'	12	77	0.448	0.710	0.380	0.572**	10	0.711	0.00050	4
Ilha do Cardoso – SP	ICvc	25°04'	47°55'	20	108	0.539	0.705	0.241	0.768	10	0.778	0.00066	4
Iporanga – SP	IPvc	24°31'	48°42'	20	117	0.647	0.710	0.091	0.724	8	0.714	0.00058	3
Intervales – SP	ITvc	24°16'	48°22'	13	75	0.323	0.703	0.553	0.565**	-	-	-	-
Bertioga – SP	BEvc	23°45'	45°55'	20	97	0.483	0.642	0.252	0.655**	13	0.667	0.00050	3
Teresópolis – RJ	TEvc	22°27'	42°57'	14	103	0.554	0.756	0.275	0.655**	4	0.500	0.00037	2
Santa Maria do Jetibá – ES	SMvc	20°10'	40°55'	20	43	0.540	0.680	0.653	0.725	9	0	0	1
Santa Teresa – ES	STvc	19°57'	40°32'	13	89	0.133	0.373	0.212	0.679**	8	0.536	0.00099	2
Total *V. carinata*				279	208	0.458	0.673	0.291	-	139	0.785	0.00787	15

Vriesea incurvata													
Maquiné – RS	MQvi	29°30'	50°14'	29	85	0.476	0.661	0.201	0.714	10	0.378	0.00015	3
Florianópolis – SC	FLvi	27°31'	48°30'	17	64	0.267	0.554	0.531	0.649**	3	0.667	0.00025	2
Antônio Carlos – SC	ACvi	27°27'	48°51'	16	74	0.398	0.644	0.403	0.699	2	1.000	0.00037	2
Corupá – SC	COvi	26°24'	49°20'	16	91	0.588	0.653	0.104	0.701	7	0.286	0.00011	2
Joinville – SC	JVvi	26°10'	48°59'	-	-	-	-	-		1	0	0	1
Matinhos – PR	MTvi	25°47'	48°31'	17	92	0.379	0.725	0.486	0.685	8	0.607	0.00051	3
Morretes – PR	MOvi	25°20'	48°52'	44	124	0.478	0.675	0.295	0.752	14	0.659	0.00041	4
Ilha do Cardoso – SP	ICvi	25°04'	47°55'	-	-	-	-	-		3	0.667	0.00246	2
São Paulo – SP	SPvi	23°27'	46°47'	11	80	0.458	0.685	0.343	0.716	7	0.524	0.00021	3
Cachoeira do Macacu – RJ	CMvi	22°24'	42°44'	17	98	0.430	0.787	0.465	0.677**	10	0.200	0.00007	2
Guapimirim – RJ	GPvi	22°30'	43°01'	19	104	0.552	0.770	0.291	0.680	8	0.643	0.00095	4
Total *V. incurvata*				186	192	0.452	0.695	0.317		73	0.731	0.00107	13
All populations				465	234					212			25

‡vc – *Vriesea carinata* and vi – *Vriesea incurvata*.

†All inbreeding coefficient (F_{IS}) departed significantly from Hardy-Weinberg equilibrium (HWE) at the $P < 0.001$ level.

*A population is considered to have undergone a bottleneck if its *M*-value falls below a threshold of 0.680, following the procedure described by Garza & Williamson (2001).

**Populations in which bottlenecks were detected according to Garza & Williamson (2001).

794 **Table 2** Analyses of Molecular Variance (AMOVA) based on 14 nuclear microsatellites
795 and cpDNA (*matK* + *trnL-trnF*) sequences.

Source variation	d.f.	Sum. of squares	Variance composition	Variation percentage	Fixation indices*
nuSSR (*V. carinata*)					
Among populations	14	116.07	0.164	6.7	F_{ST}: 0.067
Within populations	543	1238.26	2.280	93.3	
nuSSR (*V. incurvata*)					
Among populations	8	75.39	0.179	7.6	F_{ST}: 0.076
Within populations	363	796.55	2.194	92.4	
cpDNA (*V. carinata*)					
Among populations	14	57.32	0.369	33.8	F_{ST}: 0.338
Within populations	124	89.59	0.722	66.2	
cpDNA (*V. incurvata*)					
Among populations	10	72.29	1.069	70.3	F_{ST}: 0.698
Within populations	61	28.11	0.461	29.7	

796 *All fixation indices showed $P < 0.001$.

Table 3 Haplotype distribution along sampled populations of *Vriesea carinata* and *V. incurvata*. See Table 1 for population identification.

Pop	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16	H17	H18	H19	H20	H21	H22	H23	H24	H25
MQvc	10																								
CAvc		4	1																						
COvc	4	1	6	1																					
JVvc	3	2	2																						
GAvc	5	3	1																						
MTvc	1	4	8																						
MOvc	6	1	5		1	4	1	1																	
IMvc	1								1																
PAvc	3	1	5		1																				
ICvc	4	3	2							1															
IPvc	4	2	2																						
BEvc	6	2	5																						
TEvc											3	1													
SMvc													9												
STvc														5	3										
MQvi																8	1	1							
FLvi																2	1								
ACvi																1	1								
COvi																6			1						
JVvi																1									
MTvi			2													5	1								
MOvi		1														7	5			1					
ICvi																1	2								
SPvi																1	5				1				
CMvi																						9			1
GPvi												1										5	1	1	
Total	47	24	39	1	2	4	1	1	1	1	3	2	9	5	3	32	16	1	1	1	1	14	1	1	1

799 **Table 4** Summary of demographic expansion tests performed in north and southern
800 *Vriesea carinata* and *V. incurvata* populations' data set. VcS - *V. carinta* southern; VcN -
801 *V. carinata* north; ViS - *V. incurvata* southern and ViN - *V. incurvata* north distributions.

	Tajima's D	Fu and Li's (1993) F'	Fu and Li's (1993) D'	Fu's (1997) Fs
VcS	-0.526	-2.271	-2.673*	-1.210
VcN	0.281	0.115	0.027	1.738
ViS	-1.139	-1.974	-1.936	-3.497*
ViN	-0.703	0.778	1.199	-0.517

802 * Values were significant ($P < 0.05$).

803

LEGENDS OF FIGURES

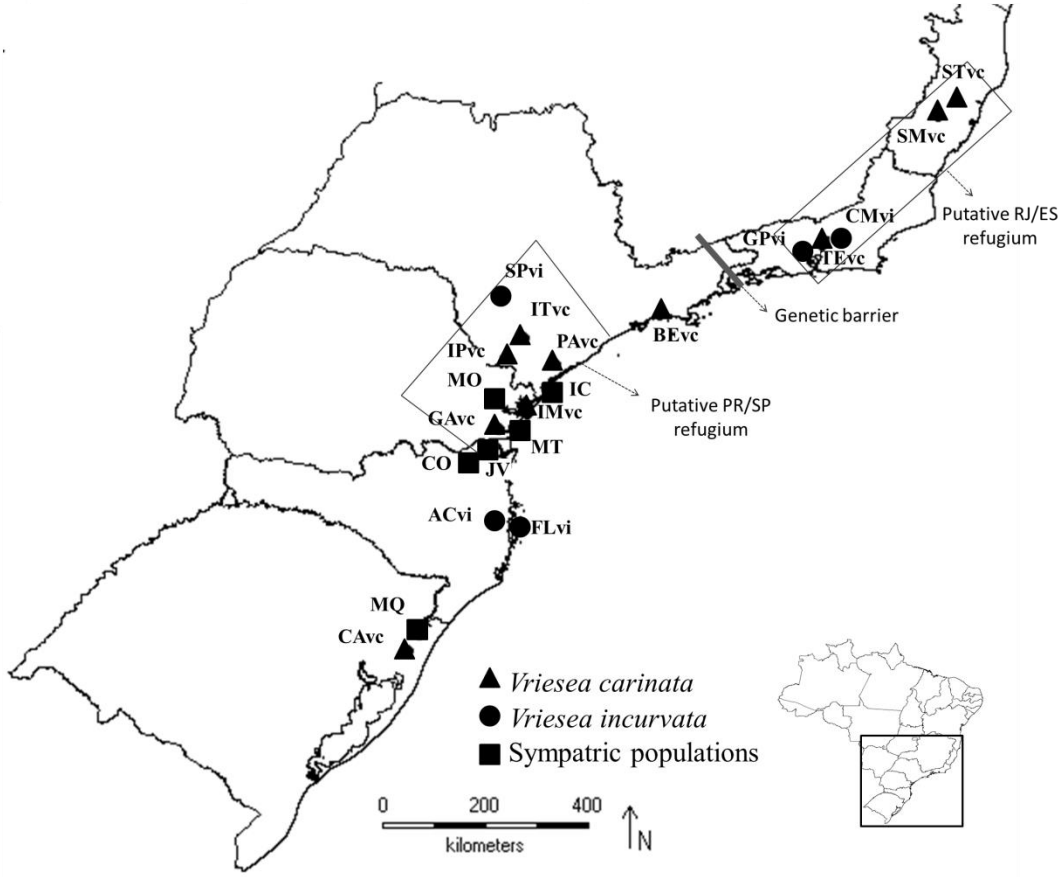
Figure 1 Map showing the geographical distribution of the 21 sampled localities for the phylogeographic study of *Vriesea carinata* and *V. incurvata*. Genetic barrier identified by Barrier software is marked on the map. Putative refugia are defined on the map. RS – Rio Grande do Sul; SC – Santa Catarina; PR – Paraná; SP – São Paulo; RJ – Rio de Janeiro; ES – Espírito Santo. See Table 1 for population identification.

Figure 2 Summary of the population structure in *Vriesea carinata* and *V. incurvata*, in Brazilian Atlantic Forest using Bayesian assignment analysis with nuSSR data set. (A) Structure results at K = 2 for *V. carinata*. (B) Bayesian admixture proportions K = 2 in *V. incurvata*. For population abbreviations, see Table 1.

Figure 3 Median-joining network (A) separating for species, *Vriesea carinata* in dark grey and *V. incurvata* in light grey. Median-joining network (B) identifying the haplotypes and distribution of cpDNA haplotypes for (C) *V. carinata*, and (D) *V. incurvata.* Circle sizes are proportional of sample size, and colors represent the different haplotypes, as show in the key. For population abbreviations, see Table 1.

Figure 4 Bar plot of the individual by populations and clusters of SAMOVA results for cpDNA markers. For population abbreviations, see Table 1.

