Universidade Federal do Rio Grande do Sul

Genética, Filogeografia e Fertilidade de populações de *Vriesea gigantea* (Bromeliaceae)

Foto: Clarisse Palma da Silva

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Genética, filogeografia e fertilidade de populações de *Vriesea gigantea* Gaud. (Bromeliaceae)

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Sumário

Resumo

Vriesea gigantea é uma espécie endêmica da Mata Atlântica, podendo ser encontrada desde o estado do Espírito Santo até o Rio Grande do Sul. A espécie é perene, diplóide e autocompatível. Suas populações naturais estão sendo rapidamente reduzidas por dois motivos principais: pela ação antrópica, que modifica seu habitat natural, e pela coleta predatória e ilegal de plantas da natureza. Os processos que formaram a extraordinária diversidade de espécies nas regiões Neotropicais ainda são pouco conhecidos. A Mata Atlântica tem sido considerada um dos maiores centros de biodiversidade, o que torna a sua conservação prioritária. A variabilidade intra-específica tem sido aceita como foco para a conservação. Além disto, a fertilidade das plantas tem grande importância conservacionista, sendo a viabilidade do pólen (qualidade do pólen) um importante componente do sucesso reprodutivo.

Com o objetivo de estudar a diversidade genética em *Vriesea gigantea*, 11 marcadores de microssatélites foram desenvolvidos e os resultados publicados conjuntamente com aqueles obtidos para outros quatro loci caracterizados para uma espécie próxima, *Alcantarea imperialis* (**Capítulo 2**). Treze novos pares de *primers* de microssatélites nucleares foram testados em 22 espécies de bromélias, indicando que estes marcadores serão úteis em inúmeros estudos com outras espécies desta mesma família.

Os marcadores nucleares de microssatélites foram utilizados para estudar os padrões de diversidade genética de *Vriesea gigantea* ao longo de toda a distribuição geográfica da espécie, em 429 indivíduos, amostrados em 13 populações (**Capítulo 3**). Os resultados indicaram uma tendência latitudinal de diminuição da diversidade genética do Norte para o Sul, consistente com o padrão histórico de expansão da Floresta Atlântica. A expansão da espécie parece ter sido impedida pela diminuição do fluxo gênico nas populações marginais. Além disso, a história evolutiva das populações marginais difere muito. O centro de diversidade genética para *V. gigantea* (São Paulo e Rio de Janeiro) coincidiu com o centro de diversidade e centro de endemismo de muitas espécies de animais e plantas da mata Atlântica. A correlação entre a diversidade genética de *V. gigantea* e a de espécies do gênero *Vriesea* indicou que ambas foram moldadas pelas mesmas forças históricas: alterações

climáticas do Pleistoceno. As análises de distância genética revelaram três grupos geograficamente separados e as análises bayesianas revelaram a presença de outros dois grupos, referentes às populações marginais, os quais são de grande importância para estabelecer estratégias de conservação da espécie.

Complementando as análises do genoma nuclear, foram seqüenciadas 21 regiões do genoma de cloroplasto, produzindo um total de cinco regiões plastidiais polimórficas (quatro microssatélites e um SNP – *Single Nucleotide Polymorphism*). As regiões de microssatélites de cloroplastos polimórficas foram isoladas pela primeira vez em Bromeliaceae. Os cinco marcadores plastidiais foram examinados em 192 indivíduos de 13 populações, com objetivo de inferir a história filogeográfica da espécie (**Capítulo 4**). Os principais resultados indicaram uma forte estrutura filogeográfica e um reduzido fluxo gênico entre as populações de *V. gigantea*. A razão de 3,3 indicou assimetria entre o fluxo de pólen e sementes, sendo o fluxo gênico via semente menos eficaz do que via pólen, resultando em uma maior estruturação do genoma do cloroplasto do que do genoma nuclear (microssatélites nucleares). A rede de haplótipos revelou dois grupos filogeográficos distintos: Norte e Centro-Sul. A análise da distribuição de *mismatch* mostrou que populações da região Norte são mais estáveis e devem ter tido um crescimento ancestral (antigas), enquanto que as populações da porção Centro-sul estão em expansão (jovens).

No **Capítulo 5**, a fertilidade de plantas das populações do sul foi analisada através do número cromossômico, comportamento meiótico e viabilidade do pólen. A maioria das células-mãe-de-pólen apresentou comportamento meiótico regular, o que está de acordo com a alta viabilidade dos grãos de pólen (84 - 98%) registrada para todas as populações investigadas. Estes resultados indicaram que as plantas das populações analisadas são potencialmente férteis. Apesar da alta viabilidade dos grãos de pólen observada, foram constatadas diferenças significantes entre populações.

Abstract

V. gigantea is a diploid, perennial and self-compatible bromeliad species endemic to Atlantic Rainforest of southeastern and southern Brazil. Its wild populations have been reduced by anthropogenic disturbance such as habitat destruction and illegal collection. The processes that have shaped the extraordinary species diversity in Neotropical rainforests are poorly understood, and knowledge about patterns of genetic diversity across species' ranges is scarce. Brazilian Atlantic Rainforest has been considered one of the major biodiversity hotspots for conservation. Intraspecific variation has increasingly been accepted as a focus for conservation. Another issue also of conservation meaning is plant fertility. The quality of pollen produced by a plant is an important component of reproductive success.

In order to start studying genetic diversity in *Vriesea gigantea* a set of 11 nuclear microsatellite markers were developed, and the results published together with four loci characterized for a closed-related species, *Alcantharea imperialis* (**Chapter 2**). These fifteen new nuclear microsatellite pair primers were tested in 22 bromeliads species indicating that these markers will be useful in numerous other taxa.

Using nuclear microsatellite markers patterns of genetic diversity on *V. gigantea* were studied all across its geographic distribution in Atlantic Rainforest in a large sampling of 429 plants from 13 populations (**Chapter 3**). The main results indicated latitudinal trend of decreasing diversity from North to South away from the equator, consistent with historical range expansion from the Northern half of the present distribution range. Further species expansion appears to be impeded by lack of gene flow at the current range margins, and the nature of range limits differs greatly between North and South. The center of genetic diversity for *V. gigantea* (São Paulo and Rio de Janeiro) coincided with centers of species diversity and endemism for most animals and plants of the Atlantic Rainforest. Significant correlation between genetic diversity in *V. gigantea* and species diversity in the *Vriesea* genus suggested that both were shaped by the same historical forces: climatic changes of the Pleistocene. Distance-based genetic analysis revealed three geographically defined clusters,

and a Bayesian analysis revealed two additional genetic clusters of conservation relevance.

To obtain a more ancient scenario, 21 chloroplast regions were sequenced and screened producing a total of five polymorphic plastid regions (four microsatellites and one SNP - Single Nucleotide Polymorphism). Polymorphic chloroplast microsatellites markers were isolated for Bromeliaceae for the first time. The five cpDNA markers were examined in 192 individuals from 13 populations to infer the phylogeographical history of the species (**Chapter 4**). The key results indicated a strong phylogeographic structure and a reduced gene flow among populations of *Vriesea gigantea.* A ratio of 3.3 indicated an asymmetry between pollen and seed flow, being gene flow via seed less efficient than via pollen, resulting in a stronger genetic structure for chloroplast genome than nuclear genome (inferred by nuclear SSR). Haplotype network revealed two major phylogeographic groups: Northern and Central-Southern. Mismatch distribution analysis showed that populations from the Northern region are stable and should have an ancestral growth ("older"), while the populations from Central-Southern region are in expansion range ("younger").

In **Chapter 5**, the plant fertility of southern populations was analyzed by assessing: chromosome number, meiotic behavior, and pollen viability. Most of pollen mother cells showed a regular meiotic behavior. In accordance, high pollen viability (84-98%) was recorded for all investigated populations. These results indicated that plants from all populations analyzed are fertile. Despite the overall high pollen viability, significant differences were detected among populations.

Capítulo 1

Introdução Geral

Introdução

I) A Família Bromeliaceae

Bromeliaceae é uma família característica da região Neotropical, suas espécies podem ser encontradas em diferentes tipos de habitat, apresentando populações numerosas de importante papel nos ecossistemas em que ocorrem. Possui, também, vários gêneros endêmicos, alguns deles encontrados exclusivamente na Floresta Atlântica (Martinelli, 1994). Diversos animais e vegetais possuem parte ou todo ciclo de vida intimamente ligado à presença de determinadas espécies de bromélias. O extenso potencial de radiação adaptativa da família se reflete na grande diversidade morfológica de suas espécies. As bromélias possuem folhas dispostas em roseta, que possibilitam o acúmulo de água, ou folhas carnosas que funcionam como órgãos de reserva. Os tricomas foliares possibilitam a absorção de água e o desenvolvimento de várias estratégias para lidar com o estresse hídrico permitindo uma extraordinária versatilidade ecológica (Benzing, 2000). Apesar das bromélias serem conhecidas como plantas parasitas, suas raízes possuem função exclusiva de suporte e fixação. Assim, as bromélias ocupam ambientes extremos com hábitos que variam de terrestres a epífitas, sendo encontradas desde o nível do mar a altitudes superiores a 4.000 metros, em regiões úmidas e desérticas, e em locais com muita ou pouca luminosidade (Benzing, 2000). As inflorescências são bastante coloridas e diversificadas, com flores nectaríferas que atraem morcegos, aves e insetos como agentes polinizadores.

Atualmente são conhecidos mais de 60 gêneros e 3000 espécies (Smith e Till, 1998), todas distribuídas no Novo Mundo. Apenas uma espécie possui distribuição diversa, *Pitcairnia feliciana,* que ocorre no oeste do continente Africano e parece ter sido um evento de recente dispersão a longa distância (Givinish *et al.,* 2004). A distribuição geográfica, da família, apresenta como limite norte de ocorrência os estados da Virgínia, Texas e Califórnia, nos Sul dos Estados Unidos (Latitude 37° N) e como limite sul o norte da Patagônia, na Argentina (Latitude 44°S) (Smith, 1934; Leme e Marigo, 1993). Informações oriundas de macro e microfósseis (pólen) indicam a existência de representantes de Bromeliaceae a partir do médio Terciário (Benzing, 2000). O surgimento da família pode ser um fenômeno relativamente recente (Smith,

1934), devendo ter ocorrido no próprio Novo Mundo, como sugere sua distribuição continental restrita (Leme e Marigo, 1993). As bromélias provavelmente surgiram no norte da América do Sul, um ambiente que é geologicamente antigo e estável, e que abriga espécies consideradas basais nas hipóteses filogenéticas existentes para o grupo (Benzing, 2000; Givinish *et al.,* 2004; Barfuss *et al.,* 2005).

A família Bromeliaceae está tradicionalmente dividida em três subfamílias: Bromelioideae (~650 sp), Pitcairnioideae (~890 sp) e Tillandsioideae (~1100 sp) (Smith e Downs, 1974, 1977 e 1979; Leme e Marigo, 1993). Bromeliaceae e suas subfamílias possuem suporte nas hipóteses filogenéticas disponíveis para o grupo (Terry *et al.,* 1997; Horres *et al.,* 2000; Crayn *et al.,* 2004; Givinish *et al.,* 2004; Barfuss *et al.,* 2005), sendo que apenas Pitcairnioideae não constitui um grupo monofilético.

O Brasil é o maior centro de diversidade de bromélias, onde podemos encontrar cerca de 50% das espécies conhecidas. Todavia, o interesse pelo cultivo de bromélias para a comercialização como plantas ornamentais é muito recente, datando do início dos anos 90. Atualmente 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 daquelas espécies com ciclos longos de vida, que levam muito tempo para atingir o tamanho ideal para o comércio, vem reduzindo drasticamente muitas populações, principalmente na Mata Atlântica (Coffani-Nunes, 2002), onde se encontram a maioria das espécies consideradas ornamentais. Outro fator relevante que põe em risco espécies de bromélias é a destruição de seu habitat devido à ação humana.

Devido a sua aparência exclusiva, as bromélias se tornaram muito populares como plantas ornamentais, e grande parte do conhecimento sobre as espécies provém de colecionadores, sendo a bibliografia científica restrita. Mesmo aspectos básicos, como o sistema reprodutivo, são desconhecidos para a maioria das espécies. Informações sobre os padrões filogeográficos, o impacto do sistema reprodutivo na estrutura genética das populações e o fluxo gênico entre populações são ainda pouco conhecidos.

II) *Vriesea gigantea*

A subfamília Tillandsioideae apresenta nove gêneros e aproximadamente 1100 espécies, sendo os gêneros *Tillandsia* e *Vriesea* os que apresentam o maior número de espécies, 518 e 230, respectivamente (Smith e Downs, 1977). A distribuição geográfica do gênero *Vriesea* é bastante ampla, sendo encontradas espécies desde a América Central até o Sul da América do Sul. O centro de diversidade do gênero é o leste Brasileiro (Smith, 1934; Smith e Downs, 1977).

Vriesea gigantea Gaud. (Anexo V), é uma espécie endêmica da Mata Atlântica, podendo ser encontrada desde o estado do Espírito Santo até o Rio Grande do Sul, o qual representa o limite austral de distribuição do gênero *Vriesea* (nas proximidades da Estação Ecológica do Taim, Latitude 31°56' S). Interessantemente, as populações de *V. gigantea* do Estado do Espírito Santo (Anexo V) possuem morfologia diferenciada das demais populações, tendo sido descrita como uma variedade - *V. gigantea* var. *seideliana* (Röth, 1992). A espécie é perene e diplóide com 2n = 50 cromossomos (Palma-Silva, 2003). Segundo Smith e Downs (1977), uma planta de *V. gigantea* pode atingir dois metros de altura, com a inflorescência (ver Anexo V). Apresenta hábito terrestre, epífito ou saxícola – Anexo V – (Smith e Downs, 1977), podendo ser encontrada numa faixa altitudinal que compreende matas ao nível do mar até 500m de altitude (Reitz, 1983). As flores são polinizadas por morcegos (Sazima *et al.,* 1995, 1999) e as pequenas sementes são dispersas pelo vento – Anexo V – (Smith e Downs, 1974), As folhas desta espécie possuem um padrão de estrias muito característico (Anexo V), o qual além de atribuir valor ornamental, possibilita o reconhecimento dos indivíduos deste grupo taxônomico mesmo na ausência da inflorescência. Após a frutificação, ocorre reprodução vegetativa por brotamento; entretanto, a espécie não se caracteriza como de reprodução clonal, uma vez que não forma touceiras, encontrando-se no máximo três indivíduos clonais (Reitz, 1983). Além disso, as populações naturais são relativamente grandes e encontradas em agrupamentos de diferentes tamanhos e densidades.

As cisternas, estruturas formadas pela disposição em roseta das folhas, são utilizadas como uma importante fonte de recursos (abrigo, alimento, sítio reprodutivo, etc.) para muitas populações naturais de espécies associadas, e alguns trabalhos sugerem uma modulação microclimática funcional para o

estabelecimento de várias espécies (Stuntz *et al.,* 2002). Assim, *V. gigantea* participa de maneira ativa em processos ecológicos das matas, como Benzing (2000) relatou também para outras espécies de bromélias. Um exemplo interessante foi o relatado por Schmidt (2003), que observou que a composição de aranhas residentes em *V. gigantea* difere substancialmente daquelas encontradas nos substratos arbóreos adjacentes da mesma região do Parque Estadual de Itapuã – RS, Brasil. Além disso, o autor observou a interação direta entre a fauna de vertebrados da região estudada e *V. gigantea*.

Assim como várias outras espécies de bromélias, *V. gigantea* possui alto potencial ornamental e faz parte da lista de espécies ameaçadas de extinção do Estado do Rio Grande do Sul (www.sema.rs.gov.br). É uma planta exuberante e seu grande porte (~ 0,95 cm de altura sem inflorescência – Paggi *et al.,* 2007) garante destaque em ambientes externos e internos. Portanto, as suas populações naturais estão sendo rapidamente reduzidas pela ação antrópica, que modifica seu habitat natural, e pela coleta predatória e ilegal de plantas da natureza. Apesar do grande potencial ornamental de *V. gigantea*, poucos são os produtores de bromélias que a cultivam para fins comerciais. Assim, grande parte do mercado é suprida pela coleta e comercialização ilegal de indivíduos da natureza (Anexo V).

V. gigantea é uma espécie não autógama (índice de autogamia = 0,19 – Paggi, 2006) e possui um sistema de cruzamento misto com baixos índices de fecundação cruzada (t_m = 0,32; GM Paggi, comunicação pessoal). Paggi *et al.* (2007) estudaram aspectos referentes ao sucesso reprodutivo de populações de *V. gigantea* do Sul do Brasil. Os autores observaram que de forma geral as populações eram viáveis com alta produção de flores, frutos e sementes. As sementes, em sua maioria, eram viáveis com média de 94% de germinação após 15 dias da semeadura. Neste mesmo trabalho, através de experimentos de polinizações manuais foi observado também que as populações analisadas eram afetadas pela limitação de pólen. A limitação de pólen provavelmente é uma conseqüência da fragmentação de habitat, e especificamente, devido à ruptura entre o mutualismo dos polinizadores e as espécies polinizadas (Paggi *et al.,* 2007).

III) Variabilidade e estrutura genética em populações naturais de bromélias

Em Bromeliaceae poucos trabalhos têm sido realizados para avaliar a diversidade genética de populações naturais. Apesar de muitas espécies possuírem uma ampla distribuição geográfica e populações abundantes, sendo um modelo interessante para estudos de genética de populações.

Inicialmente, foram utilizados marcadores isoenzimáticos para estimar a variabilidade genética entre populações de diferentes espécies de bromélias (Soltis *et al.,* 1987; Murawski e Hamrick, 1990; Izquierdo e Piñero, 2000; Sarthou *et al.,* 2001; González-Astorga *et al.,* 2004). Posteriormente, marcadores baseados em PCR, tais como o RAPD (*random amplified polymorphic DNA*) e ALFP (*amplified fragment lenght polymorphism*) começaram a ser utilizados (Ruas *et al.,* 1995; Zizka *et al.,* 1999; Sgorbati *et al.,* 2004; Cavallari *et al.,* 2006).

Soltis *et al.* (1987) foram pioneiros em estudar a variação genética em espécies de bromélias utilizando eletroforese de isoenzimas. Os autores investigaram a estrutura genética de duas espécies epífitas com diferentes sistemas de cruzamento: *Tillandsia ionantha* e *T. recurvata.* Os resultados revelaram que a estrutura genética das espécies analisadas diferiu de acordo os sistemas de cruzamento. Altos níveis de endocruzamento foram observados em *T. recurvata,* que apresentou completa ausência de heterozigotos. No entanto, *T. ionantha* exibiu características de uma espécie de fecundação cruzada, com pequena variação entre as populações nas freqüências alélicas observadas.

Murawski e Hamrick (1990) investigaram a variabilidade genética de isoenzimas em populações de *Aechmea magdalenae*, uma espécie terrestre de crescimento clonal. Apesar de ter apresentado menos variabilidade genética do que plantas com essas mesmas características, *A. magdalenae* mostrou maior variabilidade genética dentro das populações do que o relatado para as espécies epífitas *T. recurvata* e *T. ionantha* (Soltis *et al.,* 1987).

Izquierdo e Piñero (2000) descreveram a variabilidade e a estrutura genética e a diversidade clonal de uma única população endêmica de *Aechmea tuitensis* também utilizando eletroforese de isoenzimas. *A. tuitensis* apresentou alta diversidade genética, embora seja uma espécie de distribuição geográfica

restrita. Os autores acreditam que os efeitos da deriva e estruturação genética possam ter sido minimizados pelo fato da espécie apresentar reprodução vegetativa.

Sarthou *et al.,* (2001) descreveram a estrutura genética de três populações de *Pitcairnia geyskesii,* uma espécie de hábito saxícola. Esta espécie ocorre predominantemente em afloramentos rochosos isolados (*inselbergs)*, o que dificulta o fluxo gênico entre as populações. Apesar do baixo fluxo gênico observado entre os três *inselbergs*, altos níveis de variabilidade genética foram detectados nas populações amostradas. Através destas análises, informações quanto ao comportamento reprodutivo da espécie foram inferidas, demonstrando uma eficiente reprodução sexual através do recrutamento de sementes. Os níveis e a estrutura da variabilidade genética observados entre inselbergs são semelhantes às de espécies de fecundação cruzada aproximando-se da panmixia. No entanto, a reprodução vegetativa, através da dispersão clonal, também esteve envolvida no estabelecimento de novas subpopulações. Dessa forma, tanto a reprodução clonal, devido à longevidade dos genetes, quanto o recrutamento de sementes, pela reprodução sexual, são responsáveis pela alta diversidade genética dentro dos inselbergs.

González-Astorga *et al.,* 2004 estudaram a diversidade e estrutura genética em *Tillandsia achyrostachys* uma espécie endêmica das florestas tropicais secas do México. Através da análise de 16 *loci* isoenzimáticos, foi observado um déficit de heterozigozidade em todas as seis populações estudadas. Os níveis de fluxo gênico entre as populações foram considerados baixos e com grande variação indicando um padrão de isolamento por distância. Além disso, os autores também observaram que as populações fragmentadas possuíam reduzida riqueza alelica, menor diversidade genética e maior diferenciação entre as populações. Estes resultados revelaram que a fragmentação do habitat provavelmente levou a quebra da estrutura genética original.

Além das análises de eletroforese de isoenzimas, marcadores moleculares também têm sido utilizados nas avaliações de variabilidade genética em Bromeliaceae. Inicialmente, marcadores do tipo RAPD (*random amplified polymorphic DNA)* revelaram variabilidade genética moderada infra-

especifica para cinco espécies do gênero *Ananás* e uma do gênero *Pseudoananas* (Ruas *et al.,* 1995).

Sgorbati *et al.* (2004) analisaram a diversidade genética de *Puya raimondii*, uma espécie nativa dos Andes Peruanos, ameaçada de extinção. Foram utilizados marcadores do tipo ALFP e cpSSR (*cloroplast simple sequence repeat*). Os resultados indicaram baixíssima diversidade genética nessas populações, fato que foi atribuído a eventos de gargalo genético que poderiam ter ocorrido repetidas vezes nestas populações. Especificamente em relação aos *loci* de cpSSRs, os autores não encontraram polimorfismos nas amostras de *P. raimondii* estudadas.

Cavallari *et al.* (2006) estudaram três espécies do gênero *Encholirium* (*E. pedicellatum, E. biflorum e E. subsecundum*) endêmicas da Cadeia do Espinhaço, Minas Gerais, através de marcadores moleculares do tipo RAPD. As espécies são consideradas raras, possuem reprodução clonal e são encontradas em populações de poucos indivíduos. Os índices de diversidade genética observados foram maiores para a espécie *E. subsecundum* a qual tem distribuição geográfica mais ampla do que para as outras duas espécies. Os níveis de estruturação populacional variaram de Φ_{ST} = 0,16 a 0,08 mostrando que cada espécie possui diferentes padrões de distribuição da diversidade genética e fluxo gênico.

Somente há pouco tempo, marcadores do tipo microssatélites começaram a ser utilizados em estudos de genética de populações em Bromeliaceae. Inicialmente, dois grupos independentes desenvolveram os primeiros *loci* de microssatélites para a família (Cinco *loci*: Boneh *et al.,* 2003; e sete *loci:* Sarthou *et al.,* 2003). Cascante-Marín (2005) realizou o primeiro estudo utilizando os *loci* publicados por Boneh *et al.,* (2003) em duas espécies pertencentes à subfamília Tillandsioideae: *Tillandsia fasciculata* e *Guzmania monostachia.* Neste estudo o autor observou que as duas espécies epífitas possuíam diferentes níveis de variabilidade genética, sendo que *G. monostachia* apresentou baixos níveis de H_e < 0,252. Este fato foi relacionado com os altos índices de endocruzamento (F_{IS} > 0,90) detectados para a espécie. Por outro lado, *T. fasciculata* mostrou moderada diversidade gênica (H_e = 0,589) com um excesso de heterozigotos (F_{IS} = -0,411) apesar de baixa taxa de fecundação cruzada ter sido observada nesta espécie. O autor

argumentou que essa disparidade é, provavelmente, devida a heterozigozidade fixada, uma condição que está associada com espécies poliplóides. Quanto aos níveis de diferenciação entre populações os valores obtidos para *G. monostachia* e *T. fasciculata* (F_{ST} = 0,123 e 0,247, respectivamente) são significantes e estão de acordo com os altos índices de autofecundação em combinação com a limitada dispersão das sementes destas duas espécies (Cascante-Marín, 2005).

Barbará e seus colaboradores também utilizaram alguns *loci* de microssatélites nucleares descritos por Boneh *et al.,* (2003), Sarthou *et al.,* (2003) e Palma-Silva *et al.,* (2007). Estes estudos incluíram quatro espécies de bromélias endêmicas de afloramentos rochosos (*inselbergs*) da Mata Atlântica: *Alcantarea imperialis, A. geniculata* (Barbará *et al.,* 2007a)*, A. regina e A. glaziouana* (T. Barbará. *in prep*) que representam modelos para estimar os efeitos da conectividade populacional em plantas adaptadas a *inselbergs.* As quatro espécies possuem moderados níveis de diversidade gênica (H_e = 0,615; 0,429; 0,523 e 0,472, respectivamente). Os valores de diferenciação populacional foram considerados altos (F_{ST} = 0,43; 0,11; 0,195 e 0,217). O fluxo gênico entre populações foi extremamente baixo indicando que a habilidade dos polinizadores em promover o fluxo gênico entre *inselbergs* é menor do que o previamente assumido.

IV) Genética de populações e filogeografia na Mata Atlântica

A Mata Atlântica está distribuída ao longo do sul e sudeste brasileiro, sudeste do Paraguai, e nordeste da Argentina (*sensu* Oliveira-Filho e Fontes, 2000). A floresta atlântica da costa brasileira tem sofrido alto impacto pela intensa ação humana há pelo menos 500 anos (Cardoso *et al*., 1998). Atualmente, é um dos três ecossistemas mais ameaçados de extinção do mundo (Myers *et al*., 2000), não sendo mais uma floresta contínua (Heringer e Montenegro, 2000). Inicialmente apresentava uma área de distribuição que cobria 1,4 milhões de km^2 do país, hoje, os fragmentos remanescentes não excedem 7,3% da área original (Coffani-Nunes, 2002).

Informações sobre a variabilidade genética dentro e entre populações, assim como os conhecimentos do germoplasma existentes, são extremamente importantes, pois podem servir como base para a utilização e conservação dos

recursos genéticos disponíveis (Handa, 1998). O conhecimento da estrutura genética das populações permite, por exemplo, o delineamento de unidades de manejo. Um ponto fundamental é determinar se as populações diferem suficientemente, do ponto de vista genético, para justificar um manejo separado. A definição da unidade de manejo deverá levar em conta, também, a existência de fluxo gênico entre espécies. Essas informações contribuem para o estabelecimento de estratégias visando maximizar a retenção da diversidade genética e minimizar o endocruzamento (Frankham *et al.,* 2003; Barbieri, 2003). Segundo Moritiz (2002), as estratégias de conservação deveriam levar em conta os 'padrões' de distribuição da diversidade genética e também os 'processos' evolutivos envolvidos na formação de tais padrões. A reconstrução dos padrões evolutivos, especialmente em nível intrapopulacional, tem sido de grande importância para a determinação de estratégias adequadas de conservação de espécies. Neste contexto, o estudo de genética das populações e de filogeografia, possibilitam inferências de diferentes aspectos evolutivos.

A filogeografia é o estudo das relações entre populações em um contexto geográfico e disponibiliza informações históricas para o entendimento de padrões do fluxo gênico, colonização, expansão e efeitos da distribuição atual da variação genética ao longo de toda a distribuição de uma espécie (Avise 2000). As inferências filogeográficas são feitas a partir da distribuição da variação genética entre populações (Pannel e Dorken, 2006).

Estudos de filogeografia de espécies da Mata Atlântica são escassos. Alguns poucos trabalhos em plantas (Lira *et al.,* 2003; Lorenz-Lemke *et al.,* 2005; Andrade *et al.,* 2007) e em animais (Grazziotin *et al.,* 2006; Tchaicka *et al.,* 2007; Cabanne *et al.,* 2007) tem disponibilizado informações a respeito dos efeitos das expansões e contrações históricas da Mata Atlântica nos padrões de variação intra-especifica e demonstrado a complexidade da história evolutiva das espécies nesse bioma.

Análises da diversidade genética das populações de espécies nativas de vegetais da Mata Atlântica foram publicadas populações para algumas espécies (*Cryptocarya*: Moraes *et al.,* 1999; *Justicia*: Medri *et al.,* 2003; *Eugenia:* Margis *et al.,* 2002 e Salgueiro *et al.,* 2004; *Caesalpinia*: Cardoso *et al.,* 2005, *Araucaria:* Stefenon *et al.,* 2007, *Podocarpus*: Ledru *et al.,* 2007;

Euterpe: Cardoso *et al.,* 2000; *Oncidium:* Alcantara *et al.,* 2006; *Alcantarea*: Babará *et al.,* 2007a e Babará *in press*). Estes estudos mostram que os níveis de estruturação genética entre populações variaram de F_{ST} = 0.04 – 0.211 (para as espécies arbóreas) e de F_{ST} = 0,029 - 0,43 (para as herbáceas) indicando que cada espécie possui diferentes padrões de distribuição da diversidade genética e fluxo gênico.