825 Figure 1



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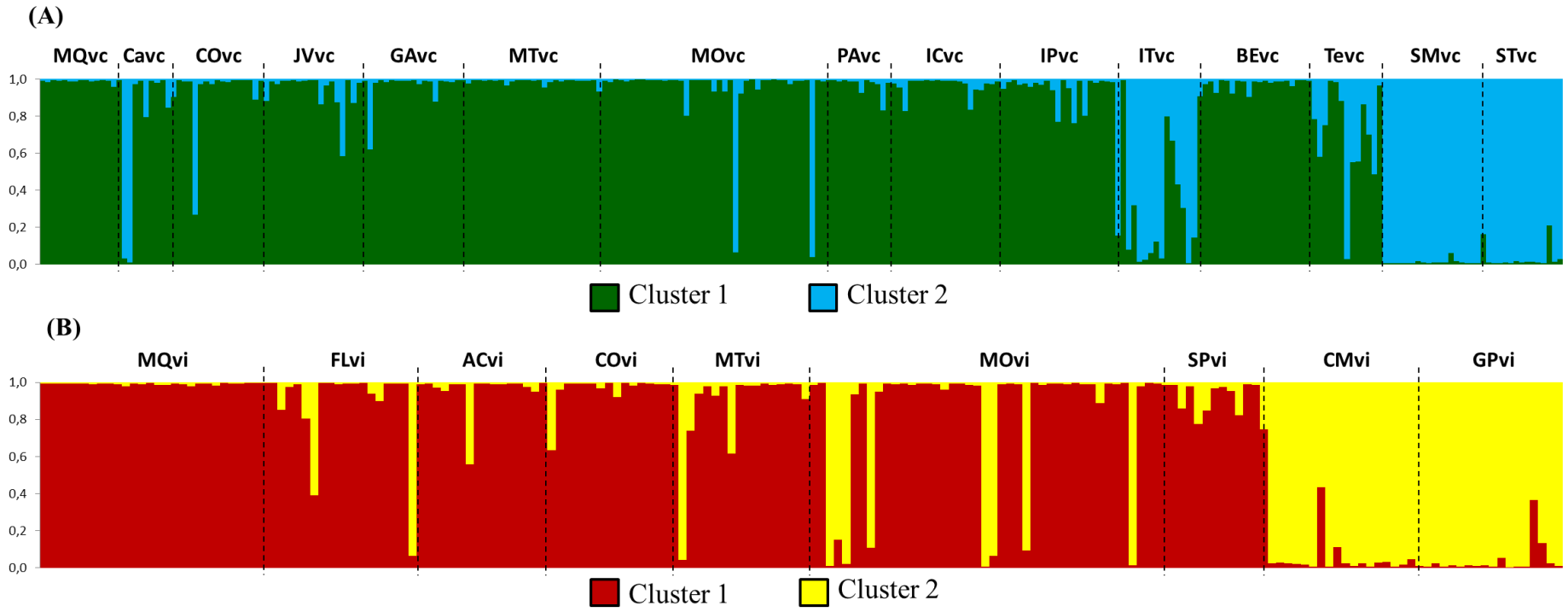
827 Figure 2



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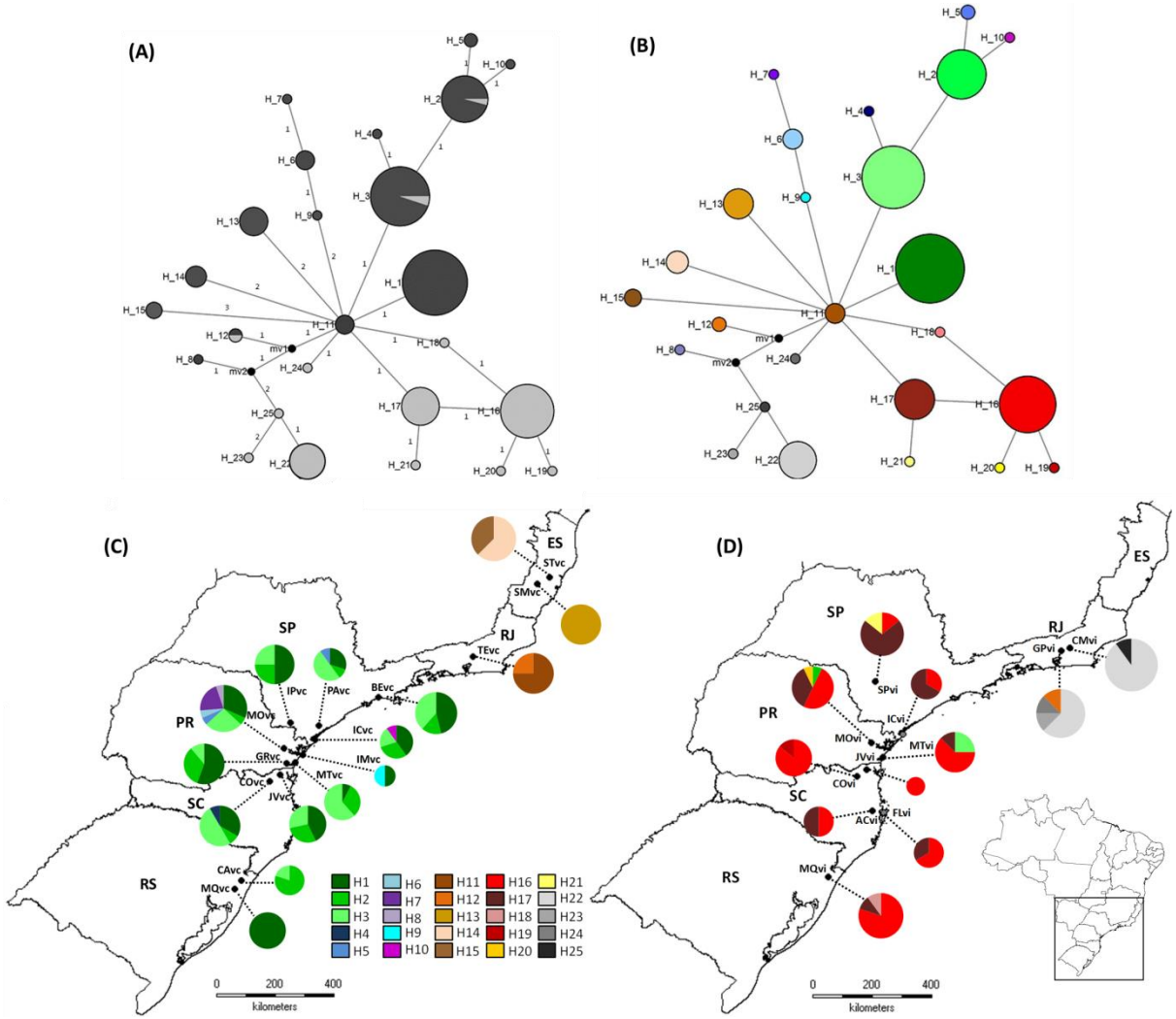
835 Figure 3



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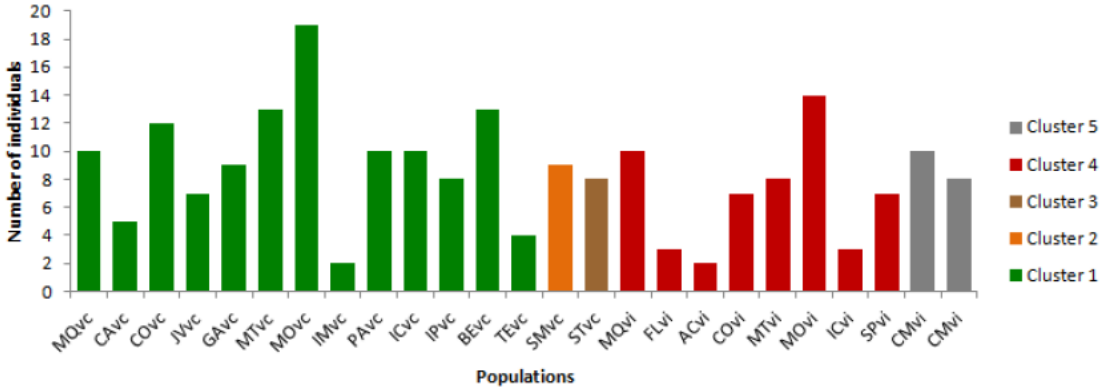
837 Figure 4



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SUPPORTING INFORMATION

Supplementary material 1 F_{ST} values for pairwise comparison between populations of *Vriesea carinata* in Brazilian Atlantic Forest based on nuclear microsatellites (below diagonal) and cpDNA sequence (above diagonal). Dashes indicate populations that were not analyzed with nuSSR and cpDNA markers. See Table 1 for population identification.

	MQvc	CAvc	COvc	JVvc	GAvc	MTvc	MOvc	IMvc	PAvc	ICvc	IPvc	ITvc	BEvc	TEvc	SMvc	STvc
MQvc	*	**0.955**	**0.539**	**0.503**	**0.359**	**0.800**	**0.247**	0.688	**0.548**	**0.471**	**0.414**	-	**0.408**	**0.812**	**1.000**	**0.639**
CAvc	**0.099**	*	**0.358**	0.279	0.349	0.192	**0.281**	0.692	0.298	0.172	0.349	-	**0.381**	**0.711**	**0.964**	**0.579**
COvc	**0.088**	**0.055**	*	-0.071	0.019	0.094	0.068	0.281	-0.078	-0.009	-0.042	-	-0.049	**0.283**	**0.768**	**0.462**
JVvc	**0.051**	0.036	**0.049**	*	-0.118	0.111	0.019	0.153	-0.117	-0.119	-0.145	-	-0.107	**0.237**	**0.783**	**0.407**
GAvc	**0.059**	**0.066**	**0.040**	**0.032**	*	0.243	0.043	0.134	-0.034	-0.074	-0.124	-	-0.064	**0.250**	**0.758**	**0.427**
MTvc	**0.054**	**0.049**	**0.038**	**0.028**	**0.019**	*	**0.207**	**0.590**	0.069	0.090	0.192	-	0.183	**0.537**	**0.863**	**0.577**
MOvc	**0.055**	**0.055**	**0.044**	**0.034**	**0.023**	**0.018**	*	-0.177	0.064	0.068	0.021	-	0.048	0.076	**0.544**	**0.343**
IMvc	-	-	-	-	-	-	-	*	0.277	0.195	0.132	-	0.207	0.172	**0.888**	0.289
PAvc	**0.094**	**0.071**	**0.059**	**0.059**	**0.039**	**0.039**	**0.044**	-	*	-0.066	-0.083	-	-0.069	**0.319**	**0.785**	**0.464**
ICvc	**0.067**	**0.066**	**0.027**	**0.048**	**0.034**	**0.035**	**0.034**	-	**0.062**	*	-0.089	-	-0.039	**0.251**	**0.731**	**0.429**
IPvc	**0.082**	**0.044**	**0.017**	**0.039**	**0.038**	**0.043**	**0.040**	-	**0.071**	**0.017**	*	-	-0.102	0.233	**0.771**	**0.414**
ITvc	**0.082**	**0.051**	**0.024**	**0.035**	0.012	**0.024**	**0.027**	-	**0.046**	**0.048**	**0.032**	*	-	-	-	-
BEvc	**0.089**	**0.070**	**0.048**	**0.039**	**0.069**	**0.069**	**0.056**	-	**0.065**	**0.084**	**0.069**	**0.047**	*	**0.255**	**0.750**	**0.455**
TEvc	**0.268**	**0.273**	**0.223**	**0.256**	**0.264**	**0.263**	**0.253**	-	**0.291**	**0.278**	**0.247**	**0.225**	**0.227**	*	**0.884**	**0.297**
SMvc	**0.203**	**0.141**	**0.137**	**0.151**	**0.153**	**0.152**	**0.157**	-	**0.158**	**0.154**	**0.149**	**0.136**	**0.119**	**0.229**	*	**0.709**
STvc	**0.121**	**0.073**	**0.081**	**0.066**	**0.077**	**0.086**	**0.068**	-	**0.060**	**0.083**	**0.086**	**0.074**	**0.056**	**0.259**	**0.101**	*

Values given in bold are significant at $P < 0.05$.

844 **Supplementary material 2** F_{ST} values for pairwise comparison between populations of
845 *Vriesea incurvata* in Brazilian Atlantic Forest based on nuclear microsatellites (below
846 diagonal) and cpDNA sequence (above diagonal). Dashes indicate populations that were
847 not analyzed with nuSSR and cpDNA markers. See Table 1 for population identification.

	MQvi	FLvi	ACvi	COvi	MTvi	MOvi	ICvi	SPvi	CMvi	GPvi
				-						
MQvi	*	-0.079	0.065	0.007	0.074	0.079	0.343	**0.538**	**0.955**	**0.763**
FLvi	**0.069**	*	-0.615	0.097	-0.144	-0.185	-0.200	0.204	**0.956**	**0.640**
ACvi	**0.066**	0.017	*	0.273	-0.262	-0.344	-0.615	-0.096	**0.955**	**0.587**
COvi	0.005	0.023	**0.028**	*	0.159	0.159	0.495	**0.625**	**0.967**	**0.756**
MTvi	**0.084**	**0.059**	0.008	**0.034**	*	-0.046	-0.062	0.193	**0.888**	**0.648**
MOvi	**0.042**	0.033	**0.033**	0.003	**0.040**	*	-0.158	0.091	**0.879**	**0.696**
ICvi	**-**	-	**-**	-	-	-	*	-0.167	**0.954**	**0.619**
SPvi	**0.103**	**0.104**	**0.069**	**0.039**	0.019	**0.057**	-	*	**0.943**	**0.691**
CMvi	**0.122**	**0.140**	**0.096**	**0.073**	**0.059**	**0.108**	-	0.028	*	0.137
GPvi	**0.161**	**0.185**	**0.139**	**0.109**	**0.090**	**0.146**	-	**0.062**	0.002	*

848 Values given in bold are significant at $P < 0.05$.

Capítulo IV

Natural hybridization between two sympatric species of bromeliads from Brazilian Atlantic Forest: evolutionary implications for species cohesion

Artigo a ser submetido para o periódico *Heredity*

Original article

Natural hybridization between two sympatric species of bromeliads from Brazilian Atlantic Forest: evolutionary implications for species cohesion.

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Short running head: Hybridization between two BAF bromeliads species

Number of words: 5,151

ABSTRACT

The knowledge of hybridization and asymmetrical patterns of interspecific gene flow are important for understanding the process of speciation, the movement of genes across species boundaries and the maintaining of species cohesion. The degree of reproductive isolation among related species is an important factor influencing species genetic integrity and hybrids formation. Multiple pre- and postzygotic components are responsible for reproductive isolation among plant species pairs. Here, we investigated the potential of

natural hybridization in four sympatric populations of *Vriesea carinata* and *Vriesea incurvata*, endemic species from Brazilian Atlantic Forest (BAF), which share life habits and pollinator, and present short time of flowering overlap. A total of 279 individuals of four sympatric populations were sampled and genotyped with 14 nuclear microsatellites and two cpDNA regions (*matK* gene and *trnL-trnF* intergenic spacer) were sequenced. All four sympatric populations analyzed presented hybrids (a total of 19) between *V. carinata* and *V. incurvata*. Bayesian assignment analysis detected the presence of F2 and backcrosses towards *V. incurvata*. cpDNA network identified bidirectional introgression between these two species. The rate of interspecific gene flow can be considered low in sympatric populations ($N_em < 0.5$) but was responsible for the forming of 10% of hybrids. The temporal difference in the flowering period of the two species has acted as a strong prezygotic reproductive barrier, being the main force responsible for species cohesion. The presence of reproductive barriers has allowed these species to persist in sympatry for extended periods of time, ensuring the maintenance of species cohesion.

Keywords: hybridization; introgression; Bromeliaceae; *Vriesea*; gene flow; reproductive barrier.

INTRODUCTION

Natural hybridization and introgression are widespread and well known phenomena, which have played a relevant role in plant evolution (Stebbins, 1959; Rieseberg and Carney, 1998), being responsible for diversification in a remarkable proportion of angiosperms (50–70%; Ellstrand *et al*. 1996; Rieseberg, 1997). The study of hybridization is important to understand the processes of speciation and the movement of genes across species boundaries, and can promote the appearance of new lineages (Seehausen, 2004) or adaptive diversification (Rieseberg *et al*., 2003). The degree of reproductive isolation among related species is an important factor influencing species genetic integrity and the probability of hybrids formation (Grant, 1981). Multiple pre- and postzygotic components can be responsible for reproductive isolation among plant species pairs, as flower color and morphology, nectar composition, flowering phenology and chromosomal divergence (see Widmer *et al*., 2009). Recent studies have tried to identify and understand how these

mechanisms of reproductive isolation, and their potentially complex interactions, have contributed to reduce or enable gene flow among populations and species (Coyne and Orr, 2004; Hersch and Roy, 2007; Pascarella, 2007; Kameyama and Kudo, 2009; Pinheiro *et al*., 2010; Palma-Silva *et al*., 2011).

The Bromeliaceae family in one of the morphologically and ecologically most diverse flowering plant families native to the tropics and subtropics of the New World (Givnish *et al*., 2011; Zanella *et al*., 2012). Bromeliads are well known for its recent adaptive radiation and have evolved to fill numerous niches, with an incredible diversity of adaptations, occupying the most diverse types of environments (Benzing, 2000). In this direction, the species have its generic limits frequently undergoing changes (Faria *et al*., 2004; de Sousa *et al*., 2007), which could suggest recent speciation processes with incipient species not completely defined (Wendt *et al*., 2008). In Bromeliaceae, the occurrence of two or more congeneric species in sympatry with sequential and overlapping blooming periods is common (Wendt *et al*. 2001, 2002; Araujo *et al*., 2004; Machado and Semir, 2006; Costa and Wendt, 2007), fact that may increase pollinator abundance. The synchrony of flowering in bromeliads allows great diversification of food supply, mainly for maintenance of pollen vectors (Machado and Semir, 2006). However, the pollinator service in mixed populations of sympatric species enables increase interspecific pollen transfer. In plants, the formation of F1 hybrids first requires that pollen from one species be transferred to the stigma of another species (Hersch and Roy, 2007). Such transfer is often mediated by a pollinator, being therefore the initial stages of hybridization influenced by pollinator movement patterns (Campbell *et al*., 1997; Wesselingh and Arnold, 2000), which can have several effects on hybridization dynamics. The degree of reproductive isolation through pre- and postzygotic mechanisms is most critical for sympatric species. In species that usually grow in sympatry, having overlapping flowering periods and non-specific pollination (Jersáková *et al*., 2006), are exposed to ample opportunities for interspecific hybridization (Lexer *et al*., 2005). Natural hybridization and weak reproductive isolation in congeneric species have been reported for some bromeliads (Luther, 1984; Gardner, 1984; Wendt *et al*., 2001, 2002, 2008; de Sousa *et al*., 2003; Palma-Silva *et al*., 2011), and artificial hybrids are easily obtained through hand pollination (Vervaeke *et al*., 2004). However, the existence of effective isolation barriers between sympatric species of Bromeliaceae should be expected, since there are few reports

of natural hybridization (Wendt *et al*., 2008), despite the low number of species studied to date (Zanella *et al*., 2012).

Sympatric populations, in which occur hybridization events, can be composed by wide variety of genotypes resultant from many generations of recombination (Lexer *et al*., 2005) and can be seen as 'natural laboratories' for studying barriers to gene flow. Also, the estimate of hybrids frequency and their geographic distribution should help directly in conservation planning (Burgess *et al*., 2005; Kothera *et al*., 2007).

The combination of different types of molecular markers provides information on different spatial and temporal scales of the hybridization–introgression dynamics. Nuclear markers have been useful to infer contemporary rates of interspecific gene exchange (Lexer *et al*., 2005; Burgarella *et al*., 2009; Pinheiro *et al*., 2010), whereas plastidial and mitochondrial DNA have been used to describe past episodes of introgression (Palmé *et al*., 2004; Heuertz *et al*., 2006; Palma-Silva *et al*., 2011).

Vriesea carinata and *V. incurvata* are typical species from Brazilian Atlantic Forest (BAF), being preferentially epiphytic. They can be found in sympatry, show similar floral morphology and share pollinator, presenting sequential flowering and short time of blooming overlap (Smith and Downs, 1977; Machado and Semir, 2006). In the present study we used admixture analysis of multilocus microsatellites genotypes and cpDNA median-joining approach from a range-wide sample of sympatric and allopatric populations of two bromeliads species, *V. carinata* and *V. incurvata*, to explore the extent and pattern of nuclear and plastidial interspecific gene flow. Our specific questions are (1) Do *V. carinata* and *V. incurvata* hybridize in wild, as suggested by field and herbarium observations? (2) If hybridization occurs, what is the genomic composition of hybrids when accessed using nuclear microsatellites markers and plastidial DNA sequence, and what do these estimates tell us about the extent and direction of introgression? (3) Does the hybridization pattern is similar among sympatric populations? (4) Which type of pre- and/or postzygotic barriers is expected? In addition, inferences are made about the potential roles of gene flow in maintaining species cohesion.

MATERIALS AND METHODS

Plant species and population sampling

Vriesea carinata Wawra and *Vriesea incurvata* Gaudichaud are epiphytic species that occur in mesophilic environments and well preserved habitat with high humidity. *Vriesea carinata* occurs from 19° to 29°S and shows a single inflorescence, with 4-12 flowers, floral bracts with apex and margins yellow or green and the rest usually bright red, being the sepals and petals yellow (Smith and Downs, 1977). The flowers usually open 7:30am and close 5:00pm, living only one day (Machado and Semir, 2006). The species blooms during the winter (from April to October), with flowering peak between June and August (Araujo *et al*., 2004; Machado and Semir, 2006). *Vriesea incurvata* presents a more restrict geographical distribution (from 22° to 29°S), showing single inflorescence with 10-35 flowers, floral bracts strongly imbricate and red with broad yellow membranaceous margins, being the sepals and petals yellow (Smith and Downs, 1977). The flowers also live only one day, opening 6:30am and closing 7:00pm (Machado and Semir, 2006). Unlike *V. carinata*, this species blooms during the summer (from October to May), with flowering peak between January and March (Machado and Semir, 2006). These two species share pollinator and display ornithophilous syndrome (Krömer *et al*., 2008), being pollinated by hummingbirds (*Phaethornis eurynome* and *Melanotrochilus fuscus*; Machado and Semir, 2006).