V) Marcadores Moleculares - microssatélites

Atualmente existem diferentes técnicas de biologia molecular que permitem a detecção da variabilidade existente ao nível do DNA. Os marcadores de microssatélites ou SSR (*simple sequence repeats*) possuem características desejáveis para serem utilizados em estudos de genética de populações (Powel *et al.,* 1996) e são atualmente os mais populares neste tipo de estudo. Estas seqüências são constituídas de DNA repetitivo, dispostas lado a lado, onde pequenos motivos (1 a 6 pares de base) são repetidos *n* vezes. Os microssatélites representam regiões instáveis do genoma, que estão sob alterações mutacionais, geralmente adições ou deleções de um número integral de repetições, com taxa muito mais elevada do que taxas observadas em seqüências de DNA não repetitivo (Jarne e Lagoda, 1996). Estão distribuídos ao longo de seqüências codificantes e não codificantes do DNA (Schlötterer e Tautz, 1992). Estas regiões repetitivas têm sido identificadas nos genomas de procariotos e eucariotos. Em eucariotos são encontrados nos três genomas: núcleo, cloroplasto (Powell *et al.,* 1995) e mitocôndria (Soranzo *et al.,* 1999).

a) Microssatélites nucleares

Os marcadores de microssatélites nucleares são de natureza codominante e possuem herança mendeliana. A grande vantagem na utilização destes marcadores está associada ao fato de, na maioria dos casos, representarem um único loco o qual é freqüentemente multialélico (Pinto, 2001). Assim, estes marcadores moleculares podem ser utilizados para resolver questões de diferentes ordens. Mais especificamente, essa ferramenta pode ajudar a resolver problemas que variam desde questões relacionadas à paternidade, à medicina forense, à estrutura genética de populações e a comparações entre

espécies (McDonald e Potts, 1997). O uso de tais marcadores permite esclarecer, também, questões quanto ao sistema de cruzamento, especialização ecológica e capacidade de colonização de populações (Boneh *et al.,* 2003).

Apesar das análises de regiões de microssatélites terem se tornado uma ferramenta muito útil para uma grande variedade de estudos de variabilidade genética em populações, sua caracterização em novas espécies é ainda um processo caro e trabalhoso. Além disso, os microssatélites nucleares são considerados espécie-específicos, pois os *primer*s que flanqueiam as ilhas de microssatélites exigem alto grau de homologia com as seqüências de DNA para que possam proporcionar a amplificação corretamente (Rossetto, 2002). No entanto, recentes evidências sugerem que muitos marcadores de microssatélites podem funcionar em espécies proximamente relacionadas (Rossetto, 2002; Barbará *et al.,* 2007b). A distribuição das taxas de amplificação heteróloga (amplificação entre espécies) de microssatélites nucleares varia muito entre táxons, sendo maior em animais e altamente variável em plantas. O potencial de sucesso de transferência parece ser maior em espécies com tempo de geração longo, sistemas de cruzamento misto ou alógamo e onde o tamanho do genoma das espécies alvo é menor do que o da espécie para a qual os *loci* foram desenvolvidos (Barbará *et al.,* 2007b).

Em Bromeliaceae os trabalhos realizados até o momento mostram que existe um grande sucesso na amplificação heteróloga de *loci* de microssatélites nucleares mesmo entre espécies pertencentes a diferentes subfamílias (Babará *et al.,* 2007a; Palma-Silva *et al.,* 2007; Cascante-Marín, 2005; Paggi *et al., in press*).

b) Microssatélites plastidiais

As seqüências do genoma de cloroplastos têm sido consideradas como uma ferramenta universal para avaliar a variabilidade dentro e entre populações (Taberlet *et al.,* 1991; Dumolin-Lapegue *et al.,* 1995). Os genomas plastidiais são tipicamente não recombinantes, possuem modo de herança uniparental, e em angiospermas geralmente a herança é maternal, além de serem efetivamente haplóides (Olsmstread e Palmer, 1994).

As regiões de microssatélites de cloroplasto (cpSSR) tem cada vez mais ganhado popularidade em estudos de genética de populações, principalmente em espécies de coníferas (Navascués e Emerson, 2005 e referências). Além disso, *primers* universais para a amplificação destas regiões também têm sido descritos para monocotiledôneas (gramíneas - Provan *et al.,* 2004) e para dicotiledôneas (Weising e Gardner, 1999). Recentemente, estudos utilizando microssatélites de cloroplastos foram publicados para várias espécies de plantas (*Caesalpinia echinata*, Lira *et al.,* 2003; *Caryocar brasiliense*, Collevati *et al.,* 2003; *Anacampis palustris,* Cozzolino *et al.,* 2003; *Justicia areysiana,* Meister *et al.,* 2005; *Vitis vinifera*, Grassi *et al.,* 2006)

Objetivo Geral

A presente tese está inserida em um projeto amplo que visa contribuir para os estudos genéticos e biológicos de famílias de plantas Neotropicais, com ênfase na família Bromeliaceae. Devido à falta de informações sobre a diversidade genética de espécies Neotropicais, incluindo *V. gigantea,* esta tese tem como objetivo geral investigar aspectos da genética das populações, fluxo gênico, padrões filogeográficos e aspectos da fertilidade desta espécie. O conhecimento destes itens será de grande importância para o desenvolvimento de estratégias para a conservação e manejo das populações de *Vriesea gigantea* e de espécies relacionadas.

Capítulo 2

A set of polymorphic microsatellite loci for *Vriesea gigantea* and *Alcantarea imperialis* (Bromeliaceae) and cross-amplification in other bromeliad species

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PRIMER NOTE

A set of polymorphic microsatellite loci for *Vriesea gigantea* and *Alcantarea imperialis* (Bromeliaceae) and cross-amplification in other bromeliad species

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Abstract

Fifteen polymorphic microsatellite markers were isolated and characterized in two species of Bromeliaceae: *Vriesea gigantea* and *Alcantarea imperialis*. The number of alleles observed for each locus ranged from three to 16. The loci will be used for studies of the genetic structure of natural populations, reproductive biology, and evolutionary relationships among and within these genera. A cross-amplification test in 22 taxa suggests that the markers will be useful for similar applications in numerous other bromeliad species.

Keywords: *Alcantarea*, Atlantic Rain Forest, Bromeliaceae, cross-amplification, microsatellites, *Vriesea*

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Vriesea gigantea (Gaud.) and *Alcantarea imperialis* (Carriere) Harms are bromeliad species endemic to the Brazilian Atlantic Rain Forest. *V. gigantea* is epiphytic, saxicolous, and terrestrial, while *A. imperialis* is adapted to rock outcrops (inselbergs) (Benzing 2000). Both species are appreciated as ornamental plants throughout the world and native populations have been reduced by intense illegal collection practices. Both species are also threatened by habitat loss and fragmentation. Knowledge of patterns of diversity and gene flow is essential for defining conservation management decisions, and for understanding the genetic consequences of variation in reproductive systems or pollination syndromes. The aim of this study was to develop a set of polymorphic microsatellite (simple sequence repeat; SSR) markers for the closely related genera *Vriesea* and *Alcantarea* for addressing these topics.

The SSRs were isolated and characterized using two different approaches. In both cases, total genomic DNA was extracted from fresh leaves following the protocol of Doyle & Doyle (1990). In *V. gigantea*, marker isolation involved construction of a genomic library enriched for $(CT)_n$ and $(GT)_n$ repeats. The methodology was based on biotinylated oligonucleotide sequences bound to Streptavidin-coated magnetic particles as described by Kijas *et al.* (1994) with modifications by Billote *et al.* (1999). Microsatellite-enriched DNA fragments were ligated into pGEM-T Easy vector (Promega) as described by the supplier and used to transform DH5a competent cells. A total of 192 recombinant colonies were obtained and sequenced using the BigDye terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems) and a 3100 DNA Analyser (Applied Biosystems). The presence of SSRs was analysed using the WEBTROLL software (http://wsmartins.net/webtroll/troll.html). For 43 clones containing a SSR motif, forward and reverse sequences were aligned in SEQUENCHER version 4.1.2 (Gene Codes), and primers were designed for 15 loci using the PRIMER 3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

Marker isolation from *A. imperialis* was based on the 5′-anchored polymerase chain reaction method of Fisher *et al.* (1996). Total genomic DNA of *A. imperialis* was amplified using degenerated primers PCT3, PCT4, PCT5, and PCT6 (Brachet *et al.* 1999). PCR products were separated on 2% agarose gels, purified using a QIAquick PCR Purification Kit (QIAGEN), ligated into pGEM-T vector (Promega) and cloned in JM109 competent cells (Stratagene). Insert-containing plasmids were sequenced as described above.

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Table 1 Primer sequences and characteristics for microsatellite loci from *Vriesea gigantea* (Vg) and *Alcantarea imperialis* (Ai), including locus name, primer sequences, repeat type, no. of alleles, allele size range, observed (H_O) and expected (H_E) heterozygosity. GenBank Accession nos EF076654–EF076668

Locus	Primer sequences	Repeat	No of alleles	Size range (bp)	H_O	H_E
VgA04	F: CAAACCCTTCTCACCTCACC* R: CGACTCACCTGGCCCTAAT	$(TC)_6(CT)_{11}(TC)_{16}$	11	187–215	0.625	0.785
VgA06	F: GGGAAAAGCTCGATAAGGTG* R: GCTACGGCTCAACATTCCAT	$(CT)_{10}$	11	223–253	0.600	0.567
VgB01	F: AATGAAGCCGATTGTCGAAT* R: CATACACCGCAAAATCTGA	$(CT)_8(AT)_6$	4	156–162	0.394	0.580
VgB06	F: CCCTATCACTTGCCACCCTA* R: TGGGCAGAACAGTAGTTGTGA	$(TC)_{13}(AACCT)_2(CT)_9$	12	201–223	0.938	0.840
VgB10	F: CGGGGGTGTATAGGTAGCAA* R: GCCGTTTTGAAAGCTCCAT	$(AG)_{24}$	12	119–156	0.613	0.673
VgC01	F: GCTAGGGTTTCACCCCAAAT* R: TCAGCCTCTGATCCATCTCC	$(CT)_{16}$	6	208–218	0.647	0.544
VgF01	F: CATGCTCTCCTCCTTCTTGG* R: TTGGGTTCATTCCTGAAAGG	$(CT)_{17}$	13	154–199	0.765	0.787
VgF02	F: TTACACCTGCCTACCCCTTG* R: TTGGAGATCAAAACCCGAAG	$(CT)_{12}(CT)_{17}$	12	176–204	0.700	0.727
VgG02	F: GAAGGCCCATCTTTGTTTGA* R: AATGCCAAAGTCGGTGACTC	$(CT)_{14}$	16	241–259	0.647	0.758
VgG03	F: GCAAGGCAATTTCTTGTGCT* R: AGGCGCAACTTACAGGAAAA	$(AT)_6(GT)_8$	3	209–213	0.257	0.227
VgG05	F: TGATCAGCTCGTCTGCAGTT* R: CGGGAAGTATGCAGGTGACT	$(AG)_9$	10	152–204	0.636	0.711
Ai5.18	F: CACCATCTCAGTTAAAGGCATTC* R: TGCTCCTTAAATCCAGCTAAATG	$(CT)_{10}(CT)_7$	5	222–257	0.281	0.335
Ai4.11	F: GAGGAATCACCGAATCCTGA* R: TTGAGCGGCTCTCTCTCT	$(GTTTGA)_2(AG)_5(AG)_{13}$	5	290–320	0.783	0.707
Ai4.10	F: CCCCTCGATATATTGATCTACACT* R: TAAACAGAAGCAGGGGGAAA	$(AT)_7(CATG)_5$	3	184–188	0.105	0.102
Ai4.03	F: TGGCTTGTGGAGTTCTCACT* R: AACAAGGAGGTTGATCAAGAGG	$(AT)_5(GA)_5(TC)_4(TA)_5$	3	191–195	0.267	0.343

*Asterisks mark primers that were M13-tailed at the 5′ end. None of the loci departed significantly from HWE at the 0.05 level.

From a total of 86 microsatellite-containing sequences, primers were designed for 20 loci using the PRIMER 3 program.

For each SSR, the forward primer was synthesized with a 19 base-pair (bp) M13 tail (5′-CACGACGTTGTAAAACGAC-3′) following the method of Schuelke (2000), which involved three primers: a forward SSR-specific primer with the M13 tail at its 5′ end, a reverse locus-specific primer, and a universal M13 primer labelled with one of the two fluorescent dyes, FAM or JOE (Applied Biosystems). All PCR amplifications were performed in a PE Applied Biosystems 9700 thermocycler in 10 μL reactions containing: 10 ng DNA template, 1X Bioline *Taq* buffer, 2 mM Bioline $MgCl_2$, 100 μM dNTPs, 5 pmol forward primer, 10 pmol reverse primer, 1 pmol universal M13 primer and 0.5 U *Taq* polymerase (Bioline). A 'touchdown' cycling program was used: 95 °C for 3 min, then 10 cycles of 94 °C for 30 s, 58 °C decreasing to 48 °C at 1 °C per cycle for 30 s, 72 °C for 30 s followed by 30 cycles of 94 °C for 30 s, 48 °C for 30 s, 72 °C for 30 s, followed by a final extension of 10 min at 72 °C. For three SSR loci (VgA04, VgB01 and Ai4.03), a standard cycling program was used in order to reduce the number of stutter bands: 95 °C for 3 min, 40 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s and a final elongation step at 72 °C for 10 min. Microsatellite alleles were resolved on a 3100 DNA Analyser (Applied Biosystems) and were precisely sized against ROX molecular size standard using GENESCAN 3.7 and GENOTYPER 3.7 software (Applied Biosystems).

A total of 80 individuals from seven populations of *V. gigantea* and 30 individuals from four populations of *A. imperialis* were used to evaluate SSR polymorphism. Observed (H_O) and expected (H_E) heterozygosities and tests for departure from Hardy–Weinberg equilibrium (HWE) were reported either for 37 individuals from one population of *V. gigantea* — Florianopolis (SC), Brazil or for 20 individuals from one population of *A. imperialis* — Araras (RJ), Brazil.

Eleven SSRs from *V. gigantea* and four SSRs from *A. imperialis* were polymorphic, with number of alleles per locus ranging from three to 16 with an average of 8.4 and no more than two bands per individual (Table 1). Hardy–Weinberg

Table 2 Cross-amplification of 13 microsatellite markers isolated from *Vriesea gigantea* and *Alcantarea imperialis* across all three subfamilies of Bromeliaceae

Species (sample size)	Subfamily	VgA04	VgA06	VgB01	VgB06	VgB10	VgC01	VgF01	VgF02	VgG02	VgG03	VgG05	Ai4.10	Ai4.03
V. gigantea (2)	Tillandsioideae	+	+	+	+	+	+	+	+	+	+	+	+	+
Vriesea platynema (1)	Tillandsioideae	–	–	–	+	–	+	–	–	–	–	+	*	*
Vriesea psittacina (1)	Tillandsioideae	+	+	+	+	+	+	+	+	+	+	+	*	*
Vriesea splendens (1)	Tillandsioideae	W	–	W	+	+	+	–	+	–	W	+	*	*
Vriesea phillippocoburgii (1)	Tillandsioideae	+	+	+	–	+	–	W	+	+	+	–	*	*
Alcantarea imperialis (2)	Tillandsioideae	+	W	–	+	+	+	W	+	+	+	+	+	+
Alcantarea geniculata (2)	Tillandsioideae	+	–	–	+	+	+	W	+	–	+	+	+	+
Alcantarea regina (2)	Tillandsioideae	+	–	–	+	+	+	W	+	W	W	+	+	+
Alcantarea glaziouana (2)	Tillandsioideae	+	–	–	+	+	+	W	+	W	+	+	+	+
Tillandsia albida (1)	Tillandsioideae	+	–	–	–	–	+	–	+	W	–	+	*	*
Catopsis morreniana (2)	Tillandsioideae	W	–	–	–	–	+	–	+	–	–	W	–	+
Portea platysepala (2)	Bromelioideae	+	–	–	–	–	+	–	+	–	–	W	–	+
Aechmea blanchetiana (2)	Bromelioideae	+	–	–	–	–	+	–	+	–	–	–	–	+
Edmundoa lindenii (1)	Bromelioideae	+	–	–	–	–	+	–	+	–	–	–	*	*
Hohenbergia eriostachya (1)	Bromelioideae	+	–	–	–	–	+	–	+	–	–	–	*	*
Orthophytum maracaensis (1)	Bromelioideae	–	–	–	–	–	+	–	–	–	–	–	*	*
Orthophytum disjunctum (1)	Bromelioideae	+	–	–	–	–	+	–	+	–	–	–	*	*
Ananas frizmuelleri (1)	Bromelioideae	+	–	–	–	–	+	–	+	–	–	–	*	*
Bromelia antiacantha (1)	Bromelioideae	+	–	–	–	–	+	–	+	–	–	–	*	*
Cryptantus microglazioui (1)	Bromelioideae	+	–	–	–	–	+	–	+	–	–	+	–	–
Fosterella penduliflora (2)	Pitcairnioideae	W	–	–	+	–	+	–	+	+	–	–	–	+
Pitcairnia atrorubens (2)	Pitcairnioideae	+	–	–	–	–	+	–	–	–	–	–	–	+

+, successful amplification with single band visualized; ++, successful amplification with more than one band visualized; W, weak amplification; –, unsuccessful amplification; *, not tested.

equilibrium (HWE) and linkage disequilibrium tests in GENEPOP (Raymond & Rousset 1995; web version 3.4) indicated no significant departure for any of the loci. The observed heterozygosity for the polymorphic loci ranged between 0.11 and 0.94 with an average of 0.55.

Thirteen of the markers were tested for cross-amplification in individuals of 22 species belonging to all three subfamilies of Bromeliaceae: Tillandsioideae (11 species), Bromelioideae (nine species), and Pitcairnioideae (two species) (Table 2). As expected, the markers were most effectively transferred to species belonging to the same subfamily (Tillandsioideae). Four primers pairs (VgA04, VgC01, VgF02, and Ai4.03) were successfully amplified among almost all species from all three subfamilies, suggesting that these markers will be useful for population genetic studies of many species in the Bromeliaceae. These markers may also help resolve phylogenetic relationships wherever sequence data are not of sufficient resolution. The SSRs presented here will be invaluable tools for conservation genetics surveys in many bromeliad taxa, e.g. for assessing the effect of landscape fragmentation on historical and contemporary gene flow in *V. gigantea* and *A. imperialis* in the Brazilian Atlantic Rain Forest.

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Capítulo 3

Range-wide patterns of genetic diversity in a Neotropical forest species, *Vriesea gigantea* (Bromeliaceae)

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Abstract

The processes that have shaped the extraordinary species diversity in Neotropical rainforests are poorly understood, and knowledge about patterns of genetic diversity across species' ranges is scarce in contrast to other regions of the globe. We have conducted a range-wide study of nuclear genetic diversity in a herbaceous plant endemic to the Brasilian Atlantic Rainforest, *Vriesea gigantea* (Bromeliaceae), based on ten nuclear microsatellites typed in 429 plants from 13 populations. The results indicate a latitudinal trend of decreasing diversity from North to South away from the equator, consistent with historical range expansion from the Northern half of the present distribution range. The center of genetic diversity for *V. gigantea* (São Paulo and Rio de Janeiro) coincides with centers of species diversity and endemism for most animals and plants of the Atlantic Rainforest. A significant correlation between genetic diversity in *V. gigantea* and species diversity in the genus *Vriesea* suggests that both were shaped by the same historical forces: historical climatic changes of the Pleistocene. Distance-based genetic analysis revealed three geographically defined gene pools, and a Bayesian analysis revealed two additional genetic clusters of conservation relevance.

Introduction

Understanding the evolution of species' ranges and the limits to evolution at range margins are topics of great current interest in ecology, evolution, and conservation biology (Kirkpatrick & Barton 1997; Lenormand 2002; Alleaume-Benharira et al. 2006; Bridle & Vines 2006). Although a full understanding of these processes in natural populations requires data on the fitness effects of heritable phenotypic traits, some important questions can indeed be addressed with the molecular genetic data typically available to molecular ecologists. These include questions regarding the potential positive and negative effects of gene flow on range expansion and local adaptation of marginal populations (Kirkpatrick & Barton 1997; Lenormand 2002; Morjan & Rieseberg 2004). They also include questions regarding the roles of drift and selection in different types of range margins, e.g. 'environmental' margins in which abiotic factors limit species distributions, or 'parapatric' margins in which biotic factors and competition are more important (review by Bridle & Vines 2006). These

questions are very rarely addressed for species that occur outside the well studied regions of the globe, such as Europe or North America.

South America represents the most biodiversity-rich subcontinent on earth, and five of the world's biodiversity 'hotspots' are located here (Myers et al. 2000). Little is known about patterns of genetic variability and gene flow in the Neotropics compared to temperate regions of the world (Hewitt 2004). Biogeographic studies of groups of taxa from Amazonia (Aleixo 2004, Ribas et al. 2005), the Andes (Pennington et al. 2004, Yoke et al. 2006, Speranza 2007) and the Brazilian Atlantic Rainforest (Silva et al 2004) point out the evolutionary complexity of the quaternary history of South America, with a prominent role for range contractions and expansions due to Pleistocene climatic fluctuations. Studies addressing the evolutionary history of Brazilian Atlantic Rainforest taxa from a phylogeographic perspective are extremely rare and include few studies of animals (Grazziotin et al. 2006; Tchaicka et al. 2007, Cabanne et al. 2007) and plants (e.g. Lira et al. 2003; Andrade et al. 2007). Clearly, range-wide genetic studies are needed to allow comparisons of historical patterns among plants and animals in South America. This type of information would provide a basis for molecular ecologists interested in the evolution of species ranges and mechanisms of speciation in Neotropical faunas and floras.

Although species diversity and genetic diversity remain the domains of community ecology and population genetics, respectively, the two levels of biodiversity have long been recognized as being related phenomena (Antonovics 1976). Many population genetic models can be applied with equal validity to the species composition of a community and to the genetic composition of a population (Vellend & Geber 2005). Despite these striking parallels, species diversity and genetic diversity are rarely studied simultaneously (Moritz & Faith 1998, Vellend 2005; Vellend & Geber 2005, Johnson & Stinchcombe 2007). This is understandable, as molecular ecologists often have to focus on a small number of tractable study systems. Nevertheless, in highly species-rich regions of the world such as the Atlantic Forest 'hotspot' of Brazil, focus of the present study, the interconnection between genetic and species diversity can not be ignored. This is particularly relevant for recent adaptive radiations including large numbers of related species with parapatric distributions, such as the Bromeliaceae family (Benzing 2000).

Bromeliaceae (bromeliads) are a large and well described adaptive radiation in the Neotropics including approximately 3000 species (Benzing 2000). Systematic relationships in the family are relatively well understood compared to most other Neotropical plant families, and bromeliads are increasingly being studied by population geneticists interested in microevolution and speciation in the Neotropics (e.g. Soltis et al. 1987; Murawski & Hamrick 1990; Barbará et al. 2007). Bromeliads also display striking variation in breeding systems (from predominant inbreeding to predominant outcrossing), growth habits (epiphytic, terrestrial, or saxicolous), and distributions (continuous forest species vs. patchily distributed, rock-adapted 'inselberg' species). Their generation times are relatively short compared to rain forest trees, and thus the genetic effects of landscape fragmentation will become apparent more quickly. This can include both, natural fragmentation in marginal populations, or more recent human-mediated fragmentation, which is a great concern for conservation of Neotropical rainforests.

Vriesea gigantea Gaud. is a perennial, diploid (2n = 50 chromosomes, Palma-Silva 2003), bromeliad species endemic to the Brazilian Atlantic Rainforest. It is non-autogamous and has a mixed mating-system (Paggi 2006). Its growth habit is epiphytic, saxicolous, or terrestrial. Its distribution range spans ca. 1600 km between the Brazilian states of Rio Grande do Sul and Espírito Santo (Fig. 1; Reitz 1983). The population from Espirito Santo was described as a distinct form, *V. gigantea* var. *seideliana*, due to morphological differences compared to populations from the rest of the species range (Roth 1992). The flowers of *V. gigantea* are pollinated by bats (Sazima et al. 1999) and its small seeds are dispersed by wind. Populations are generally large and composed of patches of different sizes and densities. *Vriesea gigantea* plays an important role in its ecosystem, as its vegetative structures form tanks that are able to hold many litres of water (Benzing 2000).

Here, recently developed nuclear microsatellite markers (Boneh et al. 2003, Palma-Silva et al. 2007) were used to conduct a range-wide study of genetic variability and gene flow in this Neotropical rainforest species to address the following questions: (i) What can present-day patterns of genetic variability, differentiation and gene flow tell us about historical range expansion in this Neotropical rainforest species? (ii) What are the likely evolutionary consequences of population divergence and gene flow in the range margins?

(iii) To what extent are patterns of genetic diversity of this Brazilian Atlantic Rainforest endemic congruent with patterns of species diversity for its genus across the same geographic area? (iv) What are the practical implications for *in situ* conservation of this ecologically and horticulturally important species?

Material and Methods

Population sampling and DNA extraction

A total of 429 individuals of *Vriesea gigantea* were sampled in 13 locations distributed across the Brazilian Atlantic Rainforest (Table 1; Fig 1). *V. gigantea* do not spread *via* cloning reproduction, each plant represent a different genotype, still individuals were collected within 5 meters distance to avoid sampling close relatives. In addition, sampling was performed in such a way as to represent the complete geographical range of the species. Geographical distances between sampled populations ranged from 34 km (FLSC-VBSC) to ca.1600 km (TMRS-SLES). Fresh leaves (~5 cm^2) were collected and stored in liquid nitrogen or in CTAB - cetyltrimethyl ammonium bromide buffer - until DNA extraction. Total genomic DNA was extracted using the protocol described by Doyle & Doyle (1990).

Microsatellite analysis

Ten microsatellite loci characterized previously for the bromeliad genera *Tillandsia* and *Guzmania* (Boneh et al. 2003 – loci: e6b, e19, CT5) and for the species *Alcantarea imperialis* and *V. gigantea* (Palma-Silva et al. 2007 – loci: Ai4.3, Ai4.10, Ai4.11, Ai5.18, VgA06, VgF01, and VgG02) were selected for this study. The forward primer of each locus was labeled at the 5´ end with a fluorescent dye (6-FAM or JOE). All PCR amplifications were performed in a PE Applied Biosystems 9700 thermocycler as described by Palma-Silva et al. (2007). For seven SSR loci (CT5, e6b, e19, VgA06, VgF01, VgG02, and Ai4.10) a 'touchdown' cycling program was used: 95ºC for 3min, then 10 cycles of 94ºC for 30s, 58ºC decreasing to 48ºC at 1ºC per cycle for 30s, 72ºC for 30s followed by 30 cycles of 94ºC for 30s, 48ºC for 30s, 72ºC for 30s, followed by a final extension of 10min at 72ºC. For the remaining three SSR loci (Ai4.3, Ai4.11 and Ai5.18) a standard cycling program was used: 95ºC for 3min, 40 cycles of 94ºC for 30s, 57ºC for 30s, 72ºC for 30s and a final elongation step at 72ºC for 10min. Microsatellite alleles were resolved on an ABI PRISM® 3100 DNA

Analyzer (Applied Biosystems) making use of the different fluorescent dyes for duplexing. Molecular sizes in base pairs were precisely sized against ROX molecular size standard (Applied Biosystems) using GENESCAN 3.7 and GENOTYPER 3.7 software (Applied Biosystems).

Statistical analysis

Genetic diversity. Levels of genetic diversity within populations were described by calculating the following statistics: (1) Rs - allelic richness (El Mousadik & Petit 1996), which is a measurement of the number of alleles per locus standardized for sample size by rarefaction; (2) Var - variance in allele size; (3) H_O - observed heterozygosity; and (4) H_E - expected heterozygosity under Hardy-Weinberg equilibrium (HWE) (Nei 1978). All genetic diversity parameters were measured with the program MICROSATELLITE ANALYSER (MSA) (Dieringer & Schlötterer 2003) and FSTAT 1.2 (Goudet 1995). GENEPOP on the Web, version 3.5 (Raymond & Rousset 1995), was used to estimate the inbreeding coefficient F_{IS} (Weir & Cockerham 1984). Because microsatellite loci are highly multiallelic, departures from HWE were tested with Fisher's exact test, to protect against type I error as a result of low frequencies of some alleles (Weir 1996) in the program TFPGA (Miller 1997). These results were adjusted for multiple tests using the sequential Bonferroni procedure with $\alpha = 0.05$ (Rice 1989).

The existence of a latitudinal trend in diversity was tested with regression analyses of mean population allelic richness, variance in allele size, and gene diversity on latitude. The marginal populations (TMRS and SLES) were excluded from the analyses (see Results).