Four sympatric populations were sampled. We collected a total of 279 individuals of *V. carinata*, *V. incurvata* and putative hybrids (Table 1, Figure 1; Maquiné, Corupá, Matinhos and Morretes populations). In addition, one allopatric population of *V. carinata* (Cananéia) and one of *V. incurvata* (Florianópolis) were sampled, being analyzed as reference populations (Figure 1). Sample sizes, names and geographical co-ordinates of each population are given in Table S1 (Supporting Information). Fresh leaves of each individual sample were collected and stored in silica gel for drying. Total genomic DNA was isolated following CTAB method (Doyle and Doyle, 1990).

Nuclear microsatellite markers and genotyping assays

A total of 14 nuclear microsatellite markers (nuSSR) were used to study the patterns of genomic diversity and admixture in the sympatric and allopatric populations, seven isolated from *Vriesea gigantea* (*loci*: VgA04, VgA06, VgB10, VgB12, VgC01, VgG02,

VgG03; Palma-Silva *et al*., 2007), three from *Alcantarea imperialis* (*loci*: Ai5.18, Ai4.10, Ai4.03; Palma-Silva *et al*., 2007), three from *Tillandsia fasciculata* (*loci*: e6, p2p19, e6b; Boneh *et al*., 2003) and one from *Pitcairnia albiflos* (*locus*: PaA10; Paggi *et al*., 2008). For each SSR, the forward primers were synthesized with a 19-bp M13 tail (5′-CACGACGTTGTAAAACGAC-3′) at the 5′ end to allow labeling with a tailed fluorescent dye M13 primer during genotyping procedures, following the method of Schuelke (2000). All polymerase chain reaction (PCR) amplifications were performed in a Veriti 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA) following the protocol described by Palma-Silva *et al*. (2007). The microsatellite alleles were resolved on an *ABI 3100* DNA Analyzer Sequencer (Applied Biosystems, Foster City, CA, USA) and sized against the *GS500 LIZ* molecular size standard (Applied Biosystems, Foster City, CA, USA) using GENEMARKER Demo version 1.97 (*SoftGenetics*, State College, PA, USA).

Plastidial non-coding region: amplification and sequencing

Two cpDNA regions, *trnL-trnF* spacer (Taberlet *et al*., 1991) and *matK* gene (Schulte *et al*., 2005) were analyzed by amplification and sequencing and were selected for a large-scale survey of haplotype variation in sympatric and allopatric *V. carinata* and *V. incurvata* populations. PCR reactions for *trnL-trnF* were run using the following parameters: denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 54 °C for 1min, and 72 °C for 1 min, and a final extension for 10 min at 72 °C, using primers *trnL5*'^{UAA}F (TabC) and *trnF*^{GAA} (TabF) for PCR and sequencing as described by Taberlet *et al*. (1991). *matK* gene amplification and sequencing were carried out as described in Schulte *et al*. (2005). All PCR was carried out in a total volume of 20 µl containing 10 ng DNA template, 1x *GoTaq* buffer, 2mM $MgCl_2$, 250µM dNTP mix, 5ρmol forward and reverse primers and 1U of *GoTaq* DNApolymerase (Promega, Madison, WI, USA). PCR amplifications were performed in a Veriti 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA). Plastid PCR products were sequenced from both ends using BigDye Kit (Applied Biosystems) at the Macrogen Inc. (South Korea). Sequences were analyzed and edited to obtain the consensus using the software MUSCLE (Edgar, 2004) implemented in MEGA5 version 5.0 (Tamura *et al*., 2011), and were visually checked for original electropherograms in CHROMAS 2.33 sequence viewer (Chromas Technelysim,

Helensvale, Australia). *V. carinata* and *V. incurvata* sequences generated in this study were deposited in GenBank under the accessions numbers.

Data analysis

Nuclear microsatellite markers

Nuclear admixture analysis for hybrids identification: To identify hybrid individuals and estimate population level hybridization, we carried out admixture analyses using two different Bayesian clustering approaches, implemented in the programs STRUCTURE version 2.3.3 (Pritchard *et al*., 2000) and NEWHYBRIDS version 1.1 beta (Anderson and Thompson, 2002). Thus, individuals were classified as *V. carinata*, *V. incurvata* and hybrids using nuSSR markers. Allopatric populations of each species were used as reference samples of pure individuals of *V. carinata* and *V. incurvata*. STRUCTURE version 2.3.3 (Pritchard *et al*., 2000) were carried out under the admixture model assuming independent allele frequencies and using a burn-in period of 100 000, run length of 500 000 and 10 replicates per K ranging from 1 to 10 with all populations in data set (sympatric and allopatric). We determined the most probable number of populations, K, by using the method described by Evanno *et al*. (2005) that examines ΔK, an *ad hoc* quantity related to the change in posterior probability between runs of different K. In our data set, the greatest amount of variation was explained by separating two genetic groups (K = 2), separating the species (see Results). In the following analysis we used K = 2 model, because we assume that the two species contribute to the gene pool of the sample. We performed analyses for each sympatric population separately, in each case including the specimens from the allopatric populations as reference samples for each species, for hybrids identification and classification. In the model implemented in STRUCTURE software, following the parameters set described above, the posterior probability (q) is the proportion of a given genotype originating from each of cluster categories (K). STRUCTURE was used to classify individuals among the two parental species and hybrids, using a threshold of $q \geq 0.90$ to classify pure individuals of *V. carinata*, $q \leq 0.10$ to classify pure individuals of *V. incurvata* and $0.10 < q < 0.90$ to classify hybrids (Vähä and Primmer, 2006). In addition, to assess the rate and direction of recent gene flow among populations at each site, we used

the methods implemented in the NEWHYBRIDS version1.1 software (Anderson and Thompson, 2002). Under this model, *q* describes posterior probabilities for each individual, which can be classified as: (i) pure *V. carinata*, (ii) pure *V. incurvata*, (iii) F1 hybrid, (iv) F2 hybrid, (v) backcross towards *V. carinata*, (vi) backcross towards *V. incurvata*, using a threshold value of $q \geq 0.50$; individuals with $q < 0.50$ remained unassigned (Vähä and Primmer, 2006). We performed the software following parameters describe by Field *et al*. (2011). All other analyses with hybrids were performed with individuals identified by STRUCTURE approach.

Nuclear genetic diversity: The nuclear microsatellite loci were characterized in the *V. carinata*, *V. incurvata* and their hybrids based on the number of alleles, allelic richness, observed and expected heterozygosity and inbreeding coefficient (F_{IS} - Weir and Cockerham, 1984), calculated for each locus using the programs FSTAT (Goudet, 1995) and MSA (Dieringer and Schlötterer, 2003). The software GENEPOP on the web (Raymond and Rousset, 1995) was used to test departures from Hardy–Weinberg equilibrium (HWE) for each locus within each species and hybrids. Allopatric and sympatric populations for each species and hybrids were characterized by number of alleles, allelic richness, variance in allele size, observed and expected heterozygosity and inbreeding coefficient (F_{IS} - Weir and Cockerham, 1984) calculated by FSTAT (Goudet, 1995) and MSA (Dieringer and Schlötterer, 2003). Departures from HWE for each population were tested using exact tests in software GENEPOP on the web (Raymond and Rousset, 1995). 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) calculated in the software FSTAT version 2.9.3.2 (Goudet, 1995), considering *V. carinata*, *V. incurvata* and hybrids. Partitioning of genetic diversity within and among *V. carinata*, *V. incurvata* and hybrids groups were examined by analysis of molecular variance (AMOVA) implemented in the software ARLEQUIN 3.1 (Excoffier *et al*., 2005).

Pairwise migration rates (N_em) were estimated for sympatric populations of *V. carinata*, *V. incurvata* and hybrids following a coalescent theory and maximum-likelihood based approach using MIGRATE 3.0.3 (Beerli and Felsenstein, 1999). The computations were carried out under both the infinite allele model (IAM; Kimura and Crow, 1964) and the stepwise mutation model (SMM; Kimura and Ohta, 1978).

Plastidial DNA sequences

Sequences were aligned and polymorphisms at mononucleotide microsatellites were excluded due to ambiguous alignment and higher mutation rates. Long indels (usually with more than 5 bp) were coded as one evolutionary event (one character), and each base pair was equally weighted before analysis. For statistical analyses, the sequences of the two chloroplast regions were concatenated. Haplotype (*h*) and nucleotide (π) diversity (Nei, 1987) were estimated for each population sympatric, allopatric and hybrids using the software ARLEQUIN 3.1 (Excoffier *et al*., 2005). Genealogical relationships among haplotypes for chloroplast data set were inferred using median-joining method (Bandelt *et al*., 1999), implemented in the software NETWORK 4.6.1.1 (http://www.fluxus-engineering.com) for total cpDNA data set sequences (n = 123).

Estimates of differentiation (G_{ST} and F_{ST} statistics) were calculated in the software DNASP v5.10 (Librado and Rozas, 2009), taking into account the pairwise distance between cpDNA haplotypes. To test the hypothesis of population differentiation, the genetic structure was further examined by an AMOVA, using all populations, in which populations were grouped to each pure species and hybrids, using the software ARLEQUIN 3.1 (Excoffier *et al*., 2005).

RESULTS

Genetic composition of sympatric populations

Genomic admixture analysis with STRUCTURE identified K = 2 as the most likely number of genetic clusters for all data set (considering both species, data not shown). Also, Bayesian STRUCTURE results for each sympatric population indicated that occurs hybridization between *V. carinata* and *V. incurvata*, being observed a total of 19 hybrids (9.1% of the total individuals sampled; $0.10 < q < 0.90$). The allopatric populations, used as reference of each parental species, were composed of purebreds, considering STRUCTURE threshold of $q \geq 0.90$ to classify pure individuals of *V. carinata* and $q \leq 0.10$ to classify pure individuals of *V. incurvata* (Figure 2). Nevertheless, considering

NEWHYBRIDS results, more hybrids were identified, totalizing 43 samples. This difference is due to some *V. incurvata* individuals, which were identified as purebreds by STRUCTURE analysis and as hybrids by NEWHYBRIDS (79.1% of hybrids; Figure 2), being most of these hybrids classified as F2.