Possible correlation between patterns of genetic diversity (H_E) across the species range of *V. gigantea* and patterns of species diversity for the genus *Vriesea* in the Brazilian coastal Atlantic Rainforest (Smith 1962) was tested using a Mantel test (Sokal and Rohlf 1995) and associated randomization procedure available in FSTAT 1.2 (Goudet 1995). In order to be able to compare genetic and species diversity, populations were arranged by Brazilian states (RS, SC, PR, SP, RJ, and ES Table 1). Since population ES in the correlation analysis (corresponding the population SLES in Table 1) has previously been described as *V. gigantea* var. *seideliana* (Roth, 1992), the

correlation between species diversity and genetic diversity was performed with and without this population.

Bottleneck tests. The possibility of founder effects due to recent colonization (genetic bottleneck) was tested using the 'sign test' and 'Wilcoxon sign-rank test' in the software BOTTLENECK 1.2.2 (Piry et al. 1999). These tests are able to detect recent and pronounced reduction in effective population size due to genetic bottlenecks. Populations that have experienced a recent reduction of their effective population size exhibit a reduction of allele numbers and heterozygosities at polymorphic loci, but allelic diversity is reduced faster than heterozygosity (Piry et al. 1999). The analyses were carried out using the 'Two-phased mutation model' (TPM) as recommended for microsatellite data by the user manual. A total of 5000 simulation iterations were conducted, as suggested by Piry et al. (1999).

Isolation-by-distance. The hypothesis that populations are differentiated due to isolation by distance (Wright 1965) was tested by calculating the correlation between geographic and genetic distance matrices with a standardized Mantel test (Sokal & Rohlf 1995) using FSTAT 1.2 (Goudet 1995). Significance was assessed through a randomization test using 10 000 Monte Carlo simulations.

Genetic structure within and among populations. F statistics were calculated in order to quantify levels of genetic diversity within and among populations and to infer the degree of population subdivision. They were computed according to Weir and Cockerham (1984) by using the software MSA (Dieringer & Schlötterer 2003). The significance of each F statistics was tested by resampling with 10 000 permutations.

The possible presence of phylogeographic structure was tested by comparing R_{ST} to F_{ST} values using 10 000 random permutations (Hardy et al. 2003) using the SPAGeDi program (Hardy & Vekemans 2002). The test is based on the fact that R_{ST} is based on differences in microsatellite allele size and thus makes use of 'ordered' alleles, whereas F_{ST} does not. A phylogeographic pattern is said to be present when alleles are more closely related between nearby populations than between distant populations, which is captured by the difference between R_{ST} and F_{ST}. If $R_{ST} > F_{ST}$, then the null

hypothesis of no phylogeographic structure can be rejected. Assuming neutrality, such a pattern is expected when the mutation rate is non negligible compared to the migration rate (Hardy & Vekemans 2002, Hardy et al. 2003).

An unrooted phenogram of Nei´s unbiased genetic distances was constructed using the neighbor-joining method (Saitou & Nei 1987) as implemented in PHYLIP 3.6 (Felsenstein 2004). Analysis of Molecular Variance (AMOVA) was used to partition genetic variation at different hierarchical levels and to test groupings revealed by the neighbor-joining tree. The AMOVA analysis was performed with the program ARLEQUIN 2.0 (Excoffier et al. 2005) and significance was tested using 10 000 permutations.

Effective population sizes and migration rates. Effective population sizes (N_e) and pairwise migration rates (N_em) were estimated following a coalescent theory and maximum-likelihood-based approach using Migrate 2.0 (Beerli & Felsenstein 1999; Beerli 2004). This software was used to estimate Theta ($4N_e\mu$) for each population and bidirectional migration coefficients (N_em) between neighbouring pairs of populations. Genetic divergence between pairs of populations (F_{ST}) was used to obtain initial start values for the estimation of Theta and N_em. Effective population sizes were estimated from Theta values by assuming a microsatellite mutation rate of 10^{-3} per gamete per generation (Zhang & Hewitt 2003).

Bayesian structure analysis. The Bayesian clustering method implemented in the program STRUCTURE 2.0 (Pritchard et al. 2000) was used to probabilistically assign individuals to genetic clusters (K) and estimate admixture proportions (Q) for each individual. The proportion of membership for each cluster was calculated without consideration of sampling localities. To determine the most likely number of clusters (K) present in the data, we conditioned our data on various values of K ranging from 1 to 13. The analyses were carried out under the admixture model assuming independent allele frequencies and using a burn-in period of 50.000, run lengths of 300.000, and 10 iterations per K to confirm stabilization of the summary statistics (Pritchard et al. 2000).

Results

Variability across loci

All ten microsatellite loci were polymorphic in *V. gigantea*, with an average of 16 alleles and ranging from three (Ai4.10) to 25 (VgF01) alleles per locus. Observed and expected heterozygosity of the loci, calculated over all populations, ranged from 0.223 to 0.547 and from 0.380 to 0.747, respectively (Table 2). All loci departed significantly from Hardy-Weinberg equilibrium (HWE) within populations (Table 3). The distribution of rare alleles tended to be geographically structured. Forty alleles out of 160 were exclusive to a single population (=private alleles). Most private alleles were found in populations JASP, BESP, PARJ, RIRJ, and SLES. Populations JASP and SLES, from the centre and the northern edge of the species range, respectively, displayed the highest number of private alleles (Table 3).

Patterns of genetic variability across populations

Populations of *V. gigantea* exhibited high levels of genetic diversity (Table 3). Allelic richness (Rs) averaged over loci was 2.83 and ranged from 1.18 to 3.49, whereas the variance in allele size (Var) ranged from 1.20 to 82.71 with an average of 31.85. Observed and expected heterozygosity per population varied from 0.039 to 0.614 and 0.065 to 0.727, respectively. The inbreeding coefficients (F_{IS}) were high and significant in all populations, except for populations FLSC and SLES in which deviations from Hardy-Weinberg proportions were not significant.

A marked decrease in diversity was observed for the two marginal populations TMRS and SLES (Table 3; Fig. 2), and several loci were even fixed for a single allele in these populations (population TMRS: loci Ai.4.3, Ai.4.11, Ai.5.18, Vg.A04, VgF01, VgG02), population SLES: loci CT5, Ai4.10, Ai.4.11). Except for locus Ai4.11, different loci were fixed in opposite margins of the species range. The distribution of genetic diversity followed a strong latitudinal correlation (Fig 2 and Fig 3). The centre of genetic diversity for *V. gigantea,* indicated by populations JASP, BESP, PTRJ, and RIRJ, visibly decreased towards the South (Figs. 1 and 2). Evidence for genetic bottlenecks (significant heterozygosity excess compared to equilibrium heterozygosity $H_E > H_{EQ}$) was found in four populations in the Southern half of the distribution range: TMRS, GASC, VBSC, and MBSC.

Patterns of genetic diversity (H_E) observed in populations of *V. gigantea* were significantly correlated (Mantel's r = 0.51, p < 0.005) with patterns of species diversity of the genus *Vriesea* reported by Smith (1962) when the population from the northern margin of the distribution (population SLES), previously described as a distinct variety or form, was excluded from the analysis. The correlation was not significant (Mantel's r = 0.29, n.s.) when this population was included.

Patterns of population divergence and gene flow

Significant differentiation among populations was indicated by both F_{ST} and R_{ST}, with values of 0.21 and 0.35, respectively. R_{ST} was larger than F_{ST} ($p<0.0001$), thus indicating significant phylogeographic structure. Further, a significant Mantel correlation was observed between genetic (F_{ST}) and geographical distance ($r = 0.49$, $p<0.001$), suggesting the presence of isolation-by-distance (Fig 4). This model assumes that gene flow occurs locally among neighboring populations in continually distributed populations (Wright 1943). Likewise, the neighbor-joining phenogram of 13 populations based on Nei´s unbiased genetic distances (1978) revealed a distinct geographic structure among populations. At least three clusters can be recognized, supported by high bootstrap values (62 – 82%) (Fig 5). Populations tended to cluster according to their geographical origin: South (TMRS, ITRS, MARS), Center (GASC, FLSC, VBSC, MBSC, PAPR, JASP, BESP), and North (PTRJ, RIRJ, SLES), with the marginal populations TMRS and SLES showing the longest branches.

Hierarchical AMOVA including these three geographic partitions (South, Center, and North) detected significant differentiation at all levels ($P < 0.001$), with 13% of differentiation among regions, 12% among populations, and the largest amount of variation (75%) residing within populations (Table 4). Maximum likelihood estimates of gene flow (N_em) between neighboring populations ranged from a minimum of 0.038 (from ITRS to TMRS – c. 222 km of distance) to a maximum of 6.773 (from JASP to PAPR – c. 96km of distance) with a mean value of 1.668 migrants per generation (Table 5), in agreement with global F-statistics. Bayesian analysis with structure consistently identified K = 5 clusters: TMRS, South (ITRS, and MARS), Center (GASC, FLSC, VBSC,

MBSC, PAPR, JASP, and BESP), North (PTRJ and RIRJ), and SLES (Fig 6) which is in agreement with the neighbor-joining tree. The admixture proportions (Q) for each individual plant are shown in Fig 6. The clustering pattern confirmed geographic patterns across the species range with restricted gene flow among clusters.

Discussion

Patterns of diversity and gene flow in Vriesea gigantea across its geographic range

To our knowledge, not many studies address range-wide patterns of variability and gene flow in herbaceous plants from the Brazilian Atlantic Rainforest. In addition, few phylogeographic studies are available for plants from this part of the world. Two studies on trees (Brazil Wood*, Caesalpinia echinata*, Lira et al. 2003; *Podocarpus*, Ledru et al. 2007) and two others on herbaceous plants (*Passiflora,* Lorenz-Lemke et al., 2005; *Monstera adansonii*; Andrade et al. 2007) provide a first glimpse of the effects of historical forest expansions and contractions on present-day patterns of intraspecific variation. Nevertheless, none of these studies fully interpreted contrasts between the centers and the margins of species' ranges. Our range-wide study in the Neotropical rainforest species *V. gigantea* revealed a latitudinal geographic trend and a strong correlation with a decrease in diversity, measured as gene diversity, allelic richness, or variance in allele size, from North to South across the species' range (Fig. 2 and 3; Table 3). Superimposed on this pattern is a marked decrease in diversity and greatly reduced gene flow in both the Northern and the Southern range margins (Fig. 2; Fig. 5; Table 3; Table 5), to be discussed further below in a separate section. The overall decrease in diversity from North to South can be interpreted in the light of available knowledge on the biogeography and history of Eastern Brazil.

The biogeographic patterns in plants of the Brazilian Atlantic Rainforest were studied by Rambo (1950, 1960) and Smith (1962), who revealed a migration route for many species from the North towards the South of Brazil. The region where the two Southern Brazilian states of Santa Catarina and Rio Grande do Sul meet demarks the southernmost limit of the Atlantic Rainforest biome. The flora there is a continuation of that centering on the coastal Rainforest of Rio de Janeiro, which represents a center of diversification in

Bromeliaceae. From there, the number of bromeliad species decreases steadily towards the South. The genus *Vriesea*, for instance, reaches the Southern limit of its distribution in Rio Grande do Sul, the population of *V. gigantea* sampled in Taim Ecological Station (TMRS - 31°56' S, 52°25' W; Table 1) being the southern-most population of any species in the genus.

During the quaternary, climatic oscillations and great associated changes in vegetation patterns affected the distributions of species in the Neotropics (Rull 2006). In addition, sea level fluctuations during the Holocene have played a role in shaping the vegetation along the Southern coast of Brazil (De Oliveira et al. 2005). During the late Pleistocene, the climate was markedly drier and 5°-7°C cooler in Southern and Southeastern Brazil, and during the last glacial maximum (c. 20.000 years ago) the tropical Atlantic Rainforest in Southern Brazil was replaced by subtropical forest or grassland (Behling 2002). The Atlantic Rainforest in Southeastern Brazil probably shrunk to a narrow belt in coastal lowlands. Subsequently, the increased precipitation and temperature during the late-glacial period caused expansion of the tropical rainforest towards Southern Brazil.

The change to wetter climatic conditions started around 6000-5000 yr B.P. in Southeastern Brazil and even later, around 3000 yr B.P., in Southern Brazil (Behling 1998; Behling 2002). It is likely that present-day patterns of genetic diversity in *V. gigantea* were shaped by these late Pleistocene climatic fluctuations. This conclusion is supported by (i) the fact that genetic diversity was highest in the Northern portion of the species range, i.e. populations JASP, BESP, PTRJ and RIRJ, located in the São Paulo/Rio de Janeiro region from where the southward migration would have been initiated according to accepted biogeographic scenarios of forest expansion (Rambo 1951, Rambo 1960, Smith 1962, Behling 2002, Rull 2006, de Oliviera et al. 2005) (Fig. 2; Table 3) (ii) the detection of genetic bottlenecks in several populations from the Southern portion of the species range, indicative of recent founder events due to colonization. Although there are limits to phylogeographic inference based on microsatellite loci due to the high mutation rates typical for these genetic markers (Goldstein & Schlötterer 2000), the significant difference between population differentiation with ordered (R_{ST}) vs. unordered alleles (F_{ST}) observed in our study suggests that our data are informative with respect to phylogeographic structure (Hardy et al. 2003). More slowly evolving plastid DNA

markers would provide a more complete account of the phylogeographic history of this Neotropical plant species and the rainforest community that supports it, and a range-wide survey of plastid DNA diversity in *V. gigantea* for this purpose is currently in progress (Palma-Silva et al., unpublished data).

Patterns of gene flow in the centre and the margins of the species range

Bridle & Vines (2006) outline two different ways in which gene flow, or the lack of it, may affect microevolution in range margins. On one hand, if populations in the margin remain connected to well adapted populations in the centre of a species' range, then expansion may be prevented because marginal populations are 'swamped' by gene flow from the centre (Lenormand 2002; Kirkpatrick & Barton 1997). On the other hand, increased drift resulting from reduced gene flow between marginal populations combined with reduced density (Allee effect) and reduced mutational input in marginal populations, may prevent adaptation at range margins and thus limit further range expansion (Courchamp et al. 1999; Bridle & Vines 2007). The results of the present study indicate that the latter of these two scenarios is more likely in the case of the Neotropical rainforest species *V. gigantea*.

The two peripheral populations (TMRS – southern margin, SLES – northern margin; Fig 1.) displayed genetic features consistent with the second scenario: (i) decreased genetic diversity (Table 3), (ii) increased differentiation (neighbor-joining and Bayesian analyses, Figs 4 and 5, respectively), (iii) smaller effective population size N_e (Table 5), and (iv) limited gene flow compared to central populations (Table 5). This pattern has been predicted for populations in range margins (Soule 1973; Lammi et al. 1999). As the spatial isolation of populations increases, genetic drift becomes more influential than gene flow, and the differentiation among populations is expected to become greater (Hutchison & Templeton 1999). Thus, it is likely that adaptation in the range margins of the rainforest species *V. gigantea* was limited by a lack rather than an excess of gene flow.

We note that inbreeding coefficients (F_{IS}) were significantly positive in most populations of *V. gigantea* studied (Table 3), in agreement with recent findings of self-compatibility and pollinator limitation in this bat-pollinated species (Paggi et al. 2007). So inbreeding in natural populations will effectively contribute to decreased gene flow and increased drift in *V. gigantea*. We also

note that gene flow in the range margins of *V. gigantea* (N_em = 0.375 migrants per generation in the North and 0.688 in the South; Table 5) were similar to that observed for pairs of populations of the related mixed-outcrossing bromeliad *Alcantarea imperialis* (Barbará et al. 2007). That species occurs on isolated 'inselberg' rock outcrops for which strongly reduced gene flow is expected throughout its range. In contrast, gene flow in the continuous forest species *V. gigantea* studied here is approximately an order of magnitude higher in the centre of its range compared to populations from the margins (Table 5).

Another question of great interest to range-wide studies of variability and gene flow refers to the nature of the range margin: 'environmental', i.e. caused mainly by abiotic habitat factors, or 'parapatric' *sensu* Bridle & Vines (2006), i.e. caused mainly by biotic interactions such as competition. Here, expansion at the Southern border is limited mainly by abiotic factors – cooler temperature regimes and reduced rainfall as the Atlantic rainforest biome gives way to the open landscapes and grasslands of Southern South America (population TMRS; Fig. 1). Quite differently, the Northern range margin in the Brazilian state of Espírito Santo (population SLES; Fig. 1), ca. 400 km north of Rio de Janeiro, coincides with a local centre of bromeliad diversity, with numerous congeners and species of related genera co-occurring and competing in limited space (Smith & Downs 1974, 1977, 1979). Thus, it represents a parapatric range margin, where biotic interactions such as competition may contribute to the steepening of selective gradients in addition to abiotic factors (e.g. drought). In the case of *V. gigantea*, the differences between Northern and Southern margins appear to be reflected by differences in genetic and phenotypic patterns.

In the Southern range margin, where mainly abiotic factors are likely to be limiting, reduced gene flow and increased drift did not result in speciation or origin of new divergent phenotypes – no intraspecific varieties or forms are described from this area, and populations close to the Southern range margin appear phenotypically uniform (Palma-Silva, unpublished field observations). In contrast, in the Northern range margin, where biotic factors and competition appear to contribute to the maintenance of the margin, the morphological characteristics of the peripheral population SLES are so contrasting that it was even described as a variety of *V. gigantea* named *V. gigantea* var. *seideliana* (Röth 1992). This phenotypic differentiation in population SLES is accompanied

by a reduction in gene flow (Table 5) and, consequently, increased genetic divergence (Fig. 4).

Some authors consider the species margins one of the most active regions of speciation (Mayr 1954, Stebbins & Major 1965; Lesica & Allendorf 1995), and steep selective gradients associated with abiotic or biotic selection pressures form an integral part of existing models of environmental and parapatric margins (Bridle & Vines 2006). Nevertheless, different evolutionary trajectories in the Southern and Northern margins may also stem from longer periods of drift in the North and older biogeographic patterns in this region. This hypothesis is supported by larger numbers of private alleles there, and by longer branch lengths on our neighbor-joining tree (Fig. 4). The existence of phylogeographic splits is difficult to pinpoint with fast-evolving nuclear microsatellite loci, and thus a follow-up study based on multiple plastid DNA markers is currently in progress to resolve genealogical relationships in *V. gigantea* at a deeper level (Palma-Silva et al., unpublished data). We also note that theoretical models of evolution in range margins commonly consider factors such as density, drift, gene flow, and selection along a selective gradient (Kirkpatrick & Barton 1997; Bridle & Vines 2006), i.e. at a spatial scale that is finer than the one studied here; future studies on the limits of evolution in the range margins of *V. gigantea* should explicitly focus on this scale in both the Northern and the Southern margins.

Relationship between genetic diversity and species diversity

It has been postulated by Antonovics (1976) and others (Vellend 2005; Vellend & Geber 2005) that genetic diversity and species diversity are related phenomena and should not be treated separately. Only recently, researchers have begun to study the parallels between these two hierarchical levels of diversity (Moritz & Faith 1998, Vellend 2005; Vellend & Geber 2005). The center of genetic diversity described here for *V. gigantea* – São Paulo and Rio de Janeiro States (populations JASP, BESP, PARJ, and RIRJ; Fig. 2) – coincides with the centers of species diversity and endemism for most animal and plant species from the Atlantic Rainforest (Rambo 1950, Smith 1962, Moritz & Faith, 1998). Our results demonstrate a significant correlation between patterns of genetic diversity (H_E) in *V. gigantea* and patterns of species diversity in *Vriesea* reported by Smith (1962) for this genus over the same geographic range (r =

0.51; $p < 0.005$), when the highly divergent Northern population SLES was excluded from the analysis. As alluded to earlier, this population represents a divergent variety or form or possibly even an incipient species, and steeper selective gradients associated with biotic interactions in the 'parapatric' Northern margin may contribute to the 'outlier behavior' of this population. Nevertheless, our results demonstrate that patterns of genetic and species diversity in *V. gigantea* are connected and possibly were shaped by the same historical forces: quaternary climatic changes.

Implications for conservation

This study revealed clear gene pool structuring in *V. gigantea* into three divergent geographical regions: (i) South, which comprises populations from the Brazilian state of Rio Grande do Sul; (ii) Centre, comprising populations from Santa Catarina to São Paulo; and (iii) North, comprising populations located in the states of Rio de Janeiro and Espirito Santo (Fig. 4). As indicated by AMOVA, genetic differences among regions explain 13% of the variation of *V. gigantea* (Table 4). As genetic differentiation among populations was relatively high, the most effective conservation strategy would aim at preserving a maximum number of populations, including populations from each of the three major geographic clusters (Fig. 4) and, where possible, from all five clusters identified by the Bayesian genetic structure analysis (Fig. 5). Priority should be given to those central populations that showed the highest levels of diversity (Table 3; Fig. 2), and to the most divergent populations (NJ tree in Fig. 4), which includes the two peripheral populations TMRS and SLES. We note that the clear gene pool structure observed may allow the tracking of plant material used in horticulture by using genetic assignment tests (Waser & Strobeck 1998), to detect illegal harvesting of plants from nature where present. Considering the rapid ongoing fragmentation in the Atlantic Rainforest 'hotspot' of South America, analyses of contemporary gene flow using two-generation approaches (Smouse et al. 2001) are needed to better understand fluctuations in gene exchange across a highly fragmented landscape.

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Figure Legends

Fig. 1 Map showing the current geographic distribution of *Vriesea gigantea* following Reitz 1983.

Fig. 2 Genetic diversity of 13 populations of *Vriesea gigantea*.

Fig. 3 Relation between allelic richness, variance in allele size, gene diversity, and latitude per populations of *Vriesea gigantea*. X-axis: geographical latitude of each population. Y-axis: mean per population of each genetic diversity parameter (a) allelic richness, (b) variance in allele size, (c) gene diversity. Marginal populations are not included.

Fig. 4 Relationship between Slatkin's F_{ST} and geographical distances in km among populations of *Vriesea gigantea* (Mantel test correlation. r = 0.49; p <0.001)

Fig. 5 Unrooted neighbour-joining tree of populations based on Nei´s unbiased genetic distances (1978). Numbers represent bootstrap percentages based on 1000 replicates. Stars showing populations with the highest genetic diversity.

Fig. 6 Bayesian admixture proportions (Q) of individual plants of *V. gigantea* for a K = 5 population model. The K = 5 'genetic clusters' identified by STRUCTURE are indicated in different colors. For population abbreviations see Materials and methods.

Table 1. Sampled localities of *Vriesea gigantea* in Brazil. ID=name of the population; State=Brazilian States.

ID	State	Locality	Latitude S	Longitude W
TMRS	RS	Taim	31°56'	52°25'
ITRS	RS	Itapuã	30°25'	50°55'
MARS	RS	Maquiné	29°48'	50°16'
GASC	SC	Garopaba	28° 4'	48°39'
FLSC	SC	Florianópolis	27°26'	48°31'
VBSC	SC	Vargem do Braço	27°40'	48°46'
MBSC	SC	Morro do Baú	26°48'	48°57'
PAPR	PR	Paranaguá	25°30'	48°20'
JASP	SP	Jacupiranga	24°40'	48° 2'
BESP	SP	Bertioga	23°50'	46° 8'
PTRJ	RJ	Paraty	23°12'	44°41'
RIRJ	RJ	Rio de Janeiro	22°57'	43°14'
SLES	ES	Santa Leopoldina	20° 7'	40°30'

RS: Rio Grande do Sul; SC: Santa Catarina; PR: Paraná; SP: São Paulo; RJ: Rio de Janeiro; ES: Espírito Santo

Table 2. Genetic characteristics and summary of F statistics of 10 SSR loci in 13 populations of *Vriesea gigantea*. N=sample size; A=number of allele per locus; H_O = observed heterozygosity. H_E = expected heterozygosity. F_{IT} = overall inbreeding coefficient; F_{ST} = fixation index; F_{IS} = inbreeding coefficient.

Locus	N	A	H_O	H_E	F_{IT}	F_{ST}	F_{IS}[1]
e6b	412	12	0.449	0.521	0.301	0.153	0.175
e19	405	4	0.223	0.38	0.676	0.356	0.496
ct5	403	21	0.439	0.692	0.458	0.213	0.311
Ai4.3	406	23	0.499	0.62	0.427	0.259	0.227
Ai4.10	401	3	0.281	0.354	0.438	0.225	0.246
Ai4.11	418	16	0.405	0.608	0.43	0.193	0.294
Ai5.18	395	16	0.412	0.572	0.41	0.181	0.28
VgA06	390	17	0.507	0.635	0.304	0.153	0.179
VgF01	401	25	0.545	0.693	0.375	0.182	0.236
VgG02	396	23	0.547	0.715	0.398	0.201	0.247
Overall/Mean	429	160	0.424	0.714	0.417	0.211	0.261

[1]All inbreeding coefficients (F_{IS}) departed significantly from HWE at the 0.05 level.

Table 3. Genetic diversity in populations of *Vriesea gigantea* observed at 10 SSR loci. ID=name of each population; N=sample size; P=percentage of polymorphic loci; Apr=number of private alleles; Rs=allelic richness; Var=variance in allele size; H_O=observed heterozygosity; H_E=expected heterozygosity; and F_{IS}=inbreeding coefficient.

ID	N	P	Apr	Rs	Var	H_O	H_E	F_{IS}
TMRS	42	40	0	1.18	1.199	0.039	0.065	0.408***
ITRS	44	100	0	2.43	15.387	0.274	0.533	0.489***
MARS	42	100	1	2.57	14.803	0.440	0.566	0.225***
GASC	31	100	1	2.86	29.721	0.455	0.614	0.263***
FLSC	37	100	0	2.75	30.473	0.549	0.580	0.053
VBSC	20	100	1	3.08	18.236	0.508	0.669	0.245***
MBSC	47	100	0	3.15	29.918	0.466	0.676	0.313***
PAPR	34	100	1	3.08	32.273	0.448	0.647	0.312***
JASP	40	100	11	3.49	35.486	0.496	0.718	0.313***
BESP	29	100	4	3.49	54.807	0.518	0.715	0.278***
PTRJ	40	100	7	3.38	82.709	0.614	0.727	0.157***
RIRJ	7	100	4	3.49	50.575	0.539	0.699	0.249***
SLES	18	70	10	1.82	18.523	0.251	0.318	0.244

Departures of within-population inbreeding coefficients (F_{IS}) from HWE are indicated by asterisks: *** $p<0.001$.

Table 4. Partitioning of genetic variability for 429 individuals of *Vriesea gigantea* from 13 populations nested in three regions in Brazil using the AMOVA procedure. See text for details about the partitioning of genetic variance into regions.

Source	d.f	Sum of square	Components of variance	%	P
Among regions	2	279.141	0.434	12.93	< 0.001
Populations within regions	10	278.387	0.395	11.78	< 0.001
Individuals/within population	845	2.133.486	2.524	75.28	< 0.001

	Ne1	Ne2	Nem1-Nem2	Nem2-Nem1
SLES[1] / RIRJ[2]	340.992	1257.920	0.152	0.375
RIRJ[1] / PTRJ[2]	1241.955	1358.484	2.642	1.561
PTRJ[1] / BESP[2]	1584.744	791.916	1.993	1.283
BESP[1] / JASP[2]	1514.946	1215.133	2.943	2.422
JASP[1] / PAPR[2]	1656.667	1142.284	6.773	1.392
PAPR[1] / MBSC[2]	978.580	587.231	1.985	1.451
MBSC[1] / FLSC[2]	628.850	361.591	0.932	3.190
FLSC[1] / VBSC[2]	521.454	515.743	2.178	1.380
VBSC[1] / GASC[2]	473.860	765.886	1.244	1.867
GASC[1] / MARS[2]	451.888	513.554	0.817	1.196
MARS[1] / ITRS[2]	285.713	376.243	0.812	0.707
ITRS[1] / TMRS[2]	424.546	89.301	0.038	0.688

Table 5. Effective population size (Ne) and Gene flow (Nem) for pairs of neighbouring populations of *Vriesea gigantea*.

1 and 2: indicating the direction of gene flow.