Analyzing populations separately, sympatric Maquiné population showed only one hybrid considering STRUCTURE and NEWHYBRIDS results, with high isolation between species. Corupá sympatric population showed only two hybrids using STRUCTURE analysis, however NEWHYBRIDS classified 15 individuals as hybrids and only one *V. incurvata* purebred. On the other hand, Matinhos and Morretes sympatric populations showed a higher number of hybrids. STRUCTURE Bayesian results identified nine hybrids in Matinhos and seven in Morretes population ($0.10 < q < 0.90$), and NEWHYBRIDS analysis presented 14 hybrids in Matinhos and 13 in Morretes population ($q \geq 0.50$; Figure 2). Considering all the 43 hybrids identified by NEWHYBRIDS analysis, 34 were classified as F2 and six as backcross with *V. incurvata*; three individuals were not classified into any of the classes with threshold $q < 0.50$. The hybridization patterns recovered indicated unidirectional introgression towards *V. incurvata*, and variation in hybrid genomic composition among sympatric populations. F2 hybrids were detected in all populations and no F1 and backcrosses towards *V. carinata* were detected in the populations studied (Figure 2). Most individuals identified as pure *V. incurvata* in the field showed intermediated genetic composition, being classified as hybrids.

Nuclear microsatellite diversity

Of a total of 14 nuclear SSR all were polymorphic, with number of alleles ranging from 4 to 24 per locus. *V. carinata* showed a total of 177 alleles (ranging from 4 to 24 per locus), and *V. incurvata* presented 144 alleles (ranging from 4 to 17). The hybrids presented a total of 97 alleles, ranging from 4 to 12 (Table 2). The total observed and expected heterozygosities per population were 0.535 and 0.720 for *V. carinata*, 0.460 and 0.665 in *V. incurvata* and 0.462 and 0.752 for hybrids, respectively (Table 2). The inbreeding coefficient (F_{IS}) was high and departed from HWE significantly in almost all loci ($P < 0.01$; Table 2). Most loci displayed high numbers of unique alleles, with 55 private alleles (out of 177 alleles) in *V. carinata* and 21 private alleles (out of 144 alleles)

in *V. incurvata* (data not shown). Hybrids showed, in average, similar genetic diversity index of purebreds' species (Table 2).

Genetic diversity was similar in *V. carinata* and *V. incurvata* across all populations and parameters (Table 1). Most of the populations displayed significant departures from HWE because of heterozygote deficits. *Vriesea carinata* and *V. incurvata* showed low levels of nuclear genetic differentiation among species, F_{ST} = 0.088 and G_{ST} = 0.072. AMOVA results showed that 9.4% of variation were among species, with F_{CT} = 0.094 (Table 3). Maximum-likelihood-based estimates of migration rates (N_em) for sympatric populations of *V. carinata* and *V. incurvata* (Maquiné, Corupá, Matinhos and Morretes) were extremely low (Figure 3), indicating restricted gene flow between species. Interspecific gene flow were low (N_em < 0.5), but sufficient to generate approximately 10% of hybrids in sympatric populations studied.

Plastidial DNA diversity and haplotype network

A total of 2708 bp from two plastidial DNA regions (*matK* gene - 1835 bp, and *trnL-trnF* intergenic spacer - 873 bp) were sequenced, being 68 individuals of *V. carinata*, 44 of *V. incurvata* and 11 of hybrids. The molecular diversity indexes are shown in Table 1. Fourteen haplotypes were identified in all samples. Haplotype diversity (*h*) in populations ranged from 0.00 to 1.00 and the nucleotide diversity (π) from 0.000 to 0.00185 (Table 1). One population was monomorphic (Maquiné – *V. carinata*) and the highest haplotype number was observed in Morretes (*V. carinata*, seven haplotypes), whereas the remaining populations showed two to four (Table 1). Two major groups could be recognized in the haplotype network (Figure 4), one with nine haplotypes typical of *V. carinata*, and the other one composed of four haplotypes of *V. incurvata*. The haplotype H14 was unique and identified in one hybrid. When cpDNA was considered, hybridization occurred in both directions, because hybrids populations showed haplotypes from both groups/species. The haplotypic sharing and distribution along the sympatric and allopatric populations studied can be seen in Figure 1. Differentiation measures across all populations were moderate, with F_{ST} = 0.456 and G_{ST} = 0.321. AMOVA results showed that 42.6% of variation found were among species, (F_{CT} = 0.427) and 12.3% of variation were among populations (Table 3).

DISCUSSION

Evidence of hybridization between *V. carinata* and *V. incurvata*

Bromeliaceae is a well known example of recent adaptive radiation (Benzing, 2000), and thus we can expect the occurrence of recent speciation processes as well as the occurrence of species not completely separated and reproductively isolated. The occurrence of hybrids between *V. carinata* and *V. incurvata* has been speculated and reported from morphological observations in the field, from herbarium notes and also reported in Flora Neotropical Monograph about Bromeliaceae (Smith and Downs, 1977). Moreover, Matos (2011) found individuals of *V. carinata* and *V. incurvata* with intermediate morphology, proposing the occurrence of hybridization between these two species. The plants from Bromeliaceae family present important ornamental value and have been receiving great attention from plant growers and collectors, being artificial hybrids easily created between species through hand pollination (Wendt *et al*., 2001, 2002; Vervaeke *et al*., 2004). In spite of this, cases of natural hybridization for bromeliads species are still poorly documented (Luther, 1984; Gardner, 1984; Wendt *et al*., 2001; 2008; de Sousa *et al*., 2003; Palma-Silva *et al*., 2011). Here, a total of 19 natural hybrids were identified in four sympatric populations through molecular approaches (Table 1 and Figure 2). *Vriesea carinata* and *V. incurvata* show similar floral morphology (Smith and Downs, 1977) and short time of flowering overlap (Araujo *et al*., 2004). Also, they share pollinator (Machado and Semir, 2006) and show the same chromosome number (2n = 50; Palma-Silva *et al*., 2004), which makes possible the occurrence of hybridization between these two species. The pattern of two or more congeneric species with blooming overlap periods and occurring in sympatry is common in Bromeliaceae family (Wendt *et al*., 2001, 2002; Kaehler *et al*., 2005; Machado and Semir, 2006; Costa and Wendt, 2007) and probably this sympatric occurrence increases pollinator abundance and interspecific pollen transfer. The present study has shown that *V. carinata* and *V. incurvata*, even with a short flowering overlap, are able to generate hybrids, despite expected effective reproductive barriers in Bromeliaceae (Wendt *et al*., 2008).

Extent of hybridization and introgression in the sympatric populations

Nuclear microsatellite loci revealed that the admixture proportions and genetic architecture are different in the studied sympatric populations of *V. carinata* and *V. incurvata*. Maquiné showed clearly separated molecular profiles and species cohesion, with only one hybrid. Corupá populations showed a different pattern, because only two hybrids were identified with STRUCTURE and 14 with NEWHYBRIDS approach (seven F2 hybrids, six backcrosses towards *V. incurvata* and two unclassified), where we observed only one pure *V. incurvata* individual (Figure 2). In this case *V. incurvata* would be suffering introgression, however we cannot exclude the possibility of hybridization with a third species in this population. Matinhos and Morretes populations showed only F2 hybrids (n = 13 in NEWHYBRIDS analysis for each population). Interestingly, populations with lower latitude showed more hybrids than populations further south, this pattern could be influenced by ecological factors, since these species show sequential flowering with a short period of overlap and pollinator sharing (Machado and Semir, 2006). A latitudinal gradient can influence the seasons, temperature, rainfall and consequently the species' flowering period (Marques and Lemes-Filho, 2008).

Different results were observed between the two methods of hybrids identification and classification. STRUCTURE and NEWHYBRIDS approaches showed a discrepancy in the identification of hybrids in the sympatric populations (Corupá, Matinhos and Morretes, Figure 2). STRUCTURE Bayesian analysis identified 19 hybrids ($0.10 < q < 0.90$), while NEWHYBRIDS results showed that 43 samples were identified as hybrids ($q \geq 0.5$). Vähä and Primmer (2006) discussed the hybrid identification efficiency of STRUCTURE and NEWHYBRIDS methods and they conclude that the two methods are equally efficient in hybrid identification when hybrids are fairly frequent (10%) in the sample (we found 9.1% of hybrids occurrence). Moreover, for NEWHYBRIDS distinguishing between backcrosses, F1, F2, hybrids and purebred parental classes, the use of a relatively large number of loci (more than 48 loci) and efficient classification of individuals to specific hybrid classes are required (Vähä and Primmer, 2006). We used 14 SSR loci, in this sense, probably the hybrids identification by STRUCTURE was more accurate than NEWHYBRIDS, and for this reason, all our analyses were performed considering the hybrids identified by STRUCTURE.

However, NEWHYBRIDS was important to classify the hybrids and for inferences about introgression.

Plastidial DNA network showed moderate haplotypic sharing among species of each sympatric population. There is a clear separation of species and bidirectional sharing of haplotypes (Table 1; Figure 1 and 4). Haplotype sharing may be potentially explained by interspecific gene flow (introgression), retention of ancestral polymorphism (incomplete lineage sorting), homoplasy (evolutionary convergence) or a combination of these factors (Palma-Silva et al., 2011). Homoplasy would not likely in Bromeliaceae, considering the low variation rate in DNA barcoding markers for this family (Maia *et al*., 2012). Tillandsioideae subfamily is monophyletic, with high bootstrap value (Givnish *et al*., 2011; Maia *et al*., 2012). The monophyly was also supported to *Vriesea* genera, however, there is low support for *V. carinata* and *V. incurvata* separation (Maia *et al*., 2012), being not possible to determine the separation time between these two species and to estimate the mode and time of speciation (Barraclough and Nee, 2001). Zanella *et al*. (*In prep*) showed a clear species separation and low haplotypic sharing between *V. carinata* and *V. incurvata*. Thus, haplotypic sharing is probably the result of interspecific gene flow (recent introgressive hybridization).

Gene flow and species cohesion

Pre- and postzygotic barriers are important to reproductive isolation and consequently to speciation and species cohesion. Recent studies concerning the relative importance of different isolating barriers between plant species pairs demonstrated that prezygotic isolation is much stronger than postzygotic isolation (Widmer *et al*., 2009). *Vriesea carinata* and *V. incurvata* showed sequential flowering with short overlapping time. In this case, pre-pollination barrier limits the transfer of pollen from individuals of one species to stigmas of another species, being the main effective isolation barrier the temporal difference of flowering, maintaining species cohesion. Temporal isolation can eliminate the possibility of interspecific pollen transfer (Grant, 1981, 1994) and sympatric taxa may achieve a high degree of isolation by opening their flowers at different times of the day or months of year (Levin, 1971). However, the short overlapping time of flowering enable hybrids formation in *V. carinata* and *V. incurvata*. This fact suggests that pre- and

448 postzygotic reproductive isolation barriers are potentially weak in theses species. Weak
449 cross-compatibility barriers are frequently present in genera with species that have radiated
450 recently (Levin, 2006), such as the bromeliads (Wendt *et al*., 2008).
451 One important question in this scenario of sequential flowering is when hybrids
452 flower, since this can influence the direction that introgression occurs (preference cross
453 towards one of the studied species) or crossing between F1 hybrids. We observed that
454 sympatric populations studied were composed by F2 individuals and few backcrosses
455 towards *V. incurvata* (Figure 2). The possibility of hybrids flowering earlier than their
456 parental species may explain why there are more F2's than backcrosses, and asymmetrical
457 introgression towards *V. incurvata* may be explained by overlap in flowering times with
458 parents of this species. Similar pattern were observed in sympatric population of *Pitcairnia*
459 *albiflos* and *P. staminea*, with predominance of F2 and backcross towards *P. albiflos*
460 (Palma-Silva *et al*., 2011).
461
462 **Conclusion and conservation implications**
463
464 In summary, hybridization was observed between *V. carinata* and *V. incurvata*, with
465 different patterns among sympatric populations studied. The short period of flowering
466 overlap enables the occurrence of hybridization and introgression over long timescale
467 between *V. carinata* and *V. incurvata*, as indicated by both nuclear and plastidial DNA.
468 The period of hybrids flowering may also influence the pattern of introgression, and in this
469 case was towards *V. incurvata*. However, there is a strong reproductive barrier, with low
470 rate of interspecific gene flow, being the temporal flowering prezygotic barrier the
471 principal force responsible for species cohesion. The presence of reproductive barriers has
472 allowed these species to persist in sympatry for extended periods of time, ensuring the
473 maintenance of species cohesion. Interspecific gene flow may contribute to the insertion of
474 new allelic combinations that can confer increased local adaptive value. Yet, it is important
475 highlight that these species are typical from BAF, and this biome currently retains only
476 7.5% of its primary vegetation (Myers et al., 2000), being the third largest hotspot of the
477 world and conservation policies need to be implemented to ensure the preservation of this
478 so threatened ecosystem.
479

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682 **Table 1** Characterization of populations of *Vriesea carinata*, *V. incurvata* and hybrids, with 14 nuclear microsatellite markers and chloroplast
683 DNA sequences (*matk* + *trnL-trnF*), including the number of individuals sampled, number of alleles (*A*), allelic richness (AR), variance in
684 allele size (Var), observed (H_O) and expected (H_E) heterozygosities, inbreeding coefficient (F_{IS}), haplotype diversity (*h*) and nucleotide
685 diversity (π) for each population.

	Nuclear microsatellites						cpDNA		
Species (sample size)	*A*	AR	Var	H_O	H_E	F_{IS}	*h*	π	Haplotypes
Allopatric									
V. carinata (40)	138	4.22	47.23	0.596	0.713	0.165*	0.712	0.00079	H1, H2, H3, H9
V. incurvata (31)	98	3.78	37.20	0.349	0.666	0.485*	0.509	0.00019	H10; H11
Sympatric									
Maquiné – RS									
V. carinata (14)	74	3.32	3.52	0.533	0.443	0.324*	0.00	0.00	H1
Hybrids (1)	-	-	-	-	-	-	-	-	-
V. incurvata (29)	83	3.36	26.81	0.509	0.635	0.201*	0.378	0.00015	H10, H11, H12
Corupá – SC									
V. carinata (19)	96	3.85	41.66	0.534	0.679	0.219*	0.682	0.00066	H1, H2, H3, H4
Hybrids (2)	-	-	-	-	-	-	-	-	-
V. incurvata (14)	88	3.72	29.28	0.585	0.645	0.097	0.333	0.00012	H10, H13
Matinhos – PR									
V. carinata (22)	103	3.96	34.38	0.518	0.696	0.263*	0.485	0.00018	H2, H3
Hybrids (9)	80	4.22	31.75	0.413	0.761	0.473*	0.733	0.00089	H1, H2, H10
V. incurvata (10)	68	3.64	29.86	0.362	0.651	0.458*	0.667	0.00025	H10, H11
Morretes – PR									
V. carinata (46)	137	4.06	33.64	0.529	0.722	0.270*	0.808	0.00012	H1, H2, H3, H5, H6, H7, H8
Hybrids (7)	65	3.88	28.23	0.529	0.729	0.291*	1.000	0.00185	H1, H3, H6, H14
V. incurvata (35)	104	3.51	26.24	0.459	0.610	0.251*	0.603	0.00037	H10, H11
Overall = 279									

686 *Inbreeding coefficient (F_{IS}) departed significantly from Hardy-Weinberg equilibrium (HWE) at the $P < 0.001$ level.

687 **Table 2** Genetic variability at 14 nuclear microsatellite loci in *Vriesea carinata*, *V. incurvata* and hybrids, including locus name, number of
688 alleles (*A*), observed (H_O)expected (H_E) and heterozygosity and inbreeding coefficient (F_{IS}) for each locus.

	Vriesea carinata (n = 141)					Hybrids (n = 19)					*Vriesea incurvata* (n = 119)				
Locus	*A*	AR	H_O	H_E	F_{IS}	*A*	AR	H_O	H_E	F_{IS}	*A*	AR	H_O	H_E	F_{IS}
Ai 4.03	9	4,5	0.391	0.450	0.132*	4	4,0	0.375	0.566	0.345	13	8,0	0.400	0.715	0.442**
Ai 4.10	9	4,8	0.356	0.695	0.488**	4	4,0	0.308	0.714	0.579*	4	3,1	0.156	0.475	0.672**
Ai 5.18	24	11,8	0.551	0.872	0.369**	6	5,7	0.400	0.772	0.491*	7	3,4	0.319	0.635	0.498**
e6	7	4,2	0.561	0.650	0.137**	5	4,8	0.437	0.709	0.391*	5	3,3	0.573	0.557	-0.028
e6b	10	6,2	0.717	0.792	0.095	6	5,9	0.667	0.768	0.136	10	6,6	0.539	0.772	0.301**
p2p19	11	6,8	0.691	0.813	0.151*	8	7,7	0.467	0.848	0.459*	10	6,2	0.491	0.561	0.125*
PaA10	4	2,9	0.179	0.269	0.334**	5	4,7	0.400	0.533	0.257	4	3,0	0.345	0.360	0.043*
VgA04	21	10,0	0.569	0.883	0.356**	9	8,4	0.687	0.871	0.216	16	10,1	0.669	0.878	0.238**
VgA06	22	12,0	0.407	0.903	0.550**	9	8,8	0.267	0.883	0.705**	16	9,9	0.384	0.857	0.553**
VgB10	20	11,4	0.721	0.909	0.207**	12	10,9	0.250	0.879	0.722**	13	9,1	0.513	0.839	0.391**
VgB12	5	3,3	0.528	0.591	0.106	4	4,0	0.562	0.719	0.224*	7	3,8	0.374	0.451	0.172**
VgC01	14	7,6	0.691	0.809	0.146**	10	9,4	0.625	0.883	0.299	17	9,2	0.721	0.833	0.135**
VgG02	16	10,7	0.647	0.899	0.281**	11	10,2	0.562	0.881	0.369*	16	10,8	0.564	0.890	0.368**
VgG03	5	3,6	0.482	0.548	0.122	4	3,4	0.467	0.499	0.067	6	4,1	0.396	0.487	0.189**
Overall	177	7,1	0.535	0.720	0.258**	97	6,6	0.462	0.752	0.393**	144	6,5	0.460	0.665	0.309**

689 Inbreeding coefficient (F_{IS}) departed significantly from Hardy-Weinberg equilibrium (HWE) are indicated by asterisks (*P < 0.01; **P <
690 0.001).

691 **Table 3** Analysis of molecular variance (AMOVA) for 14 nuclear microsatellites and cpDNA (*matK* + *trnL-trnF*) sequences data at three
692 hierarchical levels, including *V. carinata*, *V. incurvata* and hybrids individuals.

Source variation	Microsatellites			Plastidial DNA		
	Variation (%)	*F*-statistic	*P*	Variation (%)	*F*-statistic	*P*
Among species	9.41	F_{CT}: 0.094	< 0.001	42.68	F_{CT}: 0.427	< 0.001
Among population within species	3.07	F_{ST}: 0.125	< 0.001	12.33	F_{ST}: 0.550	< 0.001
Within populations	87.52	F_{SC}: 0.034	< 0.001	44.99	F_{SC}: 0.215	< 0.001

693

LEGEND OF FIGURES

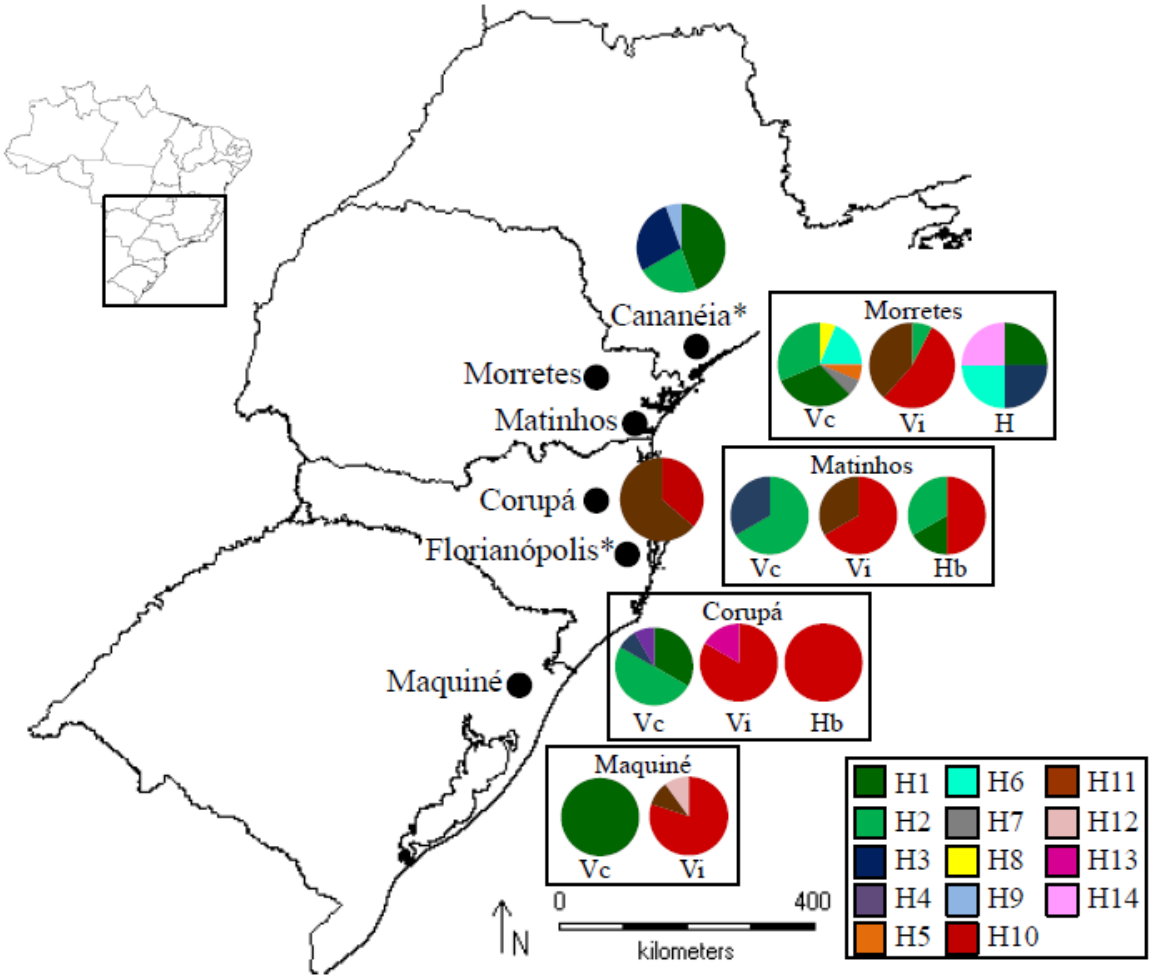
Figure 1 Map showing the geographical distribution of the four sympatric and two allopatric sampled localities of *Vriesea carinata* and *V. incurvata* and plastid DNA haplotype frequencies. Circle sizes are proportional of sample size, and colors represent the different haplotypes, as show in the key. * indicate allopatric populations. Vc – *Vriesea carinata*; Vi – *Vriesea incurvata* and Hb - hybrids identified with STRUCTURE based on nuclear markers.