Fig 1

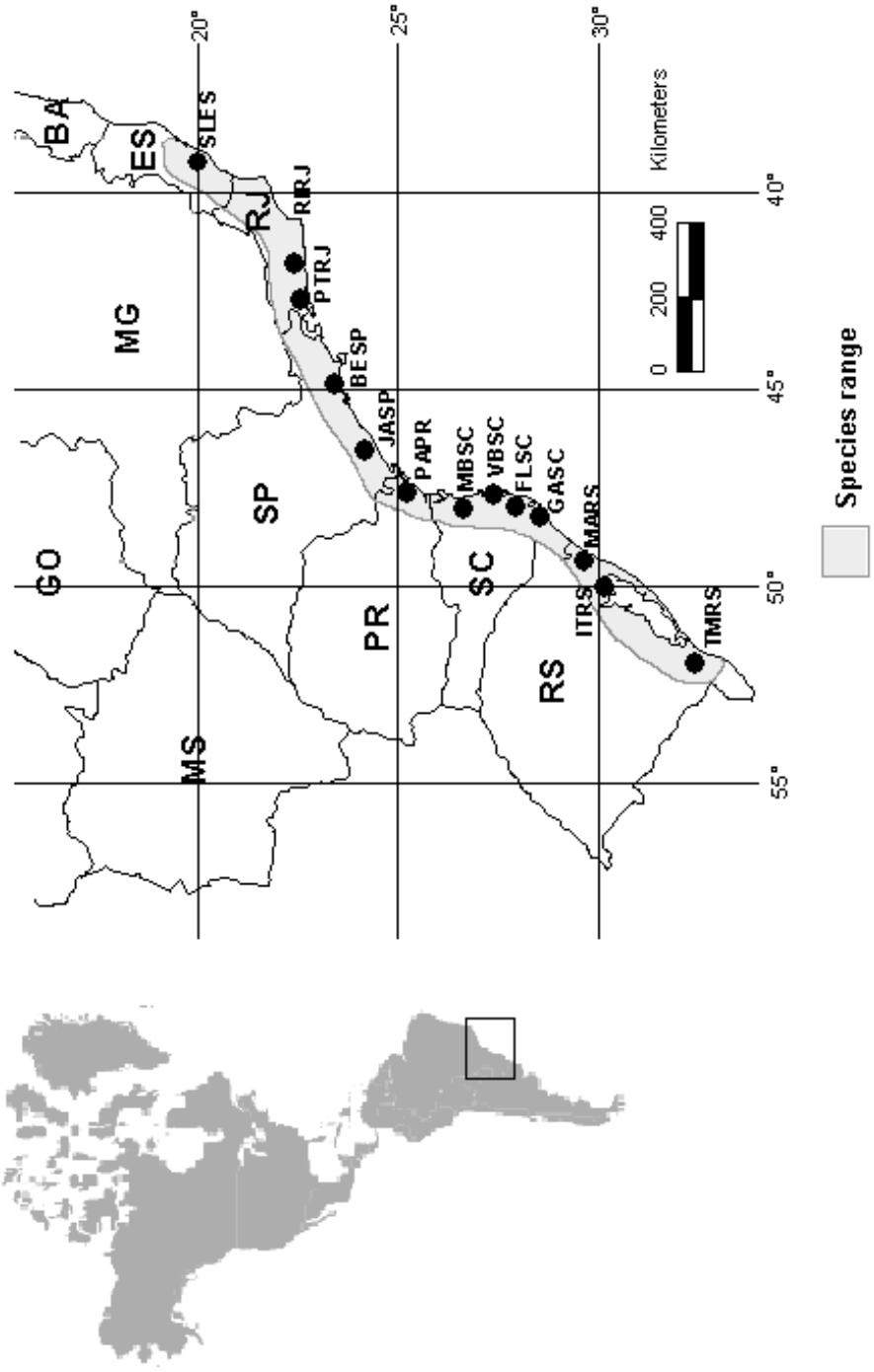

BA
ES
MG
RJ
SP
GO
MS
PR
SC
RS
SLES
RRJ
PTRJ
BESP
JASP
PAPR
MBSC
VBSC
FLSC
GASC
MARS
ITRS
TMRS
20°
25°
30°
40°
45°
50°
55°
0
200
400
Kilometers
Species range

Fig 2

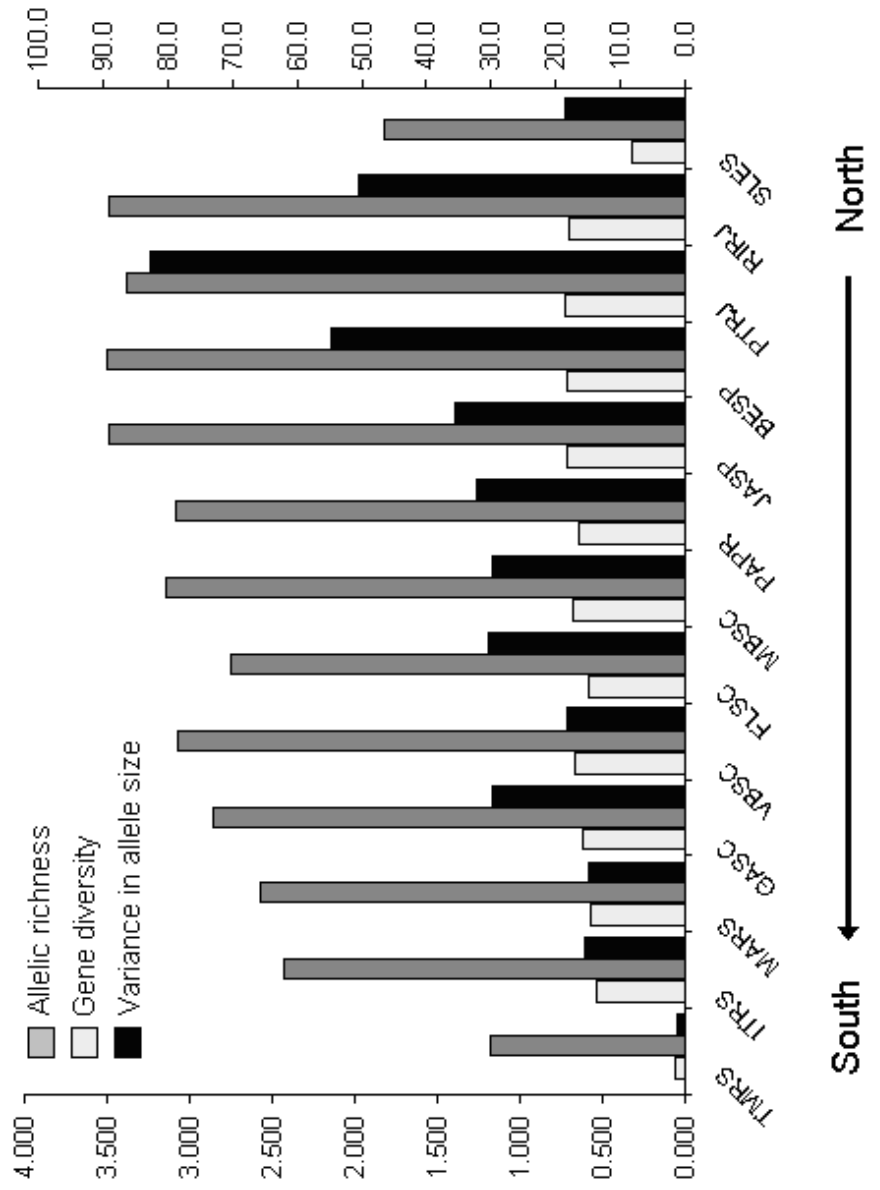

Allelic richness
Gene diversity
Variance in allele size
4.000
3.500
3.000
2.500
2.000
1.500
1.000
0.500
0.000
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
TMRS
ITRS
MARS
GASC
VBSC
FLSC
MBSC
PAPR
JASP
BESP
PTRJ
RIRJ
SLES
South
North

Fig 3

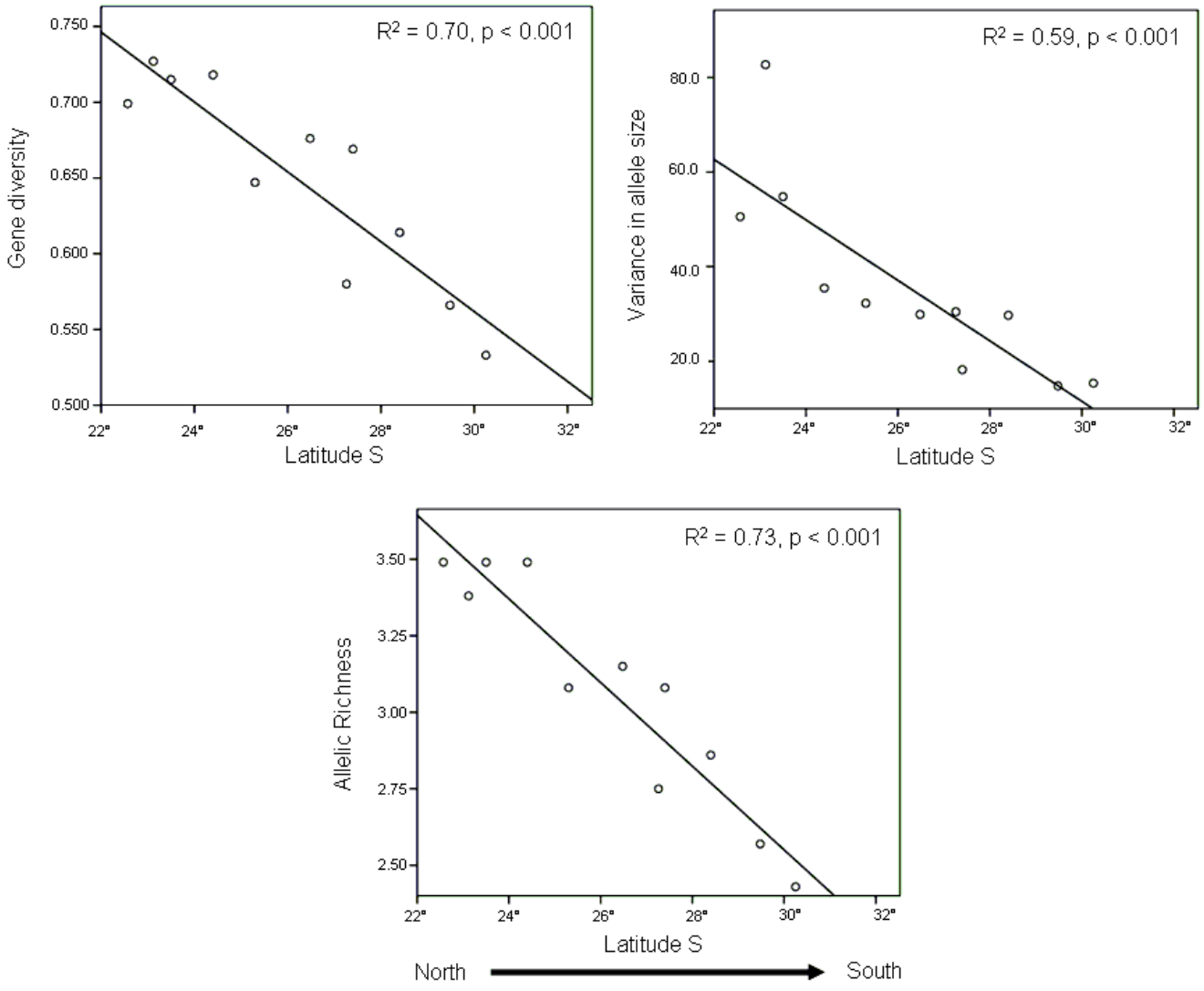

R² = 0.70, p < 0.001
Gene diversity
0.750
0.700
0.650
0.600
0.550
0.500
22°
24°
26°
28°
30°
32°
Latitude S
R² = 0.59, p < 0.001
Variance in allele size
80.0
60.0
40.0
20.0
22°
24°
26°
28°
30°
32°
Latitude S
R² = 0.73, p < 0.001
Allelic Richness
3.50
3.25
3.00
2.75
2.50
22°
24°
26°
28°
30°
32°
Latitude S
North
South

Fig 4

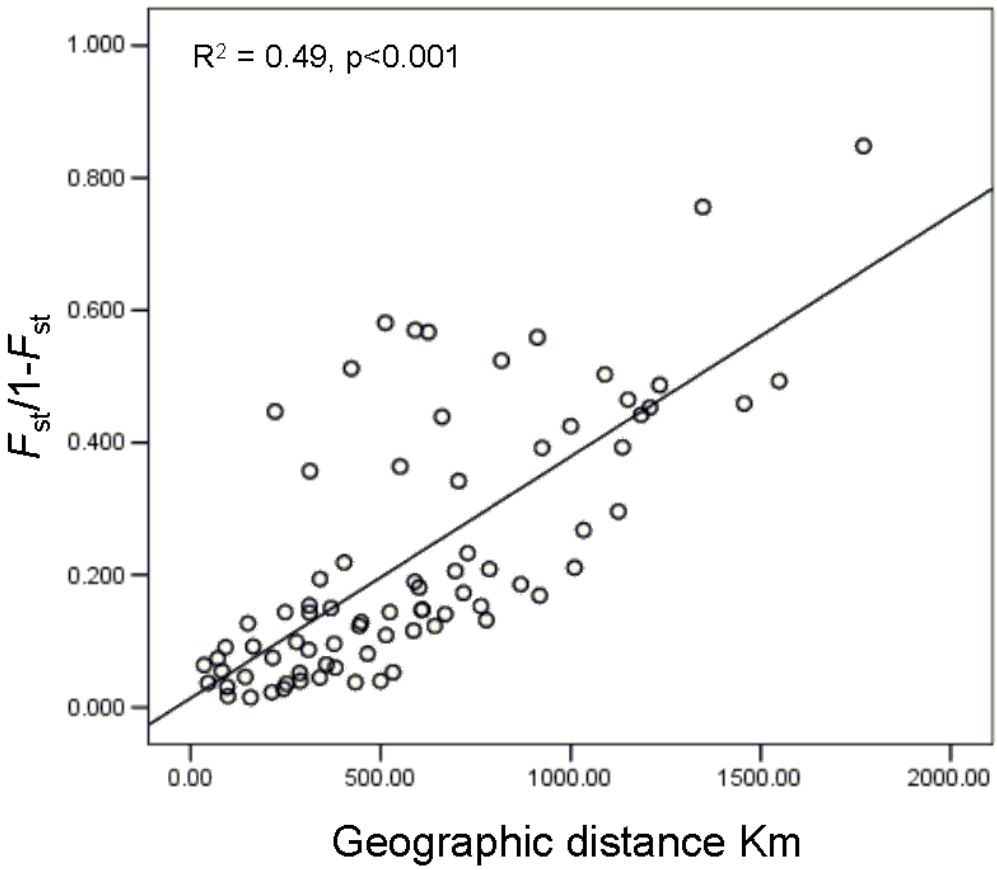

R2 = 0.49, p<0.001
1.000
0.800
0.600
0.400
0.200
0.000
Fst/1-Fst
0.00
500.00
1000.00
1500.00
2000.00
Geographic distance Km

Fig. 5

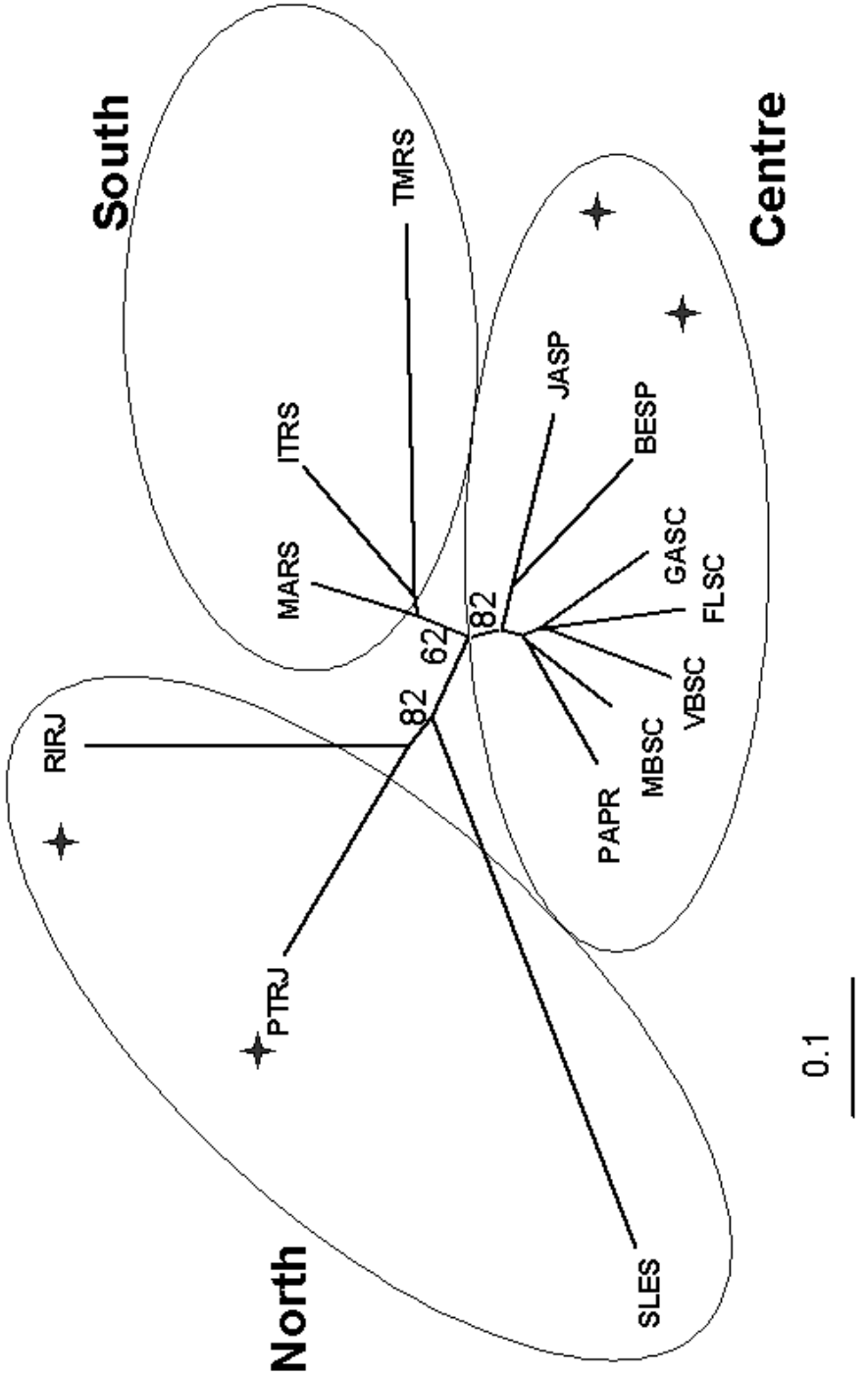
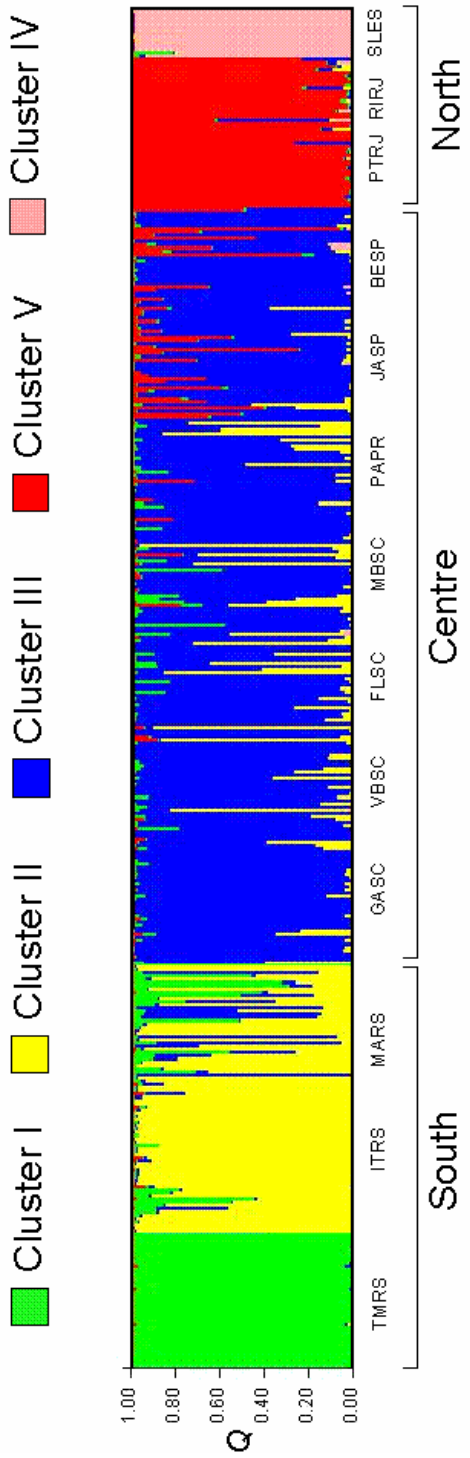

South
Centre
North
TMRS
ITRS
MARS
JASP
BESP
GASC
FLSC
VBSC
MBSC
PAPR
RIRJ
PTRJ
SLES
82
62
82
0.1

Fig 6


Cluster I
Cluster II
Cluster III
Cluster V
Cluster IV
TMRS
ITRS
MARS
GASC
VBSC
FLSC
MBSC
PAPR
JASP
BESP
PTRJ
RIRJ
SLES
South
Centre
North
Q
1.00
0.80
0.60
0.40
0.20
0.00

Capítulo 4

Intraspecific Phylogeography of *Vriesea gigantea* (Bromeliaceae) revealed by chloroplast markers

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Manuscrito submetido à Heredity

ABSTRACT

In the present study four chloroplast microsatellites and one single nucleotide polymorphism were selected after screening sequences from 21 chloroplast regions for within-species polymorphism. These five cpDNA markers were examined in 192 individuals from 13 populations to infer the phylogeographic history of the species. The key results indicate strong phylogeographic structure and restricted gene flow among populations of *Vriesea gigantea.* A pollen/seed flow ratio of 3.3 indicates an asymmetry of pollen and seed flow in *V. gigantea*, gene flow via seed being less efficient than via pollen, thus resulting in a stronger genetic structure for chloroplast compared to nuclear markers. A haplotype network revealed two major phylogeographic groups: North and Central-South. A mismatch distribution analysis showed that populations from the Northern region are stable and are thus likely to be ancestral, whereas the populations from the Central-Southern region are more likely to have experienced recent population expansion. In addition, the location of the North-South split in the gene pool of *V. gigantea* does not coincide with the latitudinal phylogeographic breaks reported for other Atlantic Rainforest species.

Introduction

The Neotropics represent the world's biodiversity-richest region, where at least five hotspots have been recognized for conservation (Myers *et al.,* 2000). However, compared with temperate regions, knowledge on patterns of genetic diversity and species diversification are very limited in the tropics (Hewitt, 2004). Studies addressing the evolutionary history of Brazilian Rainforest taxa from a phylogeographic perspective are extremely rare and include few studies of animals (Grazziotin *et al.,* 2006; Tchaicka *et al.,* 2007, Cabanne *et al.,* 2007) and plants (e.g. Lira *et al.,* 2003; Andrade *et al.,* 2007). These studies provide first insights into the effects of historical forest expansions and contractions on present-day patterns of intraspecific variation and point out the evolutionary complexity of the quaternary history of South American taxa, but major evolutionary patterns have yet to be explored (Tchaicka *et al.,* 2007).

The Brazilian Atlantic Rainforest nowadays covers only 7% of its original distribution. Habitat fragmentation and disturbance are important factors contributing to species extinction (Frankham *et al*., 2003). Because of this, intraspecific variation has increasingly been accepted as a focus for conservation. This approach is consistent with the general aim of maintaining the evolutionary potential of species (Newton *et al*., 1999). Hamrick *et al.* (1991) emphasized the importance of knowing the distribution of genetic variation within species for designing strategies for preserving biodiversity. Knowledge of the genetic structure and variability of wild populations can aid the conservation of species as dynamic entities that can evolve to cope with environmental change, and thus minimize their risk of extinction (Frankham *et al*., 2003).

Biogeographic studies have revealed that the Brazilian Atlantic Rainforest in southern Brazil was formed by the expansion of northern tropical floristic elements, with the flora being a continuation and attenuation of that centring in southeastern Brazil (Rambo, 1951, 1960 and Simth, 1962). During the Quaternary, climatic changes associated with large oscillations in the sea level and in the vegetation affected the patterns of distribution and diversification of species in the Neotropics, (De Oliveira *et al.,* 2005 and Rull, 2006). Sea level oscillations had a dramatic effect on the Brazilian coastal vegetation; in addition, many islands have recently become disconnected from each other or from the mainland (de Oliveira *et al.,* 2005). Such 'continental shelf island systems', created by rising sea levels, provide a premier setting for studying the effects of past fragmentation, dispersal, and genetic drift on taxon diversification (Bittkau and Comes, 2005).

Molecular markers based on chloroplast DNA (cpDNA) analysis have been shown to be a valuable tool for phylogeographic studies (King and Ferris, 1998). Chloroplast DNA evolves slowly compared to most well-studied nuclear DNA regions due to low mutation rates and the absence of recombination (Wolfe *et al*., 1987; Clegg & Zurawski, 1992; Zhang & Hewitt 2003). In particular, the discovery of polymorphic microsatellites in the cpDNA genome provides opportunities for analyzing the genealogies of maternally transmitted loci within and between populations and for addressing phylogeographic issues in plants

(Provan *et al*., 2001). Several studies have described varying levels of intraspecific cpDNA polymorphism in a wide range of plant species (Collevatti *et al.,* 2003, Lira *et al.,* 2003, Trapnell & Hamrick, 2004, Lorenz-Lemke *et al.,* 2005, among others).

In most angiosperms, the cpDNA genome is maternally inherited (Sears, 1980; Corriveau & Coleman, 1988; Mogensen, 1996; Ennos *et al*., 1999) and thus provides a seed-specific marker. In species where seed flow is lower than pollen flow, it is predicted that the cpDNA genome will be highly structured when compared to nuclear genes (Petit *et al*., 1993). Moreover, comparison with biparentally inherited nuclear markers has shed light on the relative importance of pollen and seed flow in structuring wild plant populations (Petit, 1992; Ennos, 1994; Hu and Ennos, 1999). Natural history and empirical studies of outcrossing plant species suggest that pollen movement contributes greatly to overall gene dispersal (Levin and Kerster, 1974; Waser, 1991; Ellstrand, 1992). However, little is known about the dispersal abilities of species that form metapopulations in unstable habitats. When local demes undergo frequent extinction and recolonization events, seed movement into an empty patch of favorable habitat may be the basic mechanism for the foundation of new subpopulations (McCauley, 1994). If so, then the pollen/seed migration ratio will tend to be smaller in such systems (McCauley, 1997).

Vriesea gigantea Gaud. is a perennial, diploid (2n = 50 chromosomes, Palma-Silva, 2003), bromeliad species endemic to the Brazilian Atlantic Rainforest. It is non-autogamous and has a mixed mating system (Paggi, 2006). The species is epiphytic, saxicolous, or terrestrial. Its distribution ranges from the Brazilian state of Rio Grande do Sul to the state of Espírito Santo (Fig. 1; Reitz, 1983). The flowers of *V. gigantea* are pollinated by bats (Sazima *et al.,* 1999) and its small seeds are dispersed by wind. Populations generally are large, formed by many individuals distributed in patches of different sizes and densities. Besides, *V. gigantea* plays an important role in ecosystems: its vegetative structures form tanks that are able to hold many litres of water, thus supporting a large number of associated animal species (Benzing, 2000). Unfortunately, *V. gigantea*, as many others bromeliad species, is severely

affected by habitat fragmentation as a consequence of logging and urban development and by illegal extraction for horticultural purposes.

In the present study we report patterns of genetic variability and phylogeography in *V. gigantea,* based on the genetic analysis of polymorphic cpDNA markers obtained from specimens sampled throughout the species' range. In particular, we ask the following questions: (i) What is the extent of genetic differentiation among regions (Central-South vs. North of the current distribution range) and which past demographic processes may have shaped the phylogeographic patterns observed? (ii) To what extent are patterns of cpDNA diversity congruent with patterns of nuclear DNA diversity and species diversity in the Brazilian Atlantic Rainforest? (iii) What is the relative contribution of seed vs. pollen flow to historical gene flow, and what are the consequences of the observed dispersal patterns for population connectivity? We discuss cpDNA phylogeographic patterns in the light of available knowledge on the biogeographic history of the Brazilian Atlantic Rainforest and comment on practical implications for conservation.

Material and Methods

Study design and DNA extraction — A total of 192 individuals from 13 wild populations of *Vriesea gigantea* were sampled from the state of Rio Grande do Sul to the state of Espírito Santo in Southern and Southeastern Brazil (Fig 1, Table 1), representing the entire geographical range of the species. Geographical distances between populations ranged from ca. 34 km (FLSC-VBSC) to ca. 1600 km (TMRS-SLES). The populations represent three main geographical regions recognized for *V. gigantea* by Palma-Silva (*in prep*) from nuclear marker-based genetic analysis of the same populations. The first region (South) is represented by populations from the state of Rio Grande do Sul: TMRS, ITRS, and MARS. The second region (Central) comprises populations from Santa Catarina, Paraná, and São Paulo States: GASC, FLSC, VGSC, MBSC, PAPR, JASP, and BESP. The third region (North) is represented by populations from the states of Rio de Janeiro and Espírito Santo: PTRJ, RIRJ, and SLES. Three (FLSC, PAPR, and PTRJ) out of 13 populations are

continental islands as shown in Table 1. Fresh leaves (~5 cm^2) were collected and stored in liquid nitrogen or in CTAB - cetyltrimethyl ammonium bromide buffer - until DNA extraction. Total genomic DNA was extracted following essentially Doyle & Doyle (1990) and stored in a -20 ºC freezer until analyses.