Figure 2 Posterior probabilities (*q*) for Maquiné, Corupá, Morretes and Matinhos sympatric populations analyzed with Structure and NewHybrids. Sympatric and allopatric localities are delimited by solid lines. The proportion of color in each bar represents an individual's assignment probability, according to different categories (pure parental species, hybrid F1, F2 and backcrosses). See Fig. 1 for details of geographical position of each locality.

Figure 3 Bidirectional migration rates (effective number of migrants, N_em) between four sympatric populations of *Vriesea carinata*, *V. incurvata* and hybrids.

Figure 4 Median-joining network among plastid DNA haplotypes. The haplotypes are indicated by the circles, the size of each circle being proportional to the observed frequency of each haplotype. The colors indicate the individuals classified as pure parental species and hybrids. Lines drawn between haplotypes represent mutation events and the numbers identify how many mutations were observed.

718 Figure1



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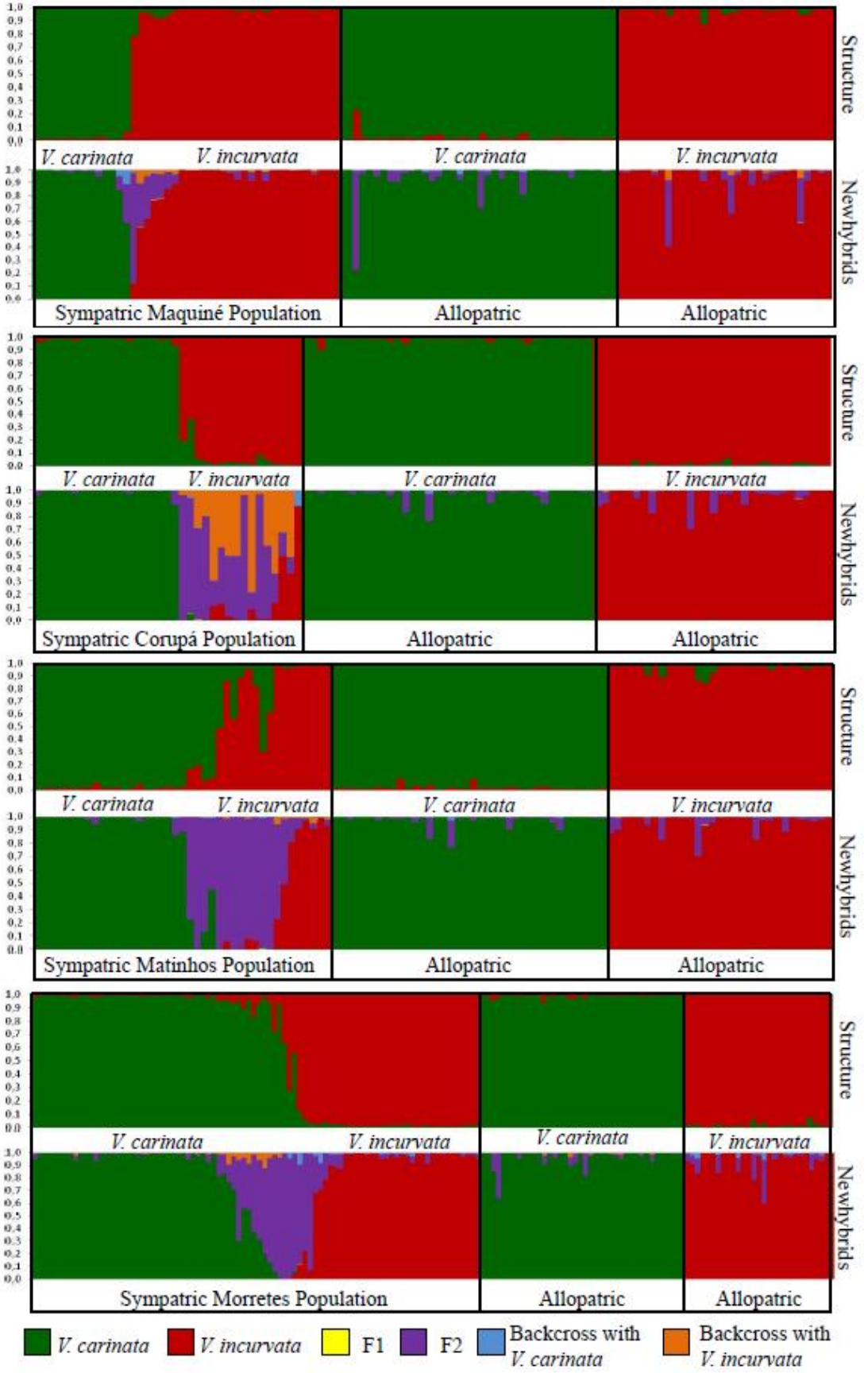
721 Figure 2



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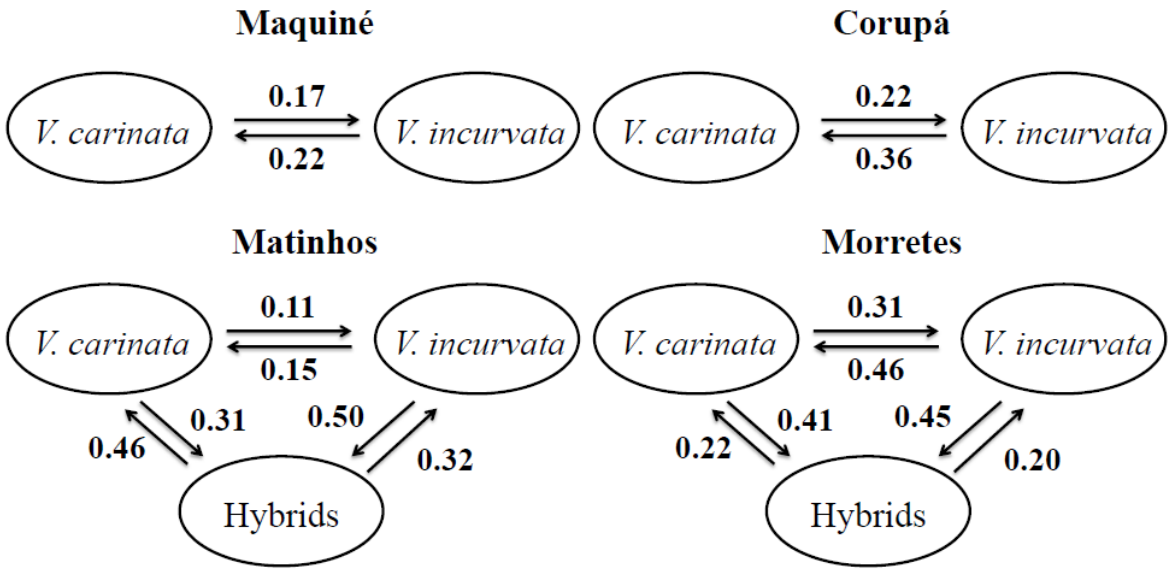
723 Figure 3



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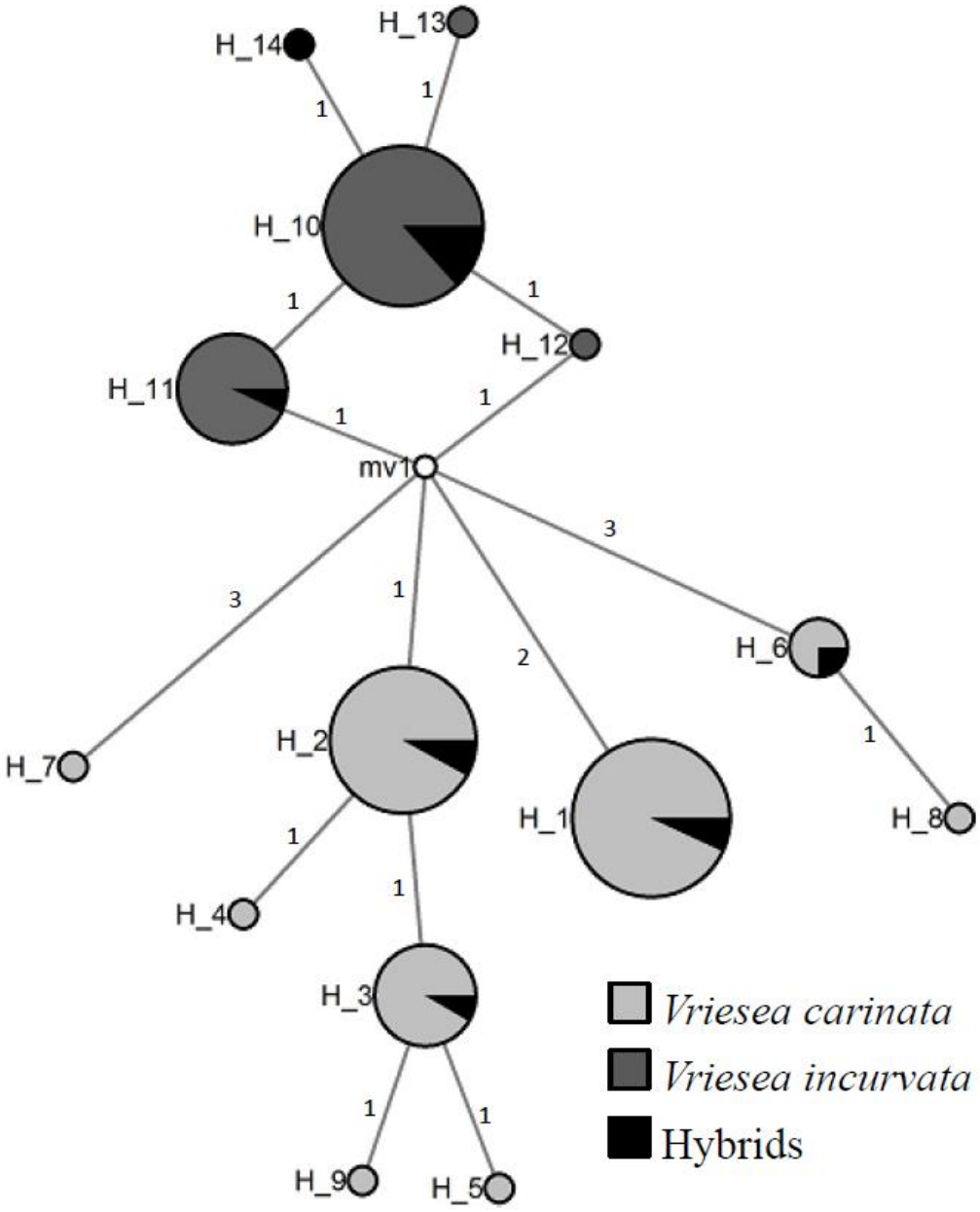
726 Figure 4



727

728 **SUPPORTING INFORMATION**

729

730 **Supplementary material 1** *Vriesea* populations sampled for the present study, including
731 population names, sample sizes for nuclear microsatellite markers (nuSSR) and chloroplast
732 DNA sequence (cpDNA - *matk* + *trnL-trnF*), and geographical coordinates of each
733 population.

	Sample size		Coordinates	
Populations	nuSSR	cpDNA	Latitude S	Longitude W
Allopatric				
Cananéia (Vc)	40	18	25°04'	47°55'
Florianópolis (Vi)	31	12	27°31'	48°30'
Sympatric				
Maquiné	44	20	29°30'	50°14'
Corupá	35	19	26°24'	49°20'
Matinhos	41	21	25°47'	48°31'
Morretes	88	33	25°20'	48°52'
Total	279	123		

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Capítulo V

Considerações Finais

CONSIDERAÇÕES FINAIS

A presente tese está dividida em três artigos relacionados a um projeto amplo que tem como objetivo principal contribuir para o entendimento de questões relacionadas à evolução de plantas Neotropicais. O conjunto de dados obtidos nestes trabalhos contribuirão para um melhor entendimento dos aspectos envolvidos na evolução da família Bromeliaceae, bem como para a compreensão da evolução dos padrões históricos da Mata Atlântica e da evolução dos mecanismos de isolamento reprodutivo desta família.

O Núcleo de Genética e Conservação de Plantas tem como linha de pesquisa central a genética e conservação de espécies de plantas Neotropicais, com principal ênfase na família Bromeliaceae, utilizando diferentes abordagens e técnicas para resolver questões relacionadas à biologia, ecologia, genética e evolução de espécies deste grupo taxonômico, desenvolvendo estudos com espécies nativas do Brasil, principalmente endêmicas da Mata Atlântica. Um artigo de revisão intitulado "Genética, evolução e conservação de Bromeliaceae" foi publicado em 2012 (Capítulo II), com intuito de agregar e compilar os trabalhos desenvolvidos com espécies desta família publicados até 2011, tendo como enfoque aspectos genéticos, adaptações evolutivas, sistemas de cruzamento e suas consequências na estruturação populacional e conservação *in situ*. Os resultados desta revisão indicam que Bromeliaceae é preferencialmente polinizada por vertebrados, com variações nos sistemas de cruzamento, tendo espécies que se reproduzem por autofecundação, sistemas mistos e espécies com fecundação cruzada obrigatória (Zanella *et al*., 2012a). Também, foi relatado que as bromélias apresentam uma constância no número cromossômico (x = 25; Marchant, 1967). A estruturação populacional observada nos trabalhos desenvolvidos até o momento, com variados marcadores moleculares, demonstrou que a diferenciação entre as populações é influenciada, principalmente, pelo modo de dispersão do pólen e das sementes, pela conectividade entre as populações e modo de reprodução clonal (Gliddon *et al*., 1987). Quanto à história evolutiva, pouco se sabe sobre a evolução da família, registros fósseis (pólen) indicaram a existência de representantes de Bromeliaceae a partir do médio Terciário (Benzing, 2000). Estudos filogenéticos e biogeográficos recentes propuseram o surgimento da família há 100 milhões de anos no Escudo das Guianas durante o Período Cretáceo e as subfamílias atuais começaram a divergir há apenas cerca de 19 milhões de anos (Givnish *et al*., 2011). São

conhecidos três centro de diversidade para a família, a qual é composta por oito subfamílias, 58 gêneros e aproximadamente 3170 espécies (Benzing, 2000, Luther, 2008, Givnish *et al*., 2007; 2011).

Vriesea carinata e *V. incurvata* foram escolhidas como objeto de estudo da presente tese devido a observações, em expedições de campo, de indivíduos com morfologia intermediária. Relatos de herbário também foram observados sobre um possível processo de hibridação entre elas, devido a características de hábito de vida compartilhadas. Para auxiliar na compreensão da evolução desses taxa, um estudo filogeográfico comparativo foi desenvolvido, com intuito de entender os padrões evolutivos históricos que atuaram sobre essas espécies e modelaram a sua distribuição atual, assim como os seus padrões de diversidade e estruturação populacional.

Vriesea carinata e *V. incurvata* são espécies endêmicas da Mata Atlântica, com hábito preferencialmente epifítico, sendo encontradas em lugares úmidos e bem preservados. São espécies de ampla distribuição ocorrendo desde o norte do Rio Grande do Sul até o sul da Bahia (*V. carinata*) e desde o norte do Rio Grande do Sul até o Rio de Janeiro (*V. incurvata*; Smith e Downs, 1977). Estas espécies apresentam morfologia floral semelhante, sendo polinizadas por beija-flores generalistas (*Phaethornis eurynome* e *Melanotrochilus fuscus*; Machado e Semir, 2006). Podem ser encontradas em simpatria e possuem florescimento sequencial, com um pequeno período de sobreposição entre elas (Araujo *et al*., 2004). Em espécies simpátricas polinizadas por agentes generalistas, pode ocorrer transferência interespecífica de pólen (Hersch e Roy, 2007).

No Capítulo III foi realizado um estudo comparativo dos padrões filogeográficos e diversidade genética de *V. carinata* e *V. incurvata*. Considerando o fluxo gênico intra-específico, ambas as espécies apresentaram níveis moderados de diversidade genética e isolamento por distância (correlação negativa significativa entre distância genética e geográfica). As espécies apresentaram padrões filogeográficos semelhantes, com uma divisão norte-sul entre os estados de São Paulo e Rio de Janeiro, considerando os dois genomas avaliados (nuSSR e cpDNA), corroborando com registros encontrados na literatura (Harris *et al*., 2005; Pellegrino *et al*., 2005; Grazziotin *et al*., 2006; Cabanne *et al*., 2007; Martins *et al*., 2007; Fitzpatrick *et al*., 2009; Palma-Silva *et al*., 2009; Ramos *et al*., 2009, Ribeiro *et al*., 2011; Silva *et al*., 2012). Provalemente essas espécies permaneceram em mais de um refúgio durante as oscilações climáticas do Pleistoceno que

atuaram sobre a Mata Atlântica. Desta forma, propomos a hipótese de múltiplos refúgios na Mata Atlântica, com descontinuidade genética entre as populações mais ao norte (RJ e ES) com as populações do sul da distribuição dessas espécies. Foram identificados dois prováveis refúgios, um localizado na região sul-sudeste (PR/SP refúgio; entre as latitudes 23°S e 25°S) e outro no sudeste do Brasil (RJ/ES; entre as latitudes 19°S e 22°S), influenciados, principalmente, pelas oscilações climáticas do Pleistoceno e por eventos orogênicos do Plioceno e Mioceno. Eventos de expansões foram observados em populações da distribuição sul, em concordância com a expansão da floresta em direção à região sul da Mata Atlântica após o último Máximo Glacial (Carnaval *et al*., 2009).

A história evolutiva da Mata Atlântica é complexa e provalmente tenha sido moldada ao longo do Pleistoceno, influenciada pelas oscilações climáticas desse período. Contudo, eventos anteriores, como elevação da costa leste brasileira durante o Terciário, também podem ter influenciado na distribuição e a diversificação dos taxons, atuando como barreiras ao fluxo gênico (Thomé *et al*., 2010; Silva *et al*., 2012; Turchetto-Zolet *et al*, 2013). Mais estudos são necessários para entender essa complexa história da Mata Atlântica, pricipalmente na porção mais ao sul do bioma.

Ao estudarmos populações simpátricas de *V. carinata* e *V. incurvata*, visando identificar padrões de fluxo gênico interespecífico natural (Capítulo IV), observamos que essas espécies hibridizam e com padrões distindos entre as populações simpátricas analizadas. Um total de 19 híbridos foram indentificados utilizando o programa STRUCTURE, e 43 foram identificados com o programa NEWHYBRIDS, sendo classificados como F2 e retrocruzamento com *V. incurvata*. Nas populações simpátricas com menor latitude encontramos um número maior de híbridos, provavelmente relacionado a um gradiente latitudinal, o qual pode influenciar as estações do ano, temperatura, precipitação e, consequentemente, período de floração da espécie (Marques e Lemes-Filho, 2008). O curto período de sobreposição da floração possibilita a ocorrência de fluxo gênico e introgressão entre *V. carinata* e *V. incurvata*, como indicado pelas análises de nuSSR e cpDNA. Apesar da identificação de alguns indivíduos com morfologia intemediária e perfil molecular característico de híbridos, uma forte barreira reprodutiva é observada entre *V. carinata* e *V. incurvata*, com baixa taxa de fluxo gênico interespecífico entre elas (N_em < 0.5), porém possibilitando a identificação de aproximadamente 10% de híbridos. A diferença no período de floração tem atuado como barreira pré-zigótica ao fluxo gênico e

pode ser considerada a principal força responsável pela coesão das espécies. A presença de barreiras reprodutivas permite que essas espécies persistam em simpatria por um longo período de tempo, assegurando a manutenção da coesão das mesmas. O fluxo gênico interespecífico é uma fonte poderosa de variabilidade, por contribuir para a inserção de novas combinações alélicas que podem conferir um maior valor adaptativo às espécies.

Em resumo, os resultados descritos nos capítulos que compreendem esta tese irão auxiliar efetivamente para que haja uma melhor compreensão dos processos biológicos, evolutivos e históricos envolvidos na família Bromeliaceae e no bioma Mata Atlântica. Além disso, ficou ainda mais evidente a necessidade de novos trabalhos com espécies animais e vegetais que ocorram neste bioma, em particular na porção sul, para que a complexa história evolutiva desta região seja compreendida.

Capítulo VI

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