Identification and characterization of cpDNA markers — an initial screening of polymorphism was performed with six individuals collected from six populations (TMRS, FLSC, PAPR, BESP, RIRJ, and SLES). In order to increase the chance to find polymorphisms, the samples chosen covered the majority of the geographical range of the species (see Fig. 1). The screening was conducted with 21 regions totaling ~ 5,500 base pairs (bp) of the cpDNA genome of *V. gigantea.* A total of four polymorphic cpDNA microsatellite loci (from here onwards, cpSSR) and one single nucleotide polymorphism (SNP) were obtained and used to identify and characterize cpDNA haplotypes. The four species-specific cpSSR primer pairs were designed by sequencing universal primers as described by Taberlet *et al.,* (1991), Sang *et al.,* (1997), and Hwang *et al.,* (2000) (Table 2). Polymerase chain reaction (PCR) was carried out in a total volume of 20µl containing: 10ng DNA template, 1X Bioline Taq buffer, 2mM Bioline $MgCl_2$, 100µM dNTPs, 20pmol forward primer, 20pmol reverse primer, 2U *Taq* polymerase (Bioline). Reactions were performed in a PE Applied Biosystems 9700 thermocycler by using a standard cycling program: 95ºC for 3min, 38 cycles of 94ºC for 30s, 53ºC for 30s, 72ºC for 30s and a final elongation step at 72ºC for 10min. Products were purified (QIAquick) and sequenced using the BigDye terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, U.S.A.) and a 3700 DNA Analyzer (Applied Biosystems). Forward and reverse sequences were aligned in SEQUENCHER version 4.1.2 (Gene Codes). The characteristics and sequences of the species-specific pair primers designed for cpSSR loci are reported in Table 2. The primers were designed using the Primer3 programme (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The SNP was identified by sequencing the plastid region *rpoB* using universal primers described in KEW RBG website (www.kew.org.uk) using the same protocol above described for the other four regions.

Genotyping of population samples — The SNP was amplified and sequenced for all samples using the same protocol as described above. For cpSSRs, all PCR amplifications were performed in a PE Applied Biosystems 9700 thermocycler in 10µl reactions containing: 10ng DNA template, 1X Bioline Taq buffer, 1.5mM Bioline $MgCl_2$, 100µM dNTPs, 10pmol either FAM or JOE labeled forward primers, 10pmol reverse primer, 0.5U *Taq* polymerase (Bioline). A standard cycling program was used: 95ºC for 3min, 30 cycles of 94ºC for 30s, 58ºC for 30s, 72ºC for 30s and a final elongation step at 72ºC for 5min. Microsatellite variants were resolved on a 3100 DNA Analyzer (Applied Biosystems) and were precisely sized against ROX molecular size standard using GENESCAN 3.7 and GENOTYPER 3.7 software (Applied Biosystems).

Data analyses

Genetic diversity estimates — the cpSSR length and SNP variation were combined to define the haplotype of each individual. In addition, each population was characterized for levels of diversity at the cpDNA markers using: (1) the number of haplotypes detected in each sample, (2) H_E – gene diversity (Nei's, 1978), and (3) allelic richness (El Mousadik & Petit, 1996) estimated using the program CONTRIB (Petit *et al*., 1996). Rarefaction uses the frequency distribution of alleles at a locus to estimate the number of alleles that would occur in smaller samples of individuals and allows for an unbiased estimate of allelic richness (Leberg, 2002).

Genetic structure analysis — Diversity and divergence statistics for unordered alleles (G_{ST}) and ordered alleles (R_{ST}) were calculated according to Ponds & Petit, (1995) using the software HAPLODIV. The possible presence of phylogeographic structure was tested by comparing R_{ST} to G_{ST} values using 10 000 random permutations (Hardy *et al*., 2003) using the program CONTRIB (Petit *et al*., 1996). If $R_{ST} > G_{ST}$, then the null hypothesis that there is no phylogeographic pattern can be rejected. R_{ST} estimates take explicit account of the mutation process that can occur at microsatellite loci (stepwise mutation model, SMM). Assuming neutrality, $R_{ST} > G_{ST}$ is expected when the mutation rate is non-negligible compared to the migration rate (Hardy and Vekemans,

2002, Hardy *et al.,* 2003). Thus, a phylogeographic pattern is said to be present in that case.

Genetic differentiation among regions, among populations within regions, and among individuals within populations, was estimated using Φ-statistics (Weir and Cockerham, 1984) within the analysis of molecular variance (AMOVA) performed in the software ARLEQUIN 3.01 (Excoffier *et al.,* 2005). To estimate the relative contribution of pollen *vs.* seed flow to total gene flow, the following formula from Ennos (1994) was applied:

$$\text{Pollen flow/seed flow} = [(1/\Phi_{SC\,(B)} - 1) - 2\,(1/\Phi_{SC\,(M)} - 1)] / (1/\Phi_{SC\,(M)} - 1)$$

Where $\Phi_{SC\,(B)}$ and $\Phi_{SC\,(M)}$ are levels of population differentiation calculated from biparentally (nuclear) and maternally (plastid) inherited markers, respectively. In most angiosperms cpDNA is predominantly maternally inherited (Ennos *et al*., 1999). The calculation of the pollen-seed flow ratio presented here is based on the assumption that maternal inheritance is the rule in *V. gigantea* as in most other angiosperms, a hypothesis that may be tested using progeny arrays.

Plastid DNA haplotype network — A median-joining haplotype NETWORK (Bandelt *et al*., 1999) was constructed based on plastid DNA haplotypes using the program NETWORK 4.1.1.1. (http://www.fluxus-engineering.com). This is a widely used method to resolve haplotype phylogenies that are too complex to be resolved with the naked eye (Posada & Crandall, 2001). The method uses parsimony criteria to identify median vectors, i.e. consensus sequences of mutually close sequences of markers that may be equivalent to unsample or extinct ancestral haplotypes. Initial runs using all the haplotype information yielded a complex structure. Hence, following the user manual, rapidly mutating characters were down weighted in subsequent runs. For all other parameters, the default settings were used.

Inference of population demographic history — In order to detect the genetic signatures of population expansion, the distribution of the observed number of differences between pairs of haplotypes was analyzed by testing whether their frequency distribution was statistically different from a distribution expected under a model of demographic expansion (Harpending *et al*., 1993).

Mismatch distributions for the total data set as well as for north region data separately were calculated using ARLEQUIN 3.01 (Excoffier *et al*., 2005). The test could not be performed for the Central and Southern regions individually because the number of pairwise differences was too small. The mismatch distribution is expected to follow a unimodal pattern for populations that underwent recent demographic changes such as recent demographic expansion (Rogers and Harpending, 1992) or a range expansion with high levels of migration between neighbouring demes (Ray *et al*., 2003, Excoffier, 2004). In contrast, a multimodal pattern is expected for populations at demographic equilibrium (Rogers and Harpending, 1992).

Results

Intraspecific cpDNA diversity – Characterization of the cpDNA markers in samples of 192 individuals from 13 populations of *V. gigantea* across its geographic range (Table 1; Fig. 1) yielded two to five alleles per polymorphic site (Table 2). The single nucleotide polymorphism found was a transversion (T-C). The combined genetic information from the five polymorphic sites resulted in 13 distinct cpDNA haplotypes (H1 – H13) (Table 3 and 4), four of which were singletons.

Haplotype distribution within and among populations – All populations were polymorphic with two to five haplotypes each, with only one exception: PTRJ (Fig 1), which displayed a single fixed haplotype (H13). The populations JASP and BESP, from the Central region, showed the highest level of genetic diversity: the largest number of haplotypes (five in each population) and the highest allelic richness (2.00 and 2.85, respectively). Among the five populations with the lowest number of haplotypes and allelic richness were the two populations from the northern and southern margin of the species' range (TMRS and SLES). Interestingly, the other three of the five less diverse populations were those from continental islands (PTRJ, with the fixed H13 haplotype: PAPR and FLSC with the two most common haplotypes - H12 and H8 (Table 3 and 4). The majority of populations showed moderate levels of gene diversity (H_E) with a mean of H_E = 0.486, with one exception (PTRJ; H_E = zero). Besides the PTRJ population, the marginal populations (TMRS and

SLES) also presented very low levels of gene diversity (He = 0.133 and 0.118, respectively) (Table 3).

The hierarchical analysis of molecular variance AMOVA (Table 5) showed that the largest proportion of the total variance was among populations (58.54%, P < 0.001), and the variance among the three geographic regions was 28.35% (*P* < 0.001). Placing the 'Central' and 'South' regions into one group versus the 'North', the total amount of variation explained by 'among regions' increased to 43% (see Table 5).

Using the value of genetic variance among populations (Φ_{SC} = 0.59) for cpDNA markers reported in this study and the value (Φ_{SC} = 0.21) for nuclear markers found by Palma-Silva *et al.* (*in prep*), the ratio of pollen flow to seed flow was estimated as ~3.3, indicating that gene flow via pollen in *V. gigantea* is three times more efficient than via seeds.

Analysis of haplotype distributions showed that population subdivision across all populations was strong with high values obtained either from ordered (G_{ST} = 0.39, h_s = 0.485; h_T = 0.795) or unordered haplotypes (R_{ST} = 0.714; v_S = 0.233; v_T = 0.814) (Table 6). The difference in genetic divergence when estimated with unordered *vs.* ordered haplotype information (G_{ST} *vs.* R_{ST}) was significant at the 0.05 level, thus indicating the presence of significant phylogeographic structure (Table 6).

Plastid DNA haplotype network

Median-joining analysis resulted in a haplotype network with two major haplogroups, one containing haplotypes typical of the Central and Southern regions, and the other one typical of the Northern region. The few exceptions were: one haplotype (H3) from the Central region, which grouped in the Northern haplogroup, and two haplotypes (H13 and H11) from the Northern region that clustered in the Central-Southern haplogroup. These two major groups were separated by three mutational steps (Fig. 2). Moreover, haplotypes from the Northern populations displayed a high degree of geographic structure, with no haplotype found in more than one population (Table 4) and a higher number of mutational steps between haplotypes compared to the Central and

Southern regions (Fig 2). In contrast, in the Central-Southern group several haplotypes were shared among populations. The most common haplotypes observed in these regions were H12 and H8 with total frequencies of 49% and 43%, respectively. The occurrence of widespread haplotypes indicates historical gene flow or shared ancient polymorphism between the Southern and Central populations. In addition, the diagram is also indicative of a relatively recent population expansion in the Central and Southern groups, where several haplotypes are connected by short branches with potentially ancestral, widespread haplotypes (H12 and H8).

Demographic history

The mismatch distribution analysis for the Northern group exhibited a multimodal curve (Fig. 3a) with observed values deviating significantly from values expected under a scenario of population expansion. The analysis for all populations yielded a significant deviation from the expectation as well, but the observed curve was clearly closer to the expectation (Fig. 3b).

Discussion

The present study is focused on the geographical distribution of cpDNA lineages within the species *V. gigantea*. Moderate levels of genetic diversity across the 13 populations of V. gigantea were detected with 13 distinct cpDNA haplotypes found. This variation was mostly due to microsatellite length variation. The distribution of cpDNA diversity within and among populations was markedly different from that observed for nuclear markers previously (Palma-Silva *et al.,* in prep), which allows us to discuss the processes that may have created these differences.

Pollen versus seed dispersal

We combined the nuclear data obtained for the same populations of *V. gigantea* by Palma-Silva *et al.* (in prep) with the current cpDNA data to estimate the pollen/seed flow ratio (Ennos, 1994). We obtained a ratio of 3.3, indicating an asymmetry between pollen and seed flow in *V. gigantea*, gene dispersal via seed being more than three times less efficient than gene dispersal via pollen.

This result can be interpreted in the light of current knowledge on the dispersal biology of bromeliads. The plumose seeds of *V. gigantea* are thought to be wind-dispersed and pollen grains are thought to be dispersed by bats (Sazima *et al.,* 1999). A previous study for other species of the same subfamily of bromeliads (Tillandsioideae) based on trapping experiments (Castante-Marín, 2005) showed restricted seed dispersal extending only a few meters from each mother plant. However, the same author also observed rare long distance dispersal, which may play an important role during colonization. Restricted seed dispersal is expected for wind-dispersal seeds (Greene and Jonhson ,1989; Bullock and Clarke, 2000). Since such seeds lack specialized features for dispersal (attachment and transport), they are dispersed rather locally (Bittkau and Comes, 2005). To our knowledge, no precise estimates are currently available for pollen dispersal distances in *V. gigantea* or even for closely related species of Tillandsioideae, however, bats are thought to be efficient pollinators and should thus be able to promote gene flow (Sazima *et al.,* 1989; Sazima *et al.,* 1999). Our genetic results support the hypothesis that pollen of *V. gigantea* is dispersed over larger distances than seeds, resulting in a stronger genetic structure for the chloroplast genome compared to the nuclear genome.

We are aware of only one other study reporting data on the relative roles of pollen and seed dispersal in bromeliads (Barbará et al., in review). In that study, the ratio of pollen *vs.* seed dispersal was estimated for four species of *Alcantarea* (the sister genus of *Vriesea*) from data on fine-scale spatial genetic structure (SGS) for biparentally inherited nuclear markers. Following Heuertz *et al.* (2003), these authors used the curvature of the kinship-distance curve (=the curve that describes the decrease of the kinship coefficient between individual plants with increasing geographic distance) to estimate whether gene dispersal was more likely to be restricted by dispersal of pollen or seed. They found that, for five out of eight populations studied, seed dispersal was more restricted than pollen dispersal, and pollen/seed dispersal ratios were estimated as being in excess of 5.7. These results are in the same order of magnitude as those obtained here, and they point at the important fact that pollen/seed flow ratios may differ between localities (for three populations studied by Barbará et al., all

three from bat-pollinated *Alcantarea* species, pollen dispersal was more restricted than seed dispersal). We also note that the pollen/seed flow ratios estimated in the present study lie well within the lower range of values reported for other plants (as reviewed by Squirrell *et al.,* 2001; Ennos *et al.,* 1999; Newton *et al.,* 1999).

Continental shelf islands (*sensus* Whittaker, 1998) have been proved to be excellent laboratories to study the effects of geographical isolation on allopatric speciation via selection and/or genetic drift (Bittkau and Comes, 2005; Grazziotin *et al.,* 2007). Such islands have recently become disconnected from each other or from the mainland by rising sea levels, making them interesting systems to study microevolutionary patterns and process (Anderson and Handley, 2002). We can compare the cpDNA diversity of the three populations sampled in continental islands (FLSC, PAPR, and PTRJ) with each other and with the remaining populations that are located on the mainland. In two of these islands (FLSC and PAPR), located in the centre of the distribution of the species, the two most common haplotypes (H8 and H12) were found (Table 4, Fig 1). In contrast, PTRJ was the only island population fixed for a single haplotype found nowhere else (H13). Assuming that the cpDNA genome is inherited maternally in these bromeliads, this indicates a recent bottleneck and/or restricted seed flow between island and mainland populations. Interestingly, population PTRJ is one of the most diverse populations of *V. gigantea* with respect to nuclear genetic diversity (Palma-Silva in prep), which indicates the ability of pollinating bats to promote gene flow between mainland and island populations. Our cpDNA data indicate that the sea represents a barrier to seed-based migration in *V. gigantea*. Thus, in both island and mainland populations restricted gene flow via seeds is the dominant force governing cpDNA genetic structure, although rare long-distance dispersal/colonization may have contributed to present-day patterns of haplotype diversity in *V. gigantea* (Fig 1).

Recent range expansion

All populations, except for PTRJ, were polymorphic for cpDNA haplotypes (Table 3). Analysis of molecular variance (AMOVA; Excoffier *et al*., 2005) showed that the differences among populations for cpDNA haplotypes were highly significant and explained 59% of total variation (Table 5). An AMOVA analysis in which regions were considered separately (Northern, Central, and Southern) showed that the variation attributed to regions ('among regions') was 28%. However, placing Central and Southern regions into one group versus the Northern region the total amount of variation attributed to the 'among regions' level increased to 43% (Table 5). The deep slip between the Northern and Central-Southern regions revealed by AMOVA is in good agreement with the median-joining analysis that resulted in a haplotype network with two major groups (Fig 2), mostly due to the major differences in haplotype composition between Central-Southern regions compared to those from the North.

We conclude that cpDNA variation is geographically structured in *V. gigantea* and that the species was possibly fragmented into two major phylogeographic groups: a Northern and a Central-Southern group. In our previous nuclear DNA analyses of *V. gigantea* (Palma-Silva et al., in preparation), the variation was found to be clearly structured across the geographical range of the species (Palma-Silva *et al.*, in prep). In that study, the split among regions (Northern, Central and Southern) was also significant with relatively high bootstrap support (64-84%) in the Neighbour-joining analysis and divergence among regions explained 13% of variance in AMOVA. However, while the nuclear DNA markers suggested the presence of three distinct groups of populations, only two groups were recognized by the present cpDNA data, with populations from Central and Southern regions presenting closely related haplotypes (Figs. 1 and 2). Nevertheless, nuclear and cpDNA markers in combination provide a congruent picture of the history of this rainforest species and its constituent populations, as both datasets highlight a historical division between two of the studied regions (Northern and Central-Southern). These data would seem to support the hypothesis that *V. gigantea* likely survived the Pleistocene climatic oscillations in two fragmented refugia.

The two major haplogroups inferred from the cpDNA data seem to have contrasting population histories in the Atlantic Rainforest. First, the shape of the haplotype network for the Central-Southern group (Fig 2), where the shorter branches were connected to the more widespread (and ancestral) haplotypes (H12 and H8), is indicative of recent population expansion. Second, the mismatch distribution analysis showed that populations from the Northern region are stable and should be ancestral ("older", although we refrain from estimating divergence times with the data currently available). In contrast, populations from the Central-Southern region may still be in their expansion phase (Figs. 2 and 3). The deep slip between the Northern and Central-Southern regions is also remarkable with respect to differences among populations within the two groups. Although in the Northern group all populations displayed different haplotypes with no overlap in their geographical representation, in the Central-Southern group populations are connected and share most of their haplotypes. The more complex demographic history in the Northern populations can be explained by the fact that these populations likely were submitted to cycles of long-term fragmentation and expansion due to climatic oscillations during the late Pleistocene. In contrast, the populations in the Central and Southern regions may have been shaped by recent expansion and colonization of the vacant territory following the last glacial maximum.

In the case of taxa with northern temperate distributions, rapid expansion from lower latitudes has been reported for many species, which explains patterns of genetic divergence among extant populations (reviewed by Hewitt 1996). In contrast, phylogeographic studies in Southern and Southeastern South America are rare and have thus far been limited to Neotropical mammals (Costa, 2003; *Cerdocyon thous:* Tchaika *et al.,* 2007), reptiles (*Bothrops jararaca:* Grazziotin *et al.,* 2006), and birds (*Xiphorhynchus fuscus*: Cabanne *et al.,* 2007). To our knowledge, only one study has reported phylogeography patterns in plants of the Southern portion of the Brazilian Atlantic Rainforest: *Passiflora actinia* and *P. elegans* from the Southern Brazilian States of Rio Grande do Sul, Santa Catarina, and Paraná (Lorenz-Lemke *et al.,* 2005). The phylogeographic patterns reported there are in agreement with those observed

for *V. gigantea* here. The authors also found a North-South split for their Atlantic Rainforest study species. In addition, for *B. jararaca, C. thous,* and *P. elegans* recent demographic expansion was inferred for the Southern groups, while Northern populations seem to have enjoyed past demographic stability (Lorenz-Lemke *et al.,* 2005, Grazziotin *et al.,* 2006, and Tchaika *et al.,* 2007). These observations are congruent with the widely discussed biogeographic scenario that the Southern part of the Atlantic Rainforest has been formed via migration of Northern tropical elements from the Rio de Janeiro region (Rambo, 1951;1960 and Simth, 1962). The dynamics of forest migration is expected to influence the demographic expansion of dependent organisms, such as the forest-dwelling *V. gigantea*.

Although there are only few similar studies on Brazilian Atlantic Rainforest species, the results currently available suggest that the climatic oscillations of the Pleistocene could explain present-day patterns of intra- and interspecific variability of Atlantic rainforest species. During the Quaternary, climatic changes and associated large oscillations in the sea level and in vegetation affected the patterns of distribution and diversification of species in the Neotropics (de Oliveira *et al.,* 2005 and Rull, 2006). The late Pleistocene was marked by drier and cooler (5°-7°C) climate. The modern tropical Atlantic Rainforest in Southern Brazil was replaced by subtropical forest or grassland during the Last Glacial Maximum (c. 20.000 years ago). The Atlantic rainforest in Southeastern Brazil probably shrunk to a narrow belt in coastal lowlands. The change to wetter climatic conditions started around 6000-5000 ^{14}C yr B.P. in Southeastern Brazil and even later, around 3000 yr B.P., in Southern Brazil (Behling, 1998, 2002). From there, increasing precipitation during the late-glacial period caused the expansion of tropical rain forest towards Southern Brazil. Therefore, the late Pleistocene climatic fluctuations most likely shaped the phylogeographic patterns of Brazilian Atlantic rainforest species such as *V. gigantea*. We note that fossil data would be required to support biogeographic scenarios such as this with confidence.

The location of the North-South split in *V. gigantea* (Fig 1) does not coincide with the latitudinal phylogeographic breaks reported by other

phylogeographic studies of Atlantic Forest species (Costa, 2003, Lorenz-Lemke *et al.,* 2005, Grazziotin *et al.,* 2006, Tchaika *et al.,* 2007, and Cabanne *et al.,* 2007). This may be explained by the simple fact that barriers to dispersal will affect different species of animals and plants in different ways. In this context, it is noteworthy that Tchaika *et al.,* (2007) considered the apparent unity of present-day biomes as potentially misleading. These authors suggested a complex history underlying the formation and biogeographic interactions of these ecosystems. Additional phylogeographic studies of species occurring in Southern and Southeastern Brazil are needed to shed light on the historical processes that have shaped the genetic diversity of the Atlantic Rainforest.

In summary, the findings obtained from the cpDNA analysis corroborate the genetic geographical structure in *V. gigantea* detected by nuclear DNA analysis (Palma-Silva *et al* in prep). In combination, the data provide some insights into the colonization history of this species endemic to the Atlantic Rainforest: historical events, such as range fragmentation and expansion appear to have influenced the present-day distribution of cpDNA lineages in *V. gigantea*. Also, much of the observed phylogeographic structure may be attributed to restriction of seed dispersal. Further phylogeographic studies are now required on a wide range of different species endemic to the Atlantic Rainforest, in order to obtain a better understanding of the factors that have influenced the evolutionary history of this critically threatened biodiversity hotspot.

Conservation implications

The present study demonstrates how the comparative analysis of maternally inherited cpDNA markers and biparentally inherited nuclear markers can provide a deeper understanding of the dynamics responsible for both ancient and more recent events that have shaped the current distribution of genetic variability in Neotropical plants. Combined datasets of this type are available for many northern temperate taxa, but information for Neotropical species is extremely scarce. Considering the highly fragmented and disturbed landscape of the Atlantic Rainforest, further analyses of contemporary gene

flow using two-generation approaches (Smouse *et al*., 2001) will help to better understand population connectivity and fluctuations in gene exchange across the fragmented landscape.

We have observed strong phylogeographic structure in *V. gigantea* with a deep phylogeographic split between the Northern and Central-Southern regions of the current distribution of this bromeliad. Considering the low levels of seed-mediated gene flow between the two haplogroups, *in situ* and *ex situ* conservation efforts should take these two geographic units into account explicitly. As indicated by AMOVA, the genetic differentiation between these two groups explains 59% of the cpDNA variation observed. Priority should be given to populations in the Central region (JASP and BESP), which displayed the highest values of haplotypic diversity. Chloroplast DNA-based assessment of genetic variability for island populations may be misleading as indicated by population PTRJ, which again highlights the need to consider nuclear and cpDNA data simultaneously.

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Figure Legends

Fig. 1. Map showing the current geographic distribution of *Vriesea gigantea* following Reitz (1983). Circles represent the frequencies of each haplotype found, each colour representing one haplotype. A black bar indicates the North-South phylogeographic split. Blue = Central-Southern group, Red = Northern group.

Fig. 2. Median-joining network of plastid DNA haplotypes. Filled circles indicate the haplotypes, the size of each circle being proportional to the observed frequency of each type. The number of mutations required to explain transitions among haplotypes is indicated along the lines connecting the haplotypes.

Fig. 3. Expected and observed mismatch distributions for cpDNA haplotype data from *V. gigantea*. **A**, populations from the North; **B**, all populations combined. For details see text.

Table 1. Sampling localities of *Vriesea gigantea* in Brazil. ID = name of the population; State = Brazilian Federal State.

ID	State[a]	Locality	Latitude S	Longitude W
TMRS	RS	Continent	31°56'	52°25'
ITRS	RS	Continental	30°25'	50°55'
MARS	RS	Continent	29°48'	50°16'
GASC	SC	Continent	28° 4'	48°39'
FLSC	SC	Island	27°26'	48°31'
VBSC	SC	Continent	27°40'	48°46'
MBSC	SC	Continent	26°48'	48°57'
PAPR	PR	Island	25°30'	48°20'
JASP	SP	Continent	24°40'	48° 2'
BESP	SP	Continent	23°50'	46° 8'
PTRJ	RJ	Island	23°12'	44°41'
RIRJ	RJ	Continent	22°57'	43°14'
SLES	ES	Continent	20° 7'	40°30'

[a] Brazilian State: RS - Rio Grande do Sul; SC - Santa Catarina; PR - Paraná; SP - São Paulo; RJ - Rio de Janeiro; ES - Espírito Santo.

Table 2. Primer sequences and characteristics for cpSSR loci isolated from *V. gigantea* (Vg) including locus name, location in the genome, primer sequences, repeat type, allele size range, and no. of alleles.

Locus	Location	Primers (5'-3')	Repeat	Size (bp)	N° of alleles
VgCP1	[1]psbA-trnH	TCTTCCTTTGTTTGATTCACATC	(T)10	137	3
		GAATTCGCGCCTACTCTGAC			
VgCP2	[2]*trn*D - *trn*T	TGCATAGGAGTTCATTCAGGA	(T)11	230	2
		CCACTAGACGATAGGGGCATA			
VgCP3	[2]*trn*L - *trn*F (e-f)	ACAAACGGATCCGAACAGAA	(T)10	209	2
		TGAGCTATCCCGACCATTTT			
VgCP4	[3]pet G	ACCTACGACATCGGGTTTTG	(A)5(T)9	185	5
		TTCTCCTATGGGCAGTAACGA			

[1] Universal primers designed by Sang *et al* (1997); [2] Universal primers designed by Taberlet *et al* (1991); [3] Universal primers designed by Hwang *et al* (2000).

Table 3. Genetic variability estimated from 13 populations of *Vriesea gigantea*, including the sample size (*N*); number of DNA plastid haplotypes, Gene diversity (H_E) and standard errors, and allelic richness.

Population	N	Nº of haplotypes	Haplotypes	H_E (SE)	Allelic richness
SLES	17	2	1.2	0.118 (0.101)	0.412
RIRJ	7	3	4.5.11	0.676 (0.160)	2.000
PTRJ	17	1	13	0.000 (0.000)	0.000
BESP	15	5	3.6.9.10.12	0.676 (0.105)	2.349
JASP	17	5	8.9.10.11.12	0.772 (0.070)	2.810
PAPR	16	2	8.12	0.458 (0.095)	0.971
MBSC	14	3	8.10.12	0.473 (0.136)	1.404
FLSC	14	2	8.12	0.440 (0.112)	0.965
VBSC	13	3	7.8.12	0.692 (0.075)	1.909
GASC	15	3	8.9.12	0.600 (0.069)	1.464
MARS	15	3	8.9.12	0.600 (0.109)	1.754
ITRS	15	3	8.9.12	0.676 (0.070)	1.857
TMRS	15	2	9.12	0.133 (0.112)	0.467
Mean	-	-	-	**0.486**	**1.412**

Table 4. cpDNA haplotype frequencies in 13 populations analyzed in this study. (*N*)= number of individuals sampled per population.

Populations	TMRS	ITRS	MARS	GASC	VBSC	FLSC	MBSC	PAPR	JASP	BESP	PTRJ	RIRJ	SLES
Haplotypes	(15)	(15)	(15)	(15)	(13)	(14)	(14)	(16)	(17)	(15)	(17)	(7)	(17)
H1													1
H2													16
H3										1			
H4												4	
H5												2	
H6										1			
H7				1									
H8		5	3	7	3	10	10	5	4				
H9	1	3	3						2	4			
H10					6		1		1	1			
H11									3			1	
H12	14	7	9	7	4	4	3	11	7	8			
H13											17		

Table 5. Analysis of molecular variance (AMOVA) for two different hierarchical levels, two and three-level modeling. (i) Two-levels, all populations. (ii) Three levels – North vs. Central vs. South. (iii) Three levels – North vs. Central plus South.

Source of variation	d.f.	Variance components	Percentage of variation	*P* value
(i)				
Among populations	12	0.55272	58.54	$P < 0.001$
Within populations	177	0.39139	41.46	$P < 0.001$
(ii)				
Among regions (north, central, and south)	2	0.29720	28.35	$P < 0.001$
Among populations	10	0.35980	34.32	$P < 0.001$
Within populations	177	0.39138	37.33	$P < 0.001$
(iii)				
Among regions (north, central-south)	1	0.55961	43.11	$P < 0.001$
Among populations	11	0.34703	26.74	$P < 0.001$
Within populations	177	0.39138	30.15	$P < 0.001$

Table 6. Analysis of cpDNA diversity in *V. gigantea* following Pons & Petit (1996), including the number of populations studied, no. of haplotypes, as well as diversity and divergence statistics for unordered and ordered haplotypes including standard errors.

No. of populations	No. of haplotypes	h_S[1]	h_T[1]	G_{ST}[1]	v_S[2]	v_T[2]	R_{ST}[2]
13	13	0.485 (±0.070)	0.795 (±0.059)	0.390 (±0.116)	0.233 (±0.043)	0.814 (±0.255)	0.714 (±0.076)

[1]Unordered haplotypes: h_S, within-population diversity; h_T, total diversity; G_{ST}: genetic divergence. [2]Ordered haplotypes: v_S, within-population diversity; v_T, total diversity; R_{ST}: genetic divergence. The difference in genetic divergence when estimated with unordered *vs.* ordered haplotype information (G_{ST} *vs.* R_{ST}) was significant at the 0.05 level indicating significant phylogeographic structure.

Fig 1

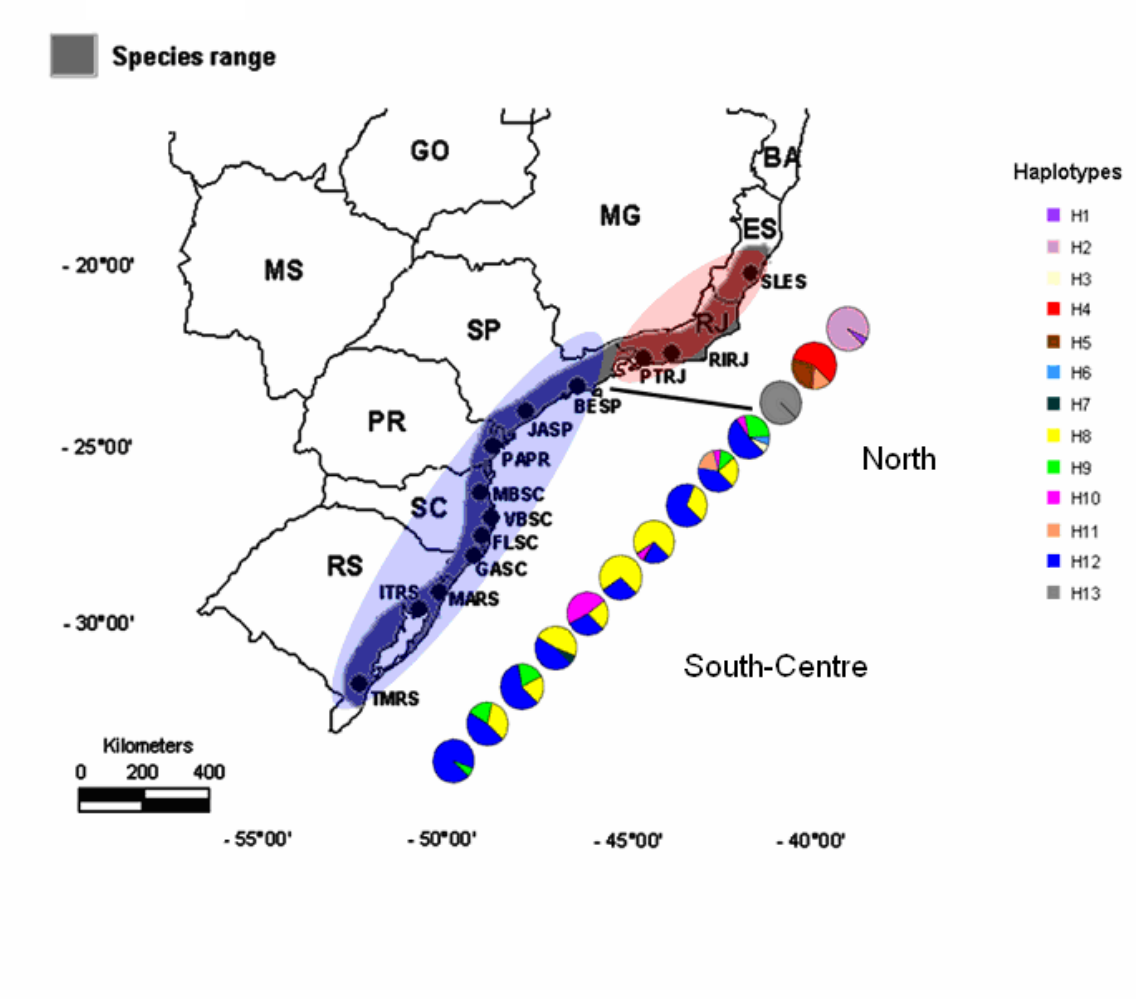

Species range
GO
BA
MG
ES
MS
SLES
- 20°00'
SP
RJ
RIRJ
PTRJ
BESP
PR
JASP
- 25°00'
PAPR
North
MBSC
SC
VBSC
FLSC
RS
GASC
ITRS
MARS
- 30°00'
South-Centre
TMRS
Kilometers
0 200 400
- 55°00'
- 50°00'
- 45°00'
- 40°00'
Haplotypes
H1
H2
H3
H4
H5
H6
H7
H8
H9
H10
H11
H12
H13

Fig 2

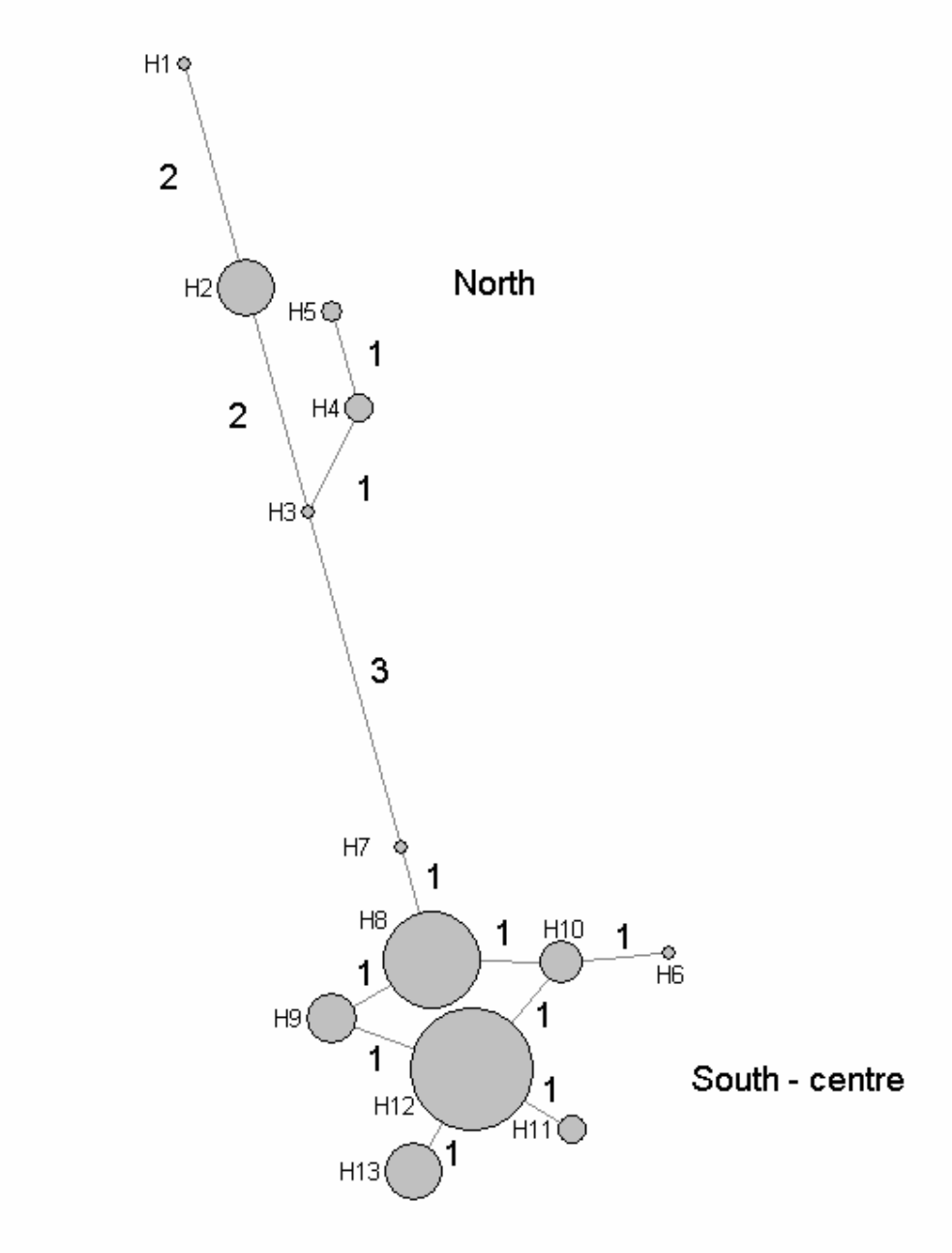
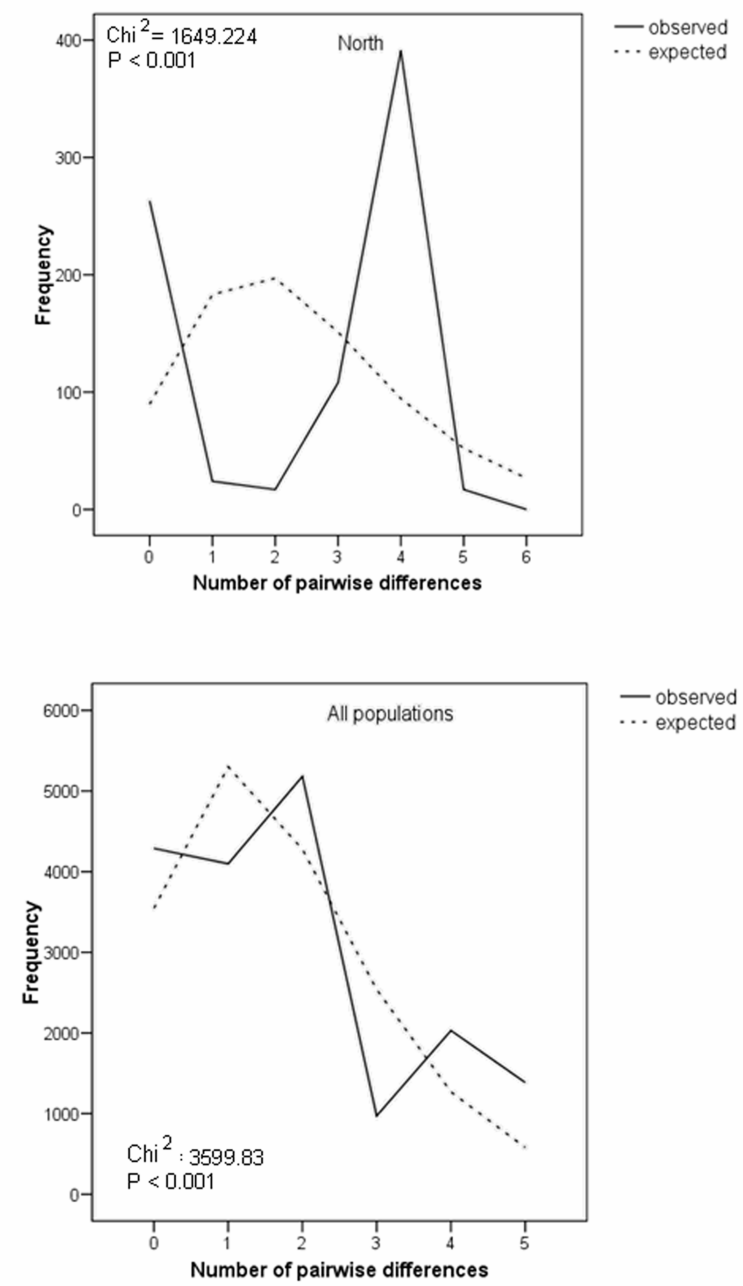

H1
2
H2
North
H5
1
H4
2
1
H3
3
H7
1
H8
1
H10
1
H6
1
H9
1
1
1
South - centre
H12
H11
1
H13

Fig 3


Chi 2= 1649.224
P < 0.001
North
observed
expected
Frequency
Number of pairwise differences
All populations
observed
expected
Frequency
Chi 2 : 3599.83
P < 0.001
Number of pairwise differences

Capítulo 5

What is the role of pollen viability and meiotic behavior in the fertility of natural populations of *Vriesea gigantea* Gaud. (Bromeliaceae)?

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Manuscrito submetido à Plant Biology

Abstract

Plant fertility can influence many aspects of population's ecology and evolution. The quantity and quality of pollen produced by a plant is an important component of reproductive success. Plant fertility was analyzed by assessing: chromosome number, meiotic behavior and pollen viability of seven native populations of *Vriesea gigantea*. All plants presented 2n = 50 chromosomes. Most of pollen mother cells showed a regular meiotic behavior with 25 bivalents and regular chromosome segregation. In accordance, high pollen viability (84-98%) was recorded for all investigated populations. These results indicated that plants from all populations analyzed are fertile. Despite the overall high pollen viability, significant differences were detected among populations. The population of Praia do Araçá showed a reduced percentage of pollen viability, although meiosis was quite regular. Reduction in pollen viability could have an important effect on plants fertility and, consequently, on fitness of this population.

Introduction

Plant fertility can influence many aspects of population's ecology and evolution. Pollination is the major step in seed formation and a central aspect of plant fertility. Pollination failure can be attributable to a combination of pollen, pollinator and plant factors (Wilcock and Neiland, 2002). The quantity and quality of pollen produced by a plant is an important component of reproductive success. In this context, pollen viability is considered as an important parameter of pollen quality (Dafni and Firmage, 2000). The chromosomes behavior at meiosis affects pollen viability. However, if meiosis is regular, *e.g.* chromosomes pair and segregate normally; sterility of the pollen grain is not expected to occur due to cytological reasons (Boff and Schifino-Wittmann, 2002). Moreover, some studies have showed that inbreeding depression can also contribute for decreasing pollen viability (Krebs and Hancock, 1990; Wills, 1993; Husband and Schemske, 1996; Goodwillie, 2000). In addition, there are many nongenetic causes of decline in pollen viability including pollen age and exposure to environmental stresses such as temperature and humidity (Stone

et al., 1995; Kelly *et al*., 2002). Although, pollen limitation and low fruit and seed set are known to be affected by pollen viability, it has received little attention in the literature (Stone *et al*., 1995).

As far as we know meiotic behavior and pollen viability studies are almost absent in Bromeliaceae. Even basic cytogenetic studies in this family are scarce, with only about 10% of the species having its chromosome numbers reported. In *Vriesea,* genus that comprises about 250 species, the chromosome numbers have been recorded for no more than 24 species (Lindschau, 1933; Gauthè, 1965; Weiss, 1965; Marchant, 1967; Brown and Gilmartin, 1989; Palma-Silva *et al*., 2004). In a previous study our research team reported constant chromosome numbers, regular meiotic behavior, and high pollen viability for 15 species of two genera, *Vriesea* and *Aechmea*, native from southern Brazil (Palma-Silva *et al*., 2004). *Vriesea gigantea* was not included in this study.

Vriesea gigantea Gaud. is a perennial bromeliad species endemic to Brazilian Atlantic Rainforest. It is nonautogamous and has a mixed mating-system (Paggi, 2006). Its distribution ranges from Espírito Santo to Rio Grande do Sul States (Reitz, 1983). The flowers of *V. gigantea* are bat pollinated (Sazima *et al*., 1999) and its small seeds are dispersed by wind. Besides, *V. gigantea* plays an important role in ecosystems, its vegetative structures form tanks that are able to hold many litters of water, providing a resource base for the associated biota (Benzing, 2000). Unfortunately, *V. gigantea* populations have been reduced due to poaching and habitat fragmentation. Because of that, this species has been included in the list of endangered species of Rio Grande do Sul State, Brazil.

The present study is part of a broad project, which aims to fill a gap in the knowledge on the basic biology of this ecologically, and horticultural important bromeliad genus. In a previous work the plant fertility of four *V. gigantea* populations was access by measuring plant and inflorescence size, flower production, fruit and seed set, flower and fruit set pattern and seed viability and germination rate (Paggi *et al*., 2007). In addition, pollination treatments showed evidence of pollen limitation. Here we aimed to complement

the previous study by evaluating the fertility in a higher number of *V. gigantea* populations, using a cytogenetics approach through meiotic behavior and pollen viability analysis. Specifically we aim to answer the following questions: (i) Do meiotic behavior and pollen viability differ among populations of *Vriesea gigantea*? If yes, what are the consequences for population's fertility? (ii) Is pollen viability affected by meiotic behavior in the studied populations? (iii) What is the role of pollen viability (quality) in the pollen limitation reported previously in *V. gigantea* (Paggi *et al*., 2007)? (iv) What are the conservationist consequences for low pollen quality for *V. gigantea*?

Materials and Methods

The populations analyzed in this study are listed in Table 1. Field collections were carried out from December to February (2004) during the reproductive flowering season of the species.

To obtain pollen mother cells (PMCs) undergoing meiosis, young inflorescences were selected when floral buds were 1.7–2.0 mm long. Floral buds were fixed for 24 h in 3:1 (absolut ethanol: glacial acetic acid) with a drop of saturated aqueous ferric chloride ($FeCl_3 \cdot 6H_2O$) at room temperature. After fixation, buds were transferred to 70% alcohol and stored in a freezer at –20 ℃. For slide preparation, the anthers were squashed in 45% acetic acid and stained with DAPI.

Pollen stainability and pollen morphology were used to access pollen viability. Flowers at anthesis were collected, fixed and stored as above described, without addition of ferric chloride. Squash preparations were stained following Alexander's method (1980), in which empty unviable pollen grain stain green and full viable pollen stain purple. Samples of 1000 pollen grains per flower were analyzed with at least three flowers per individual. Slides were examined and documented with a Zeiss Axioplan Universal photomicroscope. Statistical analyses were performed using the SAS software package (version 8, SAS Institute, Cary, North Carolina, USA).

Results and Discussion

Chromosome number — All plants analyzed by meiotic chromosome counting were diploid with a consistent number of 2n = 50 (Fig. 1). This is the first report on the chromosome number of *Vriesea gigantea.* The data are also congruent with the basic chromosome number for the Bromeliaceae family (x = 25) and *Vriesea* genus (Brown and Gilmartin, 1989; Palma-Silva *et al*., 2004).

Meiotic behavior — Meiotic behavior was analyzed in a total of 928 PMCs from 27 plants belonging to seven native populations of *V. gigantea* (Table 2). Wide ranges of meiotic stages were found in anthers within the same flower.

Most of the plants showed a regular meiotic behavior with 25 bivalents at diakinese/metaphase I (D/MI) (Fig. 1) and a regular disjunction in anaphase I/ telophase I (AI/TI). Meiotic abnormalities were observed in meiosis I and II though at low incidence (Table 1).

In the D/MI cells there was limited synapsis/desynapsis with two or four univalents (I). Due to the variation in chromosome size, it was often difficult to differentiate univalents and bivalents. However, since these plants had 25II (2n = 50), it could be assumed that the 26 or 27 elements observed in abnormal cells were 24II + 2I or 23II + 4I, respectively. In AI/TI were observed laggards (bivalent or chromosome) and unequal segregation (26/24 or 27/23). Few AI cells presented 26/26 or 25/26 elements, which could be explained by precocious chromatids separation. The migration of a bivalent to one pole could be the cause of some observed cells with 24/25 elements. Abnormal metaphase II (MII) cells presented 24, 26, 27, or 28 elements. Due to the small chromosome size it is difficult to determine whether the extra elements are chromosomes or chromatids. Missing or extra chromosome(s) in MII could be the result of unequal segregation in anaphase I.

The irregularities observed in AI/TI, as well as those recorded in MII, could be explained by the erratic behavior of univalents. The univalents, as a rule, fail to orient properly between the poles, either not segregating, randomly moving to one or the other poles, or dividing, as in mitosis, into their two chromatids (Swanson *et al*., 1981).

Despite of the abnormalities described above the meiotic behavior was quite regular, with means of 85-96% of normal cells in all populations analyzed. Statistic analysis did not detect differences in the frequencies of normal cells among populations (F=0.46, P=0.8328). Highly regular meiosis has been reported previously for other bromeliads species (Marchant, 1967; Palma-Silva *et al*., 2004).

Pollen viability — a total of 164.611 pollen grains from 56 plants from seven natural populations of *V. gigantea* were analyzed. Data are described in Table 3 and illustrated in Fig. 2.

High percentages of stained pollen grains with normal morphology were recorded (84 to 98%) for all investigated populations, indicating high pollen viability for the species. This is a reflex of the regular meiotic chromosome behavior observed in the same populations. In spite of the overall high pollen viability, differences among populations were significant (F = 8.69, P<0.0001).

The population of Praia do Araçá showed the lowest pollen viability, with values ranging from 68 - 99 % per plant. In this population a relatively high proportion of plants produced pollen grains that failed to stain and showed abnormal morphology (Fig. 2, A-D). Interestingly, as above described the meiosis was regular in this population indicating that the reduced pollen viability is likely not due to cytological abnormalities. Some studies have showed that inbreeding depression can contribute to pollen infertility (Krebs and Hancock, 1990; Wills 1993; Husband and Schemske, 1996; Goodwillie, 2000). In addition, there are a number of nongenetic causes of pollen inviability including pollen age and physical factors such as temperature and humidity (Kelly *et al*., 2002).

The relative role of each factor in affecting pollen viability in the Praia do Araçá is difficult to determine. However, lower plant fertility of Praia do Araçá population was previously reported by our research team for other parameters that measure the reproductive success: plant and inflorescence size, flower production, fruit and seed set, and germination rate (Paggi *et al*., 2007). In such study pollen supplementation experiments were performed indicating pollen limitation in *V. gigantea.* It was suggested that pollen limitation might have

contributed to the reduced plant fertility in Praia do Araçá population. In that previous study, pollen limitation was hypothesized to be due to pollinator limitation, as a result of disruption of the bat pollination mutualism. Nevertheless, pollen limitation comprises two components: quantity limitation and quality limitation. The low pollen viability (= low pollen quality) observed in the present study possibly plays a role in the pollen limitation previously reported for *V. gigantea* population of Praia do Araçá. Consequently, the low pollen viability could be responsible for the reduced fruit and seed set reported in this population (Paggi *et al*., 2007). As reviewed by Aizen and Harder (2007), many genetic studies demonstrate that poor-quality pollen can also reduce seed production, which was interpreted as a "quality limitation". Pollen limitation and low fruit and seed set are affected by pollen viability (Stone *et al*., 1995). According to Aizen and Harder (2007), *pollinator* limitation is only one component of "quantitative" pollen limitation, so these terms cannot be used as synonymous, though this practice is quite common. Similarly, in *Silene douglasii* var. *oraria* pollen quality as well as quantity might have contributed to pollen limitation due to the moderate levels of selfing and inbreeding depression reported for the species (Wilcock and Neiland, 2002).

In order to draw conclusion, detailed studies including cross- and self-pollination experiments are required to assess the extent of inbreeding depression in this mixed mating system species and its effects on pollen limitation and fitness. Moreover, molecular marker-based assessment of inbreeding coefficients and outcrossing rates in natural populations and progeny arrays (Weir and Cockerham, 1984; Ritland, 2002) will also help to evaluate the magnitude of inbreeding effects in V. *gigantea*. To understand the causes of pollination failure in plants can aid the successful conservation and management of the endangered plant species.

Acknowledgements

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Figure legends

Fig. 1 Meiotic behavior in pollen mother cell (PMCs) of *V. gigantea* stained with DAPI. A. Metaphase I, 25 bivalents, B. Metaphase II, n = 25. Bar = 10 μm.

Fig. 2 Pollen grains in *V. gigantea*. A – D, pollen grains with abnormal morphology. E, U = unviable pollen are empty and stain green; V = viable pollen are full and stain purple. Bar = 20 μm.

Table 1. Location of the seven studied populations of *Vriesea gigantea.*

Population	**Latitude S**	**Longitude W**
Torres	29°18'21"	49°43'47"
Maquiné	29°48'19"	50°16'37"
Praia do Araçá	30°22'05"	51°02'24"
Morro da Grota	30°22'14"	50°01'18"
Pedra do Fenômeno	30°21'39"	51°01'51"
Porto Alegre	30°04'12"	51°08'25"
Taim	32°29'59"	52°34'54"

Table 2. Frequency (percentage) of pollen mother cells (PMCs) showing regular meiotic behavior.

Populations	N° of plants	Total nº of cells	Regular cells%	D/MI [a]		AI/TI [b]		MII [c]	
				nº of cells	%normal	nº of cells	%normal	nº of cells	%normal
Torres	3	92	85.0 (± 8.5)	52	98.0	20	98.0	20	93
Maquiné	1	17	88.2	11	99.0	3	99.0	3	100
Praia do Araçá	9	385	93.3 (± 1.3)	236	93.0	96	88.0	53	95
Morro da Grota	2	70	92.0 (± 1.7)	46	95.0	17	99.0	7	100
Pedra do Fenômeno	5	121	91.0 (± 1.5)	102	91.0	11	100.0	8	98
Porto Alegre	1	50	96.0	21	98.0	9	100.0	18	100
Taim	6	193	91.0 (± 5.0)	116	95.0	16	100.0	59	83

[a] D/MI = diacinese/metaphase I; [b]AI/TI = anaphase I/telophase I; [c] MII = Metaphase II; [d] AII/TII = anaphase II/telophase II.

Table 3. Percentage of pollen stainability in populations of *Vriesea gigantea.*

Population	**N° of plants**	**N° of flowers**	**N° of pollen grains**	**Percentage of stained grains [a]**
Torres	5	15	15000	97.5 (± 2.09) a
Maquiné	5	15	15000	97.3 (± 1.56) a
Praia do Araçá	9	27	26591	84.0 (± 13.1) b
Morro da Grota	9	27	26566	98.4 (± 1.05) a
Pedra do Fenômeno	18	51	51454	93.3 (± 18.4) a
Agronomia	3	18	9000	97.0 (± 2.02) a
Taim	7	21	21000	93.5 (± 6.81) a

[a] Values are means with minimum and maximum ranges. Means with the same letter are not significantly different by Tukey test (5%).

Fig 1

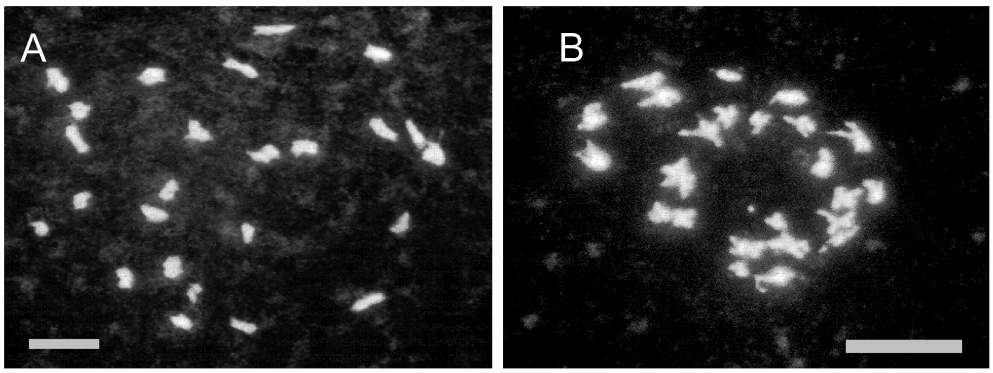
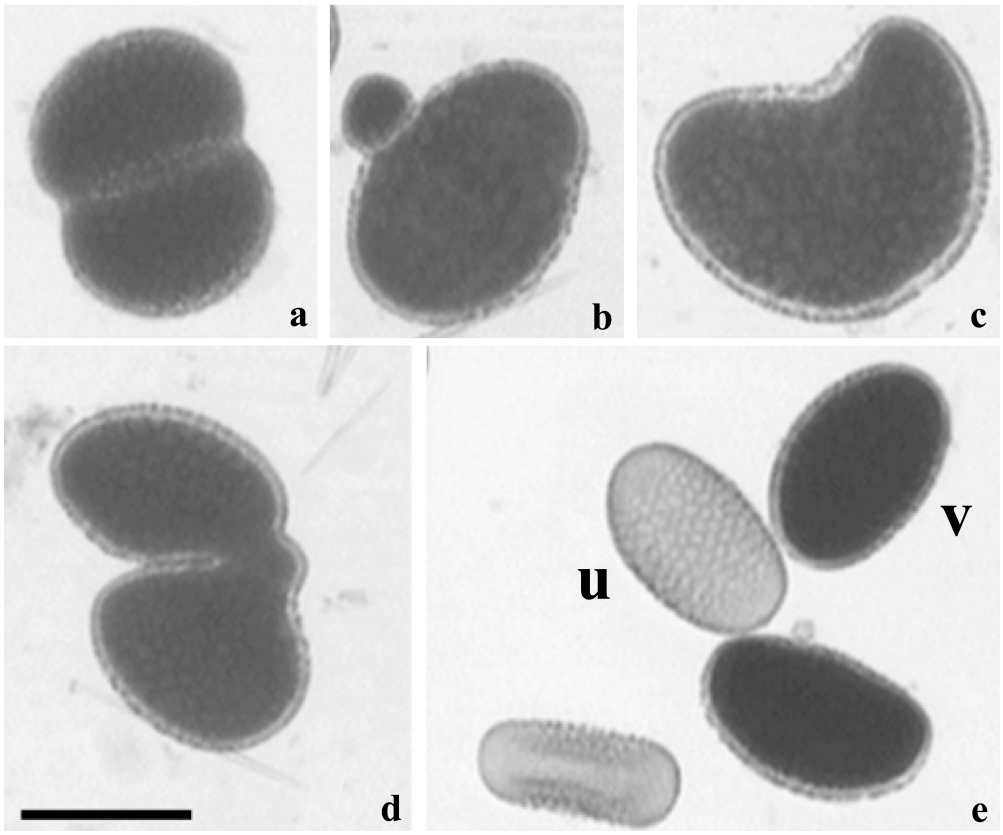


Fig 2



Capítulo 6

Considerações finais

Considerações finais

A presente tese está dividida em quatro artigos relacionados a um amplo projeto que tem como objetivo contribuir para o entendimento de questões relacionadas à evolução de famílias de plantas Neotropicais, com ênfase na família Bromeliaceae. O conjunto de dados obtidos nestes trabalhos descreve um panorama inicial que contribuirá para o esclarecimento da filogeografia e genética de populações de espécies de plantas nativas da região sul e sudeste do Brasil. Ainda, este estudo contribuirá para o preenchimento de lacunas do conhecimento existentes nesta família vegetal e, mais especificamente, auxiliará na compreensão de aspectos da história evolutiva e da dinâmica das populações de *Vriesea gigantea*.

I) Histórico

Os estudos sobre aspectos evolutivos da família Bromeliaceae iniciaram no ano de 2000, com o projeto de mestrado intitulado "Análises citogenéticas em espécies de *Vriesea* e *Aechmea* (Bromeliaceae) nativas do Rio Grande do sul". Inicialmente, foi constatado que, apesar da família Bromeliaceae ser uma das famílias de plantas mais características do Neotrópico e possuir uma enorme riqueza de espécies, estudos referentes a aspectos evolutivos, genética de populações, microevolução, filogeografia, sistema de cruzamento, biologia reprodutiva, citogenética e delimitação de espécies são muito escassos. Infelizmente, esta escassez de conhecimento não ocorre somente nesta família de plantas, mas de uma forma geral para a maioria dos organismos que ocorrem no Neotrópico, uma das regiões mais ricas em biodiversidade do mundo, onde são reconhecidos pelo menos cinco "hotspots" de biodiversidade para conservação (Myers *et al.,* 2000).

Com a preocupação de continuar a contribuir para o entendimento de questões relacionadas à evolução de famílias de plantas Neotropicais, principalmente na família Bromeliaceae, em 2004 a espécie *Vriesea gigantea* foi escolhida por nosso grupo como "planta modelo" para estudo. O objetivo foi aprender e aplicar as técnicas de biologia molecular, abrindo as portas para estudos desta natureza em outras espécies de Bromeliaceae. Este projeto

inicial foi dividido em: (a) genética de populações e filogeografia; (b) biologia reprodutiva e (c) fertilidade das populações nativas *V. gigantea*. Desta forma, foi contemplada grande parte dos assuntos relacionados aos aspectos básicos da biologia e evolução desta espécie, os quais são essenciais para a utilização em programas de manejo e conservação. Na presente tese foram incluídas investigações sobre: genética de populações, filogeografia e fertilidade das populações. Biologia reprodutiva e fertilidade são temas de estudo de outra tese de doutorado do nosso grupo. O artigo referente à fertilidade de populações de *V. gigantea* nativas do sul do Brasil está em anexo.

No final do ano de 2004 foi oficializada a colaboração com o Dr. Christian Lexer, pesquisador do Jodrell Laboratory, Royal Botanic Gardens – Kew – Londres, Inglaterra, para a realização de estudos relacionados à genética e evolução de espécies de bromélias nativas do Brasil.

A colaboração efetiva iniciou num trabalho de campo realizado em conjunto no Rio de Janeiro, no ano de 2004. O intercâmbio foi reforçado com a visita, de duas semanas, ao Jodrell Lab.-Kew, em julho de 2005, quando foi iniciada a preparação de um artigo de revisão sobre as bases biológicas da amplificação heteróloga de marcadores microssatélites publicado no periódico Molecular Ecology (Anexo 2). Em novembro do mesmo ano, recebemos em nosso laboratório a visita do Dr. Lexer e sua aluna de doutorado Thelma Barbará, cujo projeto de pesquisa está relacionado à genética de populações de quatro espécies de bromélias endêmicas de afloramentos rochosos (*Inselbergs*) da Mata Atlântica: *Alcantarea imperialis, A. geniculata, A. regina* e *A.glaziouana*, as quais representam modelos para estimar os efeitos da conectividade populacional em plantas adaptadas a *inselbergs*.

No ano de 2006, recebi um convite para desenvolver parte do projeto de doutorado no Jodrell Lab.-Kew, através do programa KLARF (Kew Latin American Research Fellowship), o qual custeou a estadia durante seis meses e todas as despesas de laboratório, nesta instituição. Durante este curto período, foram testados marcadores microssatélites desenvolvidos para *V. gigantea* (Capitulo 2) e foram realizadas as análises moleculares da diversidade genética populacional da espécie em estudo, utilizando

microssatélites nucleares (Capitulo 3). A finalização deste estudo e o desenvolvimento de uma nova etapa relacionada ao polimorfismo do genoma de cloroplasto (Capitulo 4) foram realizadas em 2007 durante o estágio de doutorado no exterior (PDEE – CAPES), também de seis meses.

II) Desenvolvimento de marcadores microssatélites nucleares para espécies de Bromélias.

Quando foi iniciado o estudo de genética de populações de *V. gigantea*, haviam sido descritos somente 12 *loci* de microssatélites para espécies de Bromeliaceae que ocorrem na América Central. Sarthou *et al*., (2003) desenvolveram sete *loci* de microssatélites para *Pitcairnia geyskesii* (subfamília Pitcairnioideae) e Boneh *et al*., (2003) descreveram microssatélites para *Tillandsia fasciculata* e *Guzmania monostachya*, ambas espécies pertencentes à subfamília. Os primeiros testes de amplificação heteróloga destes *loci* em *V. gigantea* foram positivos para os cinco *loci* descritos por Boneh *et al.* (2003), o que era esperado uma vez que estas espécies pertencem à mesma subfamília: Tillandsioideae. Já dos sete *loci* desenhados por Sarthou *et al.* (2003) somente um amplificou em *V. gigantea,* concordando com os resultados de Barbará *et al.* (2007b), que mostraram que a amplificação heteróloga é mais provável em espécies evolutivamente relacionadas. Os testes de polimorfismos destes seis *loci* que amplificaram positivamente mostraram que somente três deles eram polimórficos. Um *loci* foi monomórfico (e6), um apresentou excesso de homozigotos, provavelmente devido a alelos nulos (p2p19), e por fim o único *locus* desenvolvido para *P. geyskessi* (Pit8) que amplificou em *V. gigantea* foi também monomórfico.

Tendo em vista o número pequeno de *loci* disponíveis para o estudo de genética de populações em *V. gigantea* foram isolados e caracterizados novos *loci* para esta espécie. Os dados referentes aos 11 *loci* desenvolvidos para *V.gigantea* foram publicados em conjunto com os referentes á quatro *loci* desenvolvidos para *A. imperialis* (Palma-Silva *et al.,* 2007 - Capítulo 2).

Além do isolamento e caracterização de *loci* de microssatélites, também foram realizados testes de amplificação heteróloga em outras 22 espécies de

bromélias pertencentes as três subfamílias de Bromeliaceae. Os resultados mostraram que os *primers* desenvolvidos poderão ser úteis para estudos em outras espécies de Bromeliaceae, principalmente daquelas pertencentes à subfamília Tillandsioideae.

Com o objetivo de aumentar o número de *loci* de microssatélites para espécies de Bromélias, outros oito *loci* foram isolados e caracterizados para a espécie *Pitcairnia albiflos* (Anexo 3), a qual é endêmica de *inselbergs* do Rio de Janeiro. Os resultados de amplificação heteróloga também indicaram que esses *primers* poderão ser úteis para espécies de bromélias proximamente relacionadas.

Sendo assim, até o momento existem 35 *loci* de microssatélites desenvolvidos para espécies de bromélias pertencentes às subfamílias Tillandsioideae e Pitcairnioideae.

III) Genética das populações de *Vriesea gigantea*

Este trabalho acessou e interpretou os padrões de diversidade do genoma nuclear de uma espécie de planta da Mata Atlântica do Sul e Sudeste do Brasil, *Vriesea gigantea,* ao longo de uma ampla distribuição geográfica. A Mata Atlântica é considerada um dos 25 "hotspots" de biodiversidade em nosso planeta, no entanto são escassas as informações disponíveis no que diz respeito à ecologia molecular e filogeografia de espécies de plantas e animais que ocorrem nesta região.

Os padrões de diversidade genética evidenciaram uma tendência latitudinal de diminuição da diversidade genética nuclear do Norte para o Sul. Provavelmente estes padrões observados de diversidade genética em *V. gigantea* foram afetados pelas flutuações climáticas do Pleistoceno tardio, coincidindo com os padrões históricos de expansão relatados para a Mata Atlântica. A distribuição da vegetação na região costeira do Brasil foi fortemente influenciada pelas mudanças climáticas que ocorreram durante o Quartenário. As mudanças de temperatura influenciaram o tipo e a distribuição da vegetação e alteraram os níveis do mar, o que também teve forte influência na distribuição da vegetação ao longo da costa brasileira (de Oliveira *et al.,*

2005). Com o aumento da umidade e da temperatura durante o Quaternário, a Mata Atlântica expandiu-se, colonizando a região sul do Brasil, sendo este evento considerado recente (Behling, 1998; Behling, 2002; Rull, 2006).

A contínua expansão de *V. gigantea* parece ter sido impedida pela diminuição do fluxo gênico, observado nas margens da distribuição da espécie, aumentando assim o efeito da deriva, o que por conseqüência também pode ter ocasionado o aumento da diferenciação entre as populações. Interessantemente, em *V. gigantea*, a história evolutiva das margens (Norte e Sul) parece ter sido completamente diferente. A limitação da expansão da margem Sul parece ser afetada principalmente por fatores abióticos ("ambientais"), como o clima mais frio e seco. Já a expansão da margem Norte deve ter sido afetada por fatores bióticos ("parapátricos"), como interações entre espécies, por exemplo, competição. Apesar da diversidade de espécies e a diversidade genética serem domínios da ecologia de comunidades e genética de populações, respectivamente, estes dois níveis de biodiversidade têm sido considerados fenômenos relacionados. Os resultados do presente estudo demonstram que os padrões de diversidade de espécies do gênero *Vriesea* e de diversidade genética em *V. gigantea* estão conectados e possivelmente foram moldados pelas mesmas forças históricas, as mudanças climáticas do Quartenário.

IV) Filogeografia de *Vriesea gigantea*

O genoma do cloroplasto em espécies de Bromeliaceae é considerado pouco variável, mesmo em nível intergenérico. Por isso, poucos estudos apostaram na busca de variabilidade genética intraespecífica neste grupo (Sgorbati *et al.,* 2004 e Barbará, dados não publicados), sendo que estes dois estudos não encontraram variação suficiente para estudos populacionais. Os resultados apresentados na presente tese indicaram que a diversidade intraspecífica do genoma de cloroplasto é extremamente baixa. No entanto, devido ao grande tamanho amostral e ao grande número de regiões seqüenciadas (21 regiões), foi possível obter cinco regiões polimórficas do

cloroplasto: quatro microssatélites e um SNP (*Single Nucleotide Polimorfismo*), que puderam ser utilizadas nas análises filogeográficas de *V. gigantea*.

Para cada uma das quatro regiões que apresentaram polimorfismos de microssatélites foram desenhados *primers* específicos. Este estudo foi pioneiro no desenvolvimento de *primers* específicos de microssatélites plastidiais, para uma espécie de bromélia. Os produtos de amplificação foram genotipados em seqüenciador automático de DNA ABI 3100 (Applied Biosystems, Foster City, CA, U.S.A.) o que diminuiu o custo e o tempo das análises. Estudos de amplificação heteróloga já estão sendo realizados com essas regiões para outras duas espécies de bromélia (*Bromelia antiacantha* – Pelo nosso grupo de pesquisa do Dep. de Genética/UFRGS e *Vriesea incurvata* – por pesquisadores da Universidade de Alicante, Espanha). Os resultados preliminares indicam que, provavelmente, essas regiões poderão ser utilizadas em estudos futuros com outras espécies desta família, disponibilizando informações importantíssimas relacionadas a filogeografia e biologia evolutiva destas espécies.

Os dados obtidos para o genoma de cloroplasto foram combinados com os obtidos para o genoma nuclear indicando que o fluxo gênico através do pólen é 3,3 vezes mais eficiente do que o mediado por sementes. Estes dados estão de acordo com a dispersão restrita observada para sementes anemocóricas (Greene e Jonhson, 1989; Bullock e Clarke, 2000), assim como o observado para espécies da subfamília Tillandsioideae (Castante-Marín, 2005), a qual incluiu o gênero *Vriesea.* O restrito fluxo gênico mediado por sementes pode ser, também, observado na baixa diversidade do DNA de cloroplasto (cpDNA) encontrada nas populações de ilhas (PTRJ, PAPR, FLSC), indicando assim que o mar representa uma barreira para a dispersão de sementes. Assim sendo, nossos resultados apóiam o que é sugerido por observações biológicas, que mostram que o pólen é disperso a distâncias mais longas do que as sementes, o que resulta em uma estrutura genética mais forte para o genoma do cloroplasto do que para o genoma nuclear. Além disso, a variação do cpDNA é geograficamente estruturada em *V. gigantea.* A espécie provavelmente foi fragmentada em dois grupos filogeográficos: um

compreendendo a região Norte e o outro as regiões Sul e Centro. Esses dois grupos filogeográficos possivelmente tiveram histórias evolutivas diferentes que foram fortemente afetadas pelas flutuações climáticas durante o Pleistoceno tardio. As populações da região Norte são mais antigas e estáveis e tiveram um crescimento populacional ancestral. Já as populações das regiões sul e centro, provavelmente são mais recentes.

V) Fertilidade em populações de *V. gigantea*: viabilidade do pólen e comportamento meiótico.

A fertilidade das plantas foi, de uma forma geral, considerada alta. Todas as plantas estudadas apresentaram 2n = 50 cromossomos, pareados em 25 bivalentes e segregando regularmente. Das sete populações analisadas, somente a Praia do Araçá, mostrou valores significantemente mais baixos de viabilidade dos grãos de pólen, apesar da meiose ter sido regular. A redução na viabilidade do pólen desta população pode ter efeitos importantes na fertilidade das plantas e conseqüentemente no sucesso reprodutivo desta população. Este estudo complementa um trabalho anterior realizado por nossa equipe (Paggi *et al.,* 2007 – anexo I), no qual foi demonstrado que a população da Praia do Araçá apresentou menor produção de frutos e menores índices em todos os outros parâmetros analisados. Como conseqüência, a baixa viabilidade dos grãos de pólen pode ter um importante papel na limitação de pólen, observada através de experimentos de polinização manual, nesta população (Paggi *et al.,* 2007). Os autores apontaram a limitação do pólen como uma das causas para a menor fertilidade das plantas da Praia do Araçá.

Depressão por endocruzamento ou fatores ambientais como idade do pólen, temperatura e umidade podem ser causas de reduzida viabilidade do pólen, no entanto estudos mais aprofundados deverão ser realizados com o objetivo de avaliar as taxas de autofecundação e índices de depressão por endocruzamento para que conclusões sobre as possíveis causas e conseqüências da reduzida fertilidade das plantas na Praia do Araçá possam ser inferidas.

Capítulo 7

Referências Bibliográficas dos Capítulos 1 e 6

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Capítulo 8

Anexos

Anexos

Os trabalhos dos anexos I-IV, embora não façam parte do corpo integral da tese, foram realizados com a minha participação durante o período de doutorado. Como abordam o tema geral da tese, foram incluídos como anexos e estão dispostos de forma cronológica. O Anexo são V apresenta as figuras da tese citadas no capítulo 1 e 6.

O primeiro trabalho é um manuscrito completo intitulado "Fertility of *Vriesea gigantea* gaud. (Bromeliaceae) in southern Brazil". Este estudo mostrou que *V. gigantea* é uma espécie autocompatível e suas populações do sul do Brasil são altamente férteis. Além disso, foi observada limitação de pólen em populações desta espécie.

O segundo trabalho é um artigo de revisão intitulado "Cross-species transfer of nuclear microsatellite markers: potential and limitations". Nesta revisão foi estudada a distribuição do sucesso de amplificação heteróloga de loci de microssatélites nucleares entre animais, planta e fungos, sugerindo que a taxa de amplificação heteróloga é bastante irregular entre espécies, sendo maior em animais e altamente variável em plantas. O potencial de sucesso na amplificação heteróloga parece estar relacionado com o tempo de geração, sistema de reprodução e tamanho do genoma da espécie alvo comparado com o da espécie para a qual os *loci* foram desenvolvidos.

O terceiro é o artigo "Isolation and characterization of microsatellite loci in *Pitcairnia albiflos* (Bromeliaceae), an endemic bromeliad from the Atlantic Rainforest, and cross-amplification in other species", o qual descreve oito novos *loci* de microssatélites nucleares e a amplificação heteróloga dos mesmos em 16 outras espécies de bromélias.

Por fim, o quarto trabalho "Bromélias, beleza exótica do novo mundo" faz parte do livro "Origem e Evolução de Plantas Cultivadas" publicado pela EMBRAPA. Neste capítulo foram revisados vários aspectos referentes à taxonomia e evolução, citogenética, sistema reprodutivo e história antiga e recente da utilização de espécies de bromélias.

Anexo I

Fertility of *Vriesea gigantea* Gaud. (Bromeliaceae) in southern Brazil

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FERTILITY OF *VRIESEA GIGANTEA* GAUD. (BROMELIACEAE) IN SOUTHERN BRAZIL[1]

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Plant fertility is a central subject of many questions in plant evolutionary and conservation biology. Pollen availability, abiotic resources, and flowering pattern can limit fruit and seed production. Open pollination and pollen supplementation studies are used to estimate any pollen limitation in natural populations. To study the impact of these factors on the reproductive success of *Vriesea gigantea*, an epiphytic bromeliad in the Atlantic Rainforest in Brazil, its fertility in four natural populations in Itapuã State Park was assessed by considering plant and inflorescence size, flower production, fruit and seed set, flower and fruit set pattern, and seed viability and germination rate. Supplemental pollination in adult plants was used to determine whether fruit production in *V. gigantea* is limited by reception of pollen. The results showed that *V. gigantea* has a high production of flowers, fruits, and seeds. Seeds are highly viable in all populations, presenting an average germination rate of 94% (SE ± 3.5). Plants of *V. gigantea* from Itapuã State Park are highly fertile. The high proportion of fruit and seed set after manual hand pollination indicates that the species is self-compatible. Pollination treatments showed evidence of pollinator limitation in the Itapuã State Park population.

Key words: Bromeliaceae; flower production; fruit set; Itapuã State Park; pollen limitation; seed viability; self-compatibility.

Plant fertility and limits to fertility are issues of central importance to many questions in plant evolutionary biology and conservation biology. The degree of pollen limitation on fertility will affect the role of female mate choice in plants, the opportunity for selection on floral traits, or the possibility of changes in the mating system (Charlesworth et al., 1987; Fenster and Ritland, 1994; Dudash and Fenster, 1997). From a conservation point of view, an important question is whether fertility in plants is limited primarily by pollen or resource availability (Willson and Burley, 1983; Haig and Estoby, 1988; Huang and Guo, 2002). The disruption of plant-pollinator mutualisms has the potential of affecting the viability of populations. This phenomenon may occur when habitat fragmentation directly affects the pollinator populations or when plant population drops below the critical size threshold needed to attract and sustain pollinators (Johnson et al., 2004). Forest fragmentation and the resulting spatial isolation of plant species can modify the activity of pollinators and may have important implications for the reproductive success and reproductive systems in plants they pollinate (Byers, 1995). Moreover, pollinator limitation in natural plant populations may account for low fruit and seed set and may have profound consequence for the evolutionary ecology of plants, including, for example, the evolution of selfing (Schemske and Lande, 1985).

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Several features associated with plant fertility have been used to determine population viability, such as plant size, flower production, fruit and seed set, pattern of fruit production, pollen limitation, and seed viability (Ishii and Kadono, 2002; McIntosh, 2002; Burne et al., 2003; Ortiz et al., 2003; Buide, 2004; Clark-Tapia and Molina-Freaner, 2004; Johnson et al., 2004; Kéry and Matthies, 2004; Hampe, 2005). The fruit and seed production in plants can be limited by the availability of pollen and abiotic resources. The decrease of seed production can be the result of insufficient pollinator visits or reduction in both pollen quantity and pollen quality deposited per visit (Huang and Guo, 2002; Ashman et al., 2004; Buide, 2004). Abiotic resource limitations include inadequate soil nutrients, water, light, and climatic factors (Willson and Burley, 1983; McIntosh, 2002; Gaudeul and Till-Bottraud, 2004). Although pollen limitation and abiotic resource limitation appear to be two alternative hypotheses for inadequate seed production in plants, some authors have suggested that in equilibrium, seed production in plants should be limited by both factors (Haig and Westoby, 1988; Dogterom et al., 2000).

The flowering pattern is another intrinsic feature that may limit the fruit and seed production. When fruit set is low, the distribution of fruits and seeds within an individual plant is not uniform, and fruit maturation has frequently been found to decrease from the base to the apex of individual inflorescences (Diggle, 1995; Ortiz et al., 2003). This could be a consequence of the opening sequence because flowers that open first have the opportunity to be pollinated and fertilized earlier (Diggle, 1995).

The Bromeliaceae family has nearly 3000 species divided into three subfamilies: Pitcairnioideae, Bromelioideae, and Tillandsioideae (Smith and Downs, 1974). *Vriesea gigantea* Gaud. (Tillandsioideae) an epiphytic, saxicolous, and terrestrial bromeliad that grows in the Atlantic Rainforest, Brazil, is greatly valued as an ornamental plant. Wild populations of *V. gigantea* have been reduced by anthropogenic disturbance of

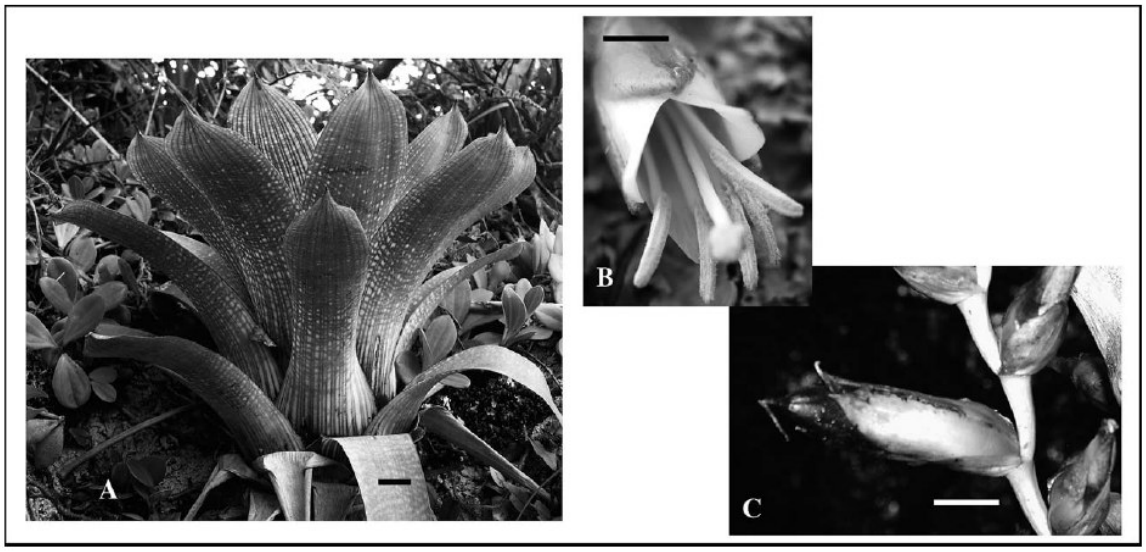

Fig. 1. *Vriesea gigantea*. (A) Aspect of plant, bar = 1 cm; (B) detail of flower, bar = 1 cm; (C) detail of fruit, bar = 0.5 cm.

their habitat and by predatory collection practices, causing inclusion of *V. gigantea* in the list of endangered species of the state of Rio Grande do Sul. In spite of this, no publications are currently available about the fertility of *V. gigantea*. Here we aim to fill this gap to aid conservation plans for this ecologically and horticulturally important species and to initiate studies on the fundamental biology of this charismatic bromeliad genus.

The investigation of the relative importance of the factors described will help elucidate their impact on the overall reproductive success of *V. gigantea*. To evaluate the fertility of *V. gigantea* in four populations from southern Brazil, we aimed (1) to record production of flowers, fruits, and seeds, the pattern of fruiting in the inflorescence, and the heights of plants and inflorescences; (2) to evaluate whether populations differ in fertility; (3) to investigate whether fruit and seed set are limited by pollen or abiotic resources; and (4) to determine the levels of seed germination and viability.

MATERIALS AND METHODS

Study species—Vogel (1969) and Sazima et al. (1999) described *Vriesea gigantea* as an outcrossing bat-pollinated bromeliad species (Fig. 1A) growing from Espírito Santo (ES) to Rio Grande do Sul (RS) in the Atlantic rainforest in southeastern and southern Brazil, respectively. Furthermore, *V. gigantea* plays an important role in ecosystems, because its vegetative structure forms reservoirs that are able to hold many liters of water, providing a resource base for the associated biota (Benzing, 2000). A typical inflorescence has a central axis with several branches on each side and one flower on each side of the lateral axis (Reitz, 1983). The flowers (Fig. 1B) are tube-shaped with three petals that are colored in accord with a chiropterophilous syndrome (Vogel, 1969). Flowers in the basal position of the inflorescence open first, whereas flowers in the center and apex position open later (Reitz, 1983; Benzing, 2000).

Study sites—The Itapuã State Park (50°50′ and 51°05′ W, 30°20′ and 30°27′ S) is located in Viamão, RS, 57 km from Porto Alegre (Fig. 2). The average temperature for the area is around 17.5°C, and annual rainfall averages 1200 mm/year. The four populations studied are located in the Trilha do Fenômeno, Praia do Araçá, Pedra da Visão, and Morro da Grota, which are 5–25 km apart (Table 1). *Vriesea gigantea* is common in vegetation predominated by *Butia capitata* (Mart.) Becc. and *Ficus organensis* (L.). In the Itapuã State Park there are more than 1000 adult plants of *V. gigantea* manly in clustered populations.

Reproductive success: flower production, fruit, and seed set—Field studies were carried out in 2004 during the reproductive period of the plants (August). Reproductive success was evaluated through flower production, fruit, and seed set with the following measures: (1) plant and inflorescence height (m); (2) the reproductive potential– (total number of flowers per plant); (3) fruit set (i.e., the fraction of flowers developing into a mature fruit) as % fruits = total number of fruits per plant/total number of flowers per plant × 100 (according to Burne et al., 2003); (4) number of seeds per fruit from five fruits randomly collected from 10 plants– (estimated by comparing the mass of 20 seeds with the mass of all seeds from each fruit); (5) flower and fruit set position (proportion of flowers and fruit set in the base, center, and apex of the inflorescence). The position of each flower and fruit was recorded for each inflorescence and was classified into three categories: apex (when found in the upper third of the inflorescence), center (in the middle third), or base (in the lower third).

Seed viability and germination rate—Seed viability and germination rate are among the less studied aspects of bromeliad fertility and reproductive biology (Benzing, 2000). These aspects vary within Bromeliaceae because most species respond differently to light and temperature (Downs, 1963; Benzing, 2000). To analyze seed viability, five plants of *V. gigantea* were randomly sampled in each population (August 2004), totaling 20 plants. Two fruits (30 seeds/fruit) were used per plant. Seeds were disinfected and placed in petri dishes with a culture medium, containing ½ MS salts (Murashige and Skoog, 1962), B5 vitamins (Gamborg et al., 1968), 3% sucrose, and 0.3% Phytagel (Sigma, Saint Louis, MO, USA), pH 6.4. The dishes were incubated in a climate-control chamber with relative humidity near 100% and photoperiod of 16 h light at 25°C and 8 h dark at 22°C. Germination was monitored daily for 30 d.

Pollen supplementation—Field studies were conducted during the plant reproductive cycle, from January to March and in August 2005. To determine whether fruit production in *V. gigantea* is limited by reception of pollen, we performed supplemental pollination in the four populations studied. The pollination treatments were accomplished as follows:

(1) Open-pollination (OP)—flowers, which opened during the day and were available to any visitor, were tagged; (2) manual self-pollination (MSP)—when flowers opened around midday and pollen was available, they were hand-

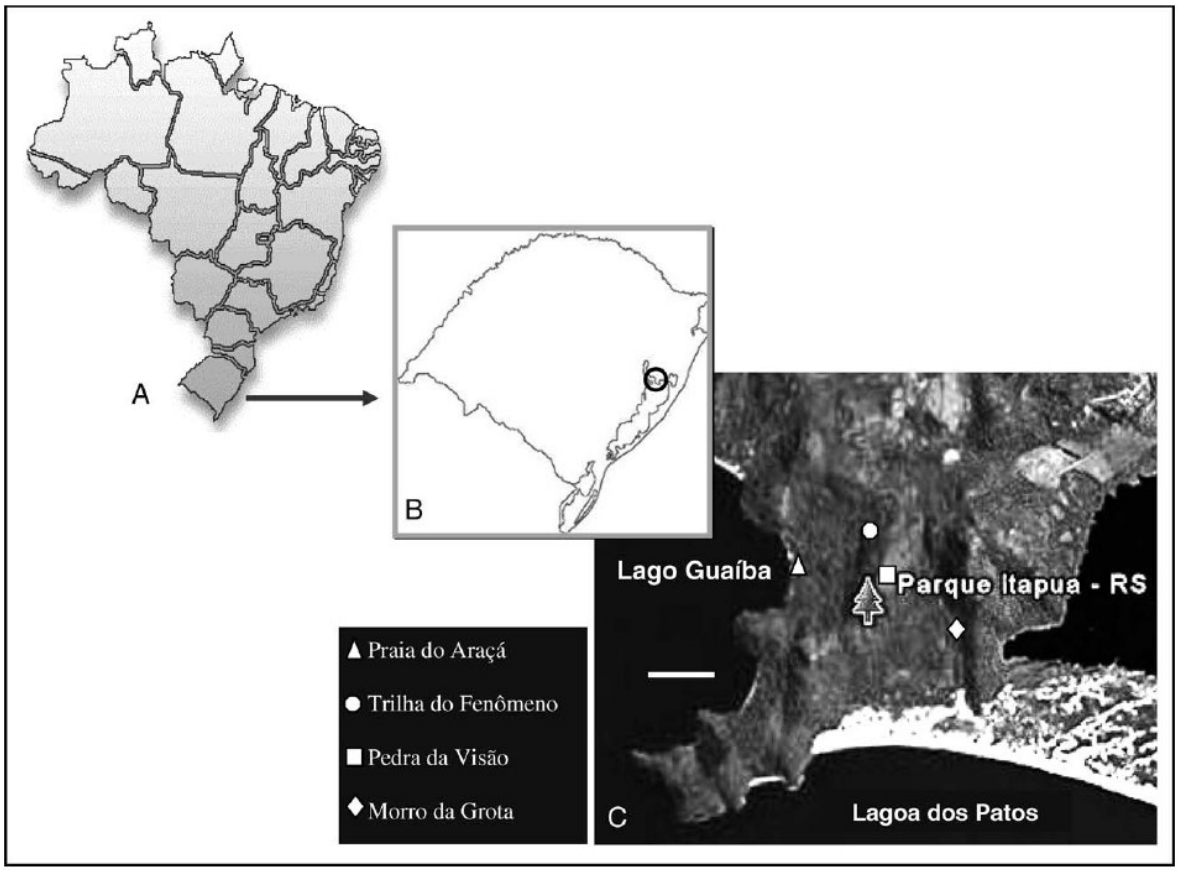

Fig. 2. (A) Map of Brazil; (B) Rio Grande do Sul state; (C) Parque Estadual de Itapuã (Itapuã State Park) and the locality of the four study populations of *Vriesea gigantea*. Bar = 25 km.

pollinated by rubbing anthers with fresh pollen in the stigma from the same flower and then bagged. Two or three flowers from 16 plants distributed throughout the populations were pollinated, whereas three other single flowers on each plant were marked as control (OP treatment). The results (i.e., aborted or developing fruit) of the pollination treatments were recorded 6 months later, and the fruit set was calculated using the formula described earlier. Mature fruits were collected and the number of seeds per fruit estimated by the mass method. To evaluate whether pollen supplementation affected seed set, the number of seeds from hand-pollinated and naturally pollinated fruits were compared.

Statistical analyses—All statistical analyses were performed using the SAS software package (version 8, SAS Institute, Cary, North Carolina, USA). When necessary, data were transformed to stabilize variances and achieve approximate normality of residuals. The plant height parameter and pollen-supplementation per population data were analyzed by the nested Kruskal-Wallis test. The effect of pollen supplementation per treatment was analyzed by the Wilcoxon two-sample test. The inflorescence height (square-root transformed), flower production, fruit set, flower/fruit set position, and the number of seeds per fruit were analyzed by the nested ANOVA test followed by the Tukey test to determine if the differences among plants or populations were significant.

TABLE 1. Location of the four study populations of *Vriesea gigantea* in Itapuã State Park, Brazil.

Population	Location
Morro da Grota	30°22′13.3″ S, 51°01′42.2″ W
Pedra da Visão	30°22′40.2″ S, 51°01′44.7″ W
Trilha do Fenômeno	30°21′40.3″ S, 51°01′51.1″ W
Praia do Araçá	30°21′31.5″ S, 51°02′41.1″ W

RESULTS

Reproductive success: flower production, fruit, and seed set—The overall means of plant and inflorescence height were 0.95 m (SE ± 0.045) and 1.51 m (SE ± 0.044), respectively, without significant differences among populations (Table 2). The mean of flowers per plant was 143.93 (SE ± 11.08), without significant differences among populations (Table 3). The fruit set per plant ranged from 9.88 to 69.92% with an overall mean of 42.71% (SE ± 8.24). Significant differences were detected among populations. Fruit set was higher in Pedra da Visão and lower in Trilha do Fenômeno and Praia do Araçá populations (Table 3). The number of seeds per fruit ranged from 0 to 839, with significant differences among plants (Fig. 3). Plants number 6, 8, and 10 had significantly lower production of seeds per fruit when compared with plant number 1; no seeds were obtained from plant 9. The architecture of the inflorescence did not affect the flower

TABLE 2. Reproductive success: means of plant and inflorescence height of the four studied populations of *Vriesea gigantea* in Brazil. Values shown are means ± 1 SE.

Population	Analyzed plants (*N*)	Plant height (m)[a]	Inflorescence height (m)[b]
Morro da Grota	6	1.04 ± 0.108	1.70 ± 0.090
Trilha do Fenômeno	8	1.03 ± 0.057	1.57 ± 0.053
Pedra da Visão	5	0.93 ± 0.103	1.46 ± 0.037
Praia do Araçá	4	0.78 ± 0.080	1.29 ± 0.053
Total	23	0.95 ± 0.045	1.51 ± 0.044

[a] Kruskal–Wallis test (5%), $P = 0.0941$.
[b] ANOVA (5%) (square-root transformed), $F_2 = 2.63$ and $P = 0.0757$.

production pattern (Fig. 4). On the other hand, fruits were not homogeneously distributed on the inflorescence: the upper third (apex) presented significantly lower fruit set than the lower third (base) (Fig. 4).

Seed viability and germination rate—The seed viability and germination rate of *V. gigantea* were very high. An average of 94% (SE ± 3.5) of seeds from open-pollinated flowers germinated in vitro. After 15 d in culture, most seeds had germinated.

Pollen-supplementation—There was a significant effect of pollen supplementation on fruit and seed set (Table 4). On average, 47.92% (SE ± 9.61) of open-pollinated flowers developed fruits. The mean fruit set of manual self-pollinated plants was 85.42 % (SE ± 5.67). Manual self-pollinated flowers produced more than twice as many seeds as naturally pollinated flowers (544.25 vs. 245.71). Data on pollen-supplementation per population are in Table 5. The mean seeds per fruit in Morro da Grota was significantly higher than in the Pedra da Visão population after manual self-pollination. Nonetheless, fruit set did not differ significantly among populations. In the open treatment (control), although the differences among populations were not significant, Praia do Araçá population yielded only a mean of 102.89 (SE ± 69.16) seeds per fruit, around 3.8 times lower than the Morro da Grota population (Table 5).

DISCUSSION

Reproductive success: flower production, fruit, and seed set—The adult individuals of *V. gigantea* presented a height general mean of 2.46 m, with inflorescences (Table 2). This is in agreement with Reitz (1983) who reported an average height of 2.00 m for adult individuals of this species. The four analyzed populations of *V. gigantea* produced a high number of flowers, fruits, and seeds. Considering mean of flowers per plant (143.93 ± 11.08), the mean of seeds per fruit (303), and taking into account that 43% of flowers developed into fruit, it is possible to estimate that one plant can produce about 18.753 seeds in each reproductive cycle. Intriguingly, although differences among populations were detected only on fruit set, plants from Praia do Araçá presented lower values in all parameters evaluated (Tables 2 and 3). These results suggest lower plant fertility in this population. Several factors may have been responsible for this phenomenon. As the Praia do Araçá population is located 5 m from Guaíba Lake (Fig. 2), local growth conditions may be quite different from those of other populations. McIntosh (2002) and Kéry and Matthies (2004) reported that the reduction in plant size, flower size, number of seeds per plant or fruit, and flower number in small populations can be a consequence of lower habitat quality, reduced pollination, or inbreeding depression. The relative importance of each factor in limiting fruit set in the Praia do Araçá population is difficult to determine. Nevertheless, we assumed those abiotic resources, such as climatic variation and soil conditions, and other factors such as pollen limitation may have contributed to the reduced plant fertility in this population.

A decrease in fruit set was observed from the base to the apex of the inflorescence in *V. gigantea* (Fig. 4). A similar result was reported by Ortiz et al. (2003) in *Stryphnodendron adstringens*. The reproductive success of flowering plants appears to be affected by a variety of factors, including the timing, frequency, duration of the flowering period, and flowering pattern (Rathcke and Lacey, 1985; Diggle, 1995; Ortiz et al., 2003). According to Thomson (1989), in some species the reproductive potential differs from one flower to another, e.g., flowers that open later may have fewer ovules, and thus they are less likely to produce fruits (usually found in the apex of the inflorescence). This is consistent with the hypothesis that plants tend to direct fewer resources to the production of ovules that have little chance of being converted into seeds (Ortiz et al., 2003).

Seed viability and germination rate—Seeds from all analyzed populations were highly viable, presenting a

TABLE 3. Reproductive success: means (±1 SE) of flower production and rate of fruit setting in four populations of *Vriesea gigantea* in Itapuã State Park in Brazil.

Population	Plants sampled (*N*)	Flower production[a]	Fruit set (%)[b]
Pedra da Visão	5	120.40 ± 10.19	69.92 ± 6.83[A]
Morro da Grota	8	182.25 ± 21.52	48.32 ± 14.53[A,B]
Trilha do Fenômeno	8	159.88 ± 17.48	42.72 ± 13.47[B]
Praia do Araçá	5	113.20 ± 26.82	9.88 ± 2.70[B]
Total	26	143.93 ± 11.08	42.71 ± 8.24

Note: Means with the same letter are not significantly different by ANOVA (5%).
[a] $F_2 = 2.50$ and $P = 0.0864$.
[b] $F_2 = 6.69$ and $P = 0.0002$.

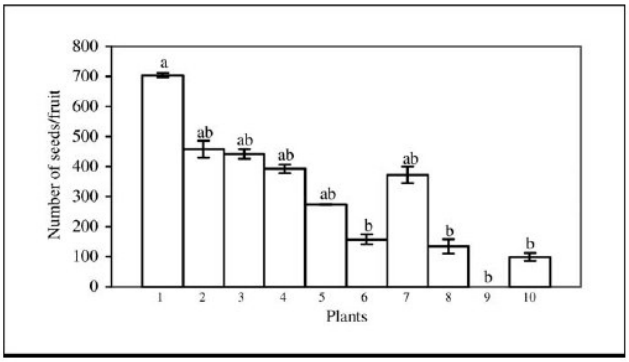

Fig. 3. Mean (±1 SE) number of seeds per fruit of *Vriesea gigantea* estimated for five fruits from 10 plants during 2004. Columns that share the same letter are not significantly different by the Tukey test (5%), $F_2 = 4.46$ and $P = 0.0004$.

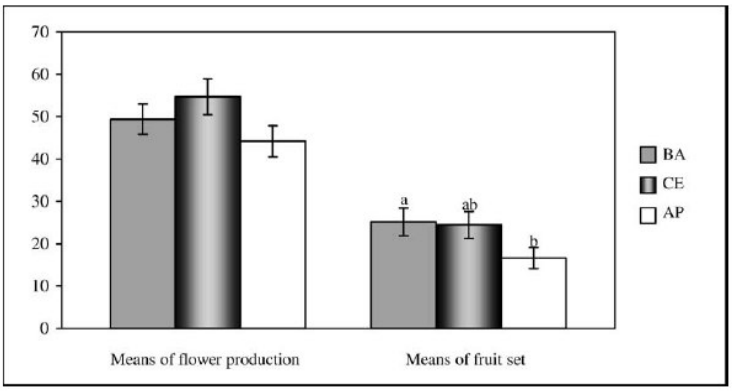

Fig. 4. Means (±1 SE) of flower production and fruit set in different positions on the inflorescence. BA = base, CE = center, AP = apex. ($N = 26$ plants). Columns in the same category that share the same letter are not significantly different by the Tukey test (5%), $F_2 = 3.88$ and $P = 0.025$.

germination rate of 94%. Downs (1963) reported similar results for other bromeliad species. Seed production, seed viability and germination rate and the longevity and mortality rate of plants may affect population dynamics (Kéry and Matthies, 2004). As far as we know, the results described here are the first reported on seed viability and germination rate in *V. gigantea*. Further studies on plant longevity, mortality rate, and recruitment of new individuals will be essential to evaluate the *V. gigantea* populations dynamic.

Pollen supplementation—The mean fruit set from manual self-pollinated plants was 85.42%, indicating that *V. gigantea* is self-compatible as described for other members of this genus (Martinelli, 1994). In natural conditions, 47.92% of open-pollinated flowers developed fruits (Table 4). Similar results were recorded by Canela and Sazima (2005) for *Bromelia antiacantha* (Bert.), whose fruit set under natural conditions, occurred in 50% of the flowers. The differences among populations regarding seed production in the pollen-supplementation experiments may be associated with the lower level of genetic variability found in Pedra da Visão population (G. M. Paggi, C. Palma-Silva, L. C. T. Silveira, J. A. T. Sampaio, E. Kaltchuk-Santos, M. H. Bodanese-Zanetinni, F. Bered, Universidade Federal do Rio Grande do Sul, unpublished manuscript). Reduced genetic variability may result in inbreeding depression and a decrease in plant size and fruit/seed set (Huang and Guo, 2002; Buide, 2004; Kéry and Matthies, 2004; Yang et al., 2005).

Under open-pollination, plants from Praia do Araçá yielded 3.8-fold less seeds than those from Morro da Grota (Table 5). It could be hypothesized that the low seed production may be due to pollen-limitation in Praia do Araçá. Similarly, Clark-Tapia and Molina-Freaner (2004) and Ishii and Kadono (2002) concluded that pollen-limitation may be the most important factor contributing to the low seed productivity for *Stenocereus eruca* and Poaceae species, respectively. Furthermore, in *Ferocactus cylindraceus* species, both extrinsic and intrinsic factors were considered important in limiting reproductive output (McIntosh, 2002).

Pollen limitation is fairly common in plants and can be considered a natural phenomenon (DiFazio et al., 1998; Thomson, 2001; Ishii and Kadomo, 2002). According to Buide (2004), evidence from pollination treatments and spatial variation in fruit set seem to indicate that pollinators limit female fecundity. As *V. gigantea* is a bat-pollinated species (Sazima et al., 1999), observations on pollinator visits could provide important information to confirm our hypotheses of pollen limitation. The occurrence of glossophagines bats, such as *Anoura caudifer* and *Glossophaga soricina*, has been recorded throughout the Itapuã State Park (Fábian et al., 1999; T. Freitas, Universidade Federal do Rio Grande do Sul, personal communication). Moreover, the largest bat population was observed in Morro da Grota (W. Silva, Secretaria do Meio Ambiente do Rio Grande do Sul, personal communication). The high abundance of bats in this population could explain the high number of seeds and fruit setting under open pollination (Table 5). However, our observation on pollinator visits are limited, and further field studies should be carried out to understand pollinator behavior and its effects on the breeding system of this species.

TABLE 4. Pollen-supplementation experiments on *Vriesea gigantea* in Itapuã State Park, Brazil: means (±1 SE) of fruit and seed set in the pollination treatments. MSP, Manual self-pollination; OP, open-pollination.

Treatment	No. plants	Fruit set (%)[a]	No. seeds per fruit[b]
MSP	16	85.42 ± 5.67 a	544.25 ± 55.72 a
OP	16	47.92 ± 9.61 b	245.71 ± 62.32 b

Note: Means with the same letter are not significantly different by the Wilcoxon two-sample test (5%).

[a] $P = 0.0034$.

[b] $P = 0.0068$.

Evolutionary and conservation considerations—The pollen-supplementation results (Table 4) agree with the pollinator-limitation hypothesis, which suggests that observed fruit production is below some optimal level that would be achieved if pollinators were more abundant (Calvo and Horvitz, 1990). Consequently, pollen limitation appears to be the case for *V. gigantea*. Genetic models of mutation and selection to analyze the joint evolution of inbreeding depression and self-fertilization in natural populations demonstrate that in many situations the two extremes (predominant outcrossing and predominant self-fertilization) represent alternative stable states of the mating system (Schemske and Lande, 1985). Which of these

TABLE 5. The effects of pollen supplementation on mean (±1 SE) fruit and seed set in four populations of *Vriesea gigantea* in Itapuã State Park, Brazil. MSP, Manual self-pollination; OP, open-pollination.

	Treatment			
	MSP		OP	
Population	Fruit set (%)	No. of seeds	Fruit set (%)	No. of seeds
Morro da Grota	90.00 ± 10.00	691.50 ± 72.10[A]	66.67 ± 14.91	395.33 ± 81.88
Praia do Araçá	100.00 ± 0	652.83 ± 18.47[A,B]	44.44 ± 29.40	102.89 ± 69.16
Trilha do Fenômeno	73.33 ± 11.30	516.42 ± 103.48[A,B]	33.33 ± 18.26	187.71 ± 88.01
Pedra da Visão	83.33 ± 16.67	276.71 ± 61.36[B]	44.44 ± 22.22	186.56 ± 82.58

Note: Means with the same letter are not significantly different; Kruskal-Wallis Test (5%), $P = 0.0179$.

two extremes will be the case depends largely on a species' history, e.g., species with a long history of occasional population bottlenecks and/or pollinator failure are expected to have relatively little inbreeding depression and to be selected for self-fertilization, whereas species with historically large, outcrossing populations are expected to have substantial inbreeding depression when forced to self and to be selected for mechanisms to prevent inbreeding. In addition to the lack of pollinators, there are several other selective factors thought to promote the evolution of selfing, such as cost of outcrossing, repeated colonization of new areas by single individuals (= the need for reproductive assurance), and selection for local adaptation (Lande and Schemske, 1985; Charlesworth et al., 1987). Although Vogel (1969) has described *V. gigantea* as an outcrossing species, our results indicate that this species is self-compatible and has the potential to tolerate selfing (Table 4). We note that occasional selfing in monoecious or hermaphroditic outcrossers is often an incidental by-product of self-compatibility (Schemske and Lande, 1987). In the future, molecular marker-based assessment of inbreeding coefficients and outcrossing rates in natural populations and progeny arrays (Weir and Cockerham, 1984; Ritland, 2002) will help us shed light on the breeding system of *V. gigantea*.

In summary, this study demonstrates that the analyzed populations are viable. Plants produce large number of flowers, fruits, and highly viable seeds. A high proportion of fruits matured, and seeds in the manual self-pollination treatment indicate that species is self-compatible. Reduction in seed set of individuals from Praia do Araçá was probably due to pollinator limitation. If pollinator abundance is reduced, outcrossing will be suppressed, and genetic variability will also be reduced. Pollen limitation is most likely a consequence of habitat fragmentation, and, specifically, a disruption of the bat pollination mutualism. Hence, to ensure the continuous survival of populations and to maintain their evolutionary potential, large-scale conservation strategy is necessary to protect plants and pollinators.

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Anexo II

Cross-species transfer of nuclear microsatellite markers: potential and limitations

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OPINION ARTICLE

Cross-species transfer of nuclear microsatellite markers: potential and limitations

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Abstract

Molecular ecologists increasingly require 'universal' genetic markers that can easily be transferred between species. The distribution of cross-species transferability of nuclear microsatellite loci is highly uneven across taxa, being greater in animals and highly variable in flowering plants. The potential for successful cross-species transfer appears highest in species with long generation times, mixed or outcrossing breeding systems, and where genome size in the target species is small compared to the source. We discuss the implications of these findings and close with an outlook on potential alternative sources of cross-species transferable markers.

Keywords: animals, cross-species amplification, DNA sequencing, fungi, microsatellites, plants

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Nuclear microsatellites are currently one of the most popular types of genetic markers for molecular ecology studies. However, molecular ecologists increasingly require universal markers that can readily be transferred between species. Such transferable markers facilitate comparisons among closely related taxa for addressing the mechanisms involved in population divergence and speciation (Noor & Feder 2006), and comparisons among multiple co-occurring species for studying how patterns of diversity at the genetic and community levels interact (Whitham *et al.* 2006).

Ongoing research on the genetic effects of habitat fragmentation in a biodiversity 'hotspot', the Atlantic Rainforest of Brazil (Myers *et al.* 2000), has confronted us with an issue well known to readers of this journal: many species need to be studied in a comparative way while time to do so is running out, in our case due to logging and urban development which reduce remaining forest fragments at a rapid pace. This has prompted us to utilize the potential of cross-species transfer of microsatellite loci in our ongoing studies (Palma-Silva *et al.* 2006; Barbará *et al.* 2007). It has also led us to investigate its potential in other taxa to inform molecular ecologists in similar situations, and the results of our literature search are discussed here. We do *not* focus on interspecific differences in microsatellite mutation rates, constraints on microsatellite evolution, or homoplasy, as these issues have been dealt with elsewhere (e.g. Estoup & Cornuet 1999; Amos 1999). Rather, we focus on the likelihood of successful cross-species transfer measured as the proportions of amplified and polymorphic markers in a large number of animals, fungi, and plants. In doing so, we expand earlier studies by Schlötterer *et al.* (1991), Primmer *et al.* (1996, 2005), Rosetto (2001), and Primmer & Merilä (2002) on the conservation of microsatellite loci in specific groups of taxa. To our knowledge, this is the first comprehensive evaluation of microsatellite cross-species transfer potential across three kingdoms.

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Review of the success of cross-species marker transfer in animals, plants, and fungi

We reviewed 64 primer notes published in *Molecular Ecology*, *Molecular Ecology Notes*, and elsewhere between 1997 and mid-2006, representing a total of 611 cross-species encounters and matching with stringent quality criteria (Table 1). Each study reported cross-transfer results for at least 10 markers and five target taxa within a fully informative table (not just partial presentation in the text). In each original study, successful marker amplification was determined by comparison to expected fragment size and /

Table 1 Summary of the reviewed studies, including the total number of studies, total number of cross-species encounters, and number of cross-species encounters for different taxonomic categories and taxonomic ranges of molecular marker assays

Category	No. of items
Total studies*	64
Total cross-species encounters	611
Cross-species encounters per taxonomic category:	
Vertebrates	311
Invertebrates	114
Plants	155
Fungi	31
Taxonomic range of cross-species amplification†:	
Among species — within genus	301
Among genera — within family	190
Among families — within order	92
Among orders — within class	21

*Each article reported a cross-amplification table based on at least 10 markers and five target taxa. †Seven cases were difficult to classify and are thus not listed.

or direct sequencing of amplified fragments. For each cross-species encounter in each study, we scored the 'percentage of markers amplified' and, among those, the 'percentage of polymorphic markers' as surrogates of transfer success. Then we sought to explain variation in cross-species transfer success in a three-step procedure.

In the *first* step, we used 'taxonomic grouping' and the 'taxonomic range' of cross-species transfer as predictor variables in linear models to inspect the taxonomic distribution of cross-species transfer success (see Table 2 for a key to these variables). The use of taxonomic variables rather than genetic distance based on DNA sequence data was necessary because there is currently no universal molecular 'barcode' across animals, fungi, and plants (Chase *et al.* 2005). The response of marker transfer success to taxonomic predictors was modelled using either least-squares analysis of variance (ANOVA) or a mixed model including 'source species' of microsatellites as a random factor. The latter approach takes into account the lack of independence in cross-species comparisons involving the same source species. In the *second* step, we added organismal and geographical predictor variables to refine the analysis and identify additional patterns. The following organismal and geographical variables were analysed: mating system, geographical distribution, distribution range, and generation time (see Table 2 for factor levels of these variables). We focused on organismal factors rather than molecular properties of individual loci, as the latter have been dealt with in a review of selected animal taxa recently (Primmer *et al.* 2005). The organismal factors we recorded may be related to rates of molecular evolution which are known to vary greatly between taxa (Zhang & Hewitt 2003). We did not include primer annealing temperature (T_a) in the analysis because lowering T_a in cross-species amplifications is now routine. Also, no contrast between anonymous and expressed sequence tag (EST)-derived markers was intended, as this topic has been addressed elsewhere (Pashley *et al.* 2006; Bouck & Vision 2007). Response to organismal and geographical factors was modelled by least-squares ANOVA, because the representation of some of the variables was not sufficiently balanced for running mixed models, and our earlier results showed that the results of both approaches are comparable. For graphical representation, means and standard errors of transfer success for different categories of organismal/geographical factors were plotted after adjusting for differences in the taxonomic range of marker transfer. Residuals from simple one-way ANOVA with taxonomic range as factor variable were used for this adjustment. In a *third* and final step, the effect of variation in genome size (*C* value) on cross-species transfer success was analysed using Pearson's correlation.

Distribution of cross-species transfer success

The results of our analysis contain information on the distribution of the effort invested by the molecular ecology community in testing the potential for cross-species marker transfer, the taxonomic distribution of cross-species transfer success that follows from these tests, and the relationship between transfer success and key organismal and geographical factors.

With respect to distribution of investment, it seems clear that the potential of cross-species marker transfer has been utilized the least where it is needed most: in narrowly endemic and tropical taxa. Our review of 64 informative primer note articles published over a 10-year period revealed only 88 cross-species encounters for tropical target taxa vs. 243 for temperate ones (Table 2 — second column from the left), despite the fact that approximately half of the source species in the reviewed studies were tropical. Likewise, our review revealed only 42 cross-species encounters for narrow endemics vs. 250 for widespread and 96 for regionally distributed taxa (Table 2). The under-representation of endemic taxa is surprising, as endemics should be of great interest to conservation biologists. We suspect that failure to test the transferability of markers in narrow endemics is primarily due to the difficulty of obtaining samples from suitable specimens for the necessary laboratory tests at short notice.

With respect to taxonomic distribution of transfer success, we found a significant effect of 'taxonomic group' and, as expected from previous reports (e.g. Primmer *et al.* 1996; Steinkellner *et al.* 1997), 'taxonomic range of marker assay' on the proportions of amplified and polymorphic markers (Table 3). Reptiles, birds, mammals, and invertebrates other than arthropods clearly fared best within

Table 2 Percentages of amplified and polymorphic markers by taxonomic group, taxonomic range of cross-species amplification, mating system, geographical distribution, distribution range, and generation time

	Amplification***			Polymorphism		
		%			%	
Variable	*n*†††	Mean ± SE	Median	*n*†††	Mean ± SE	Median
Taxonomic group						
Conifers*	5	71 ± 6	66	0	—	—
Monocots†	23	58 ± 7	64	9	27 ± 7	35
Eudicots‡	127	71 ± 2	80	49	48 ± 4	46
Mammals§	84	64 ± 4	82	68	52 ± 4	61
Birds¶	100	53 ± 3	59	38	44 ± 6	41
Reptiles**	5	85 ± 4	88	5	69 ± 11	76
Fishes††	122	57 ± 2	58	68	57 ± 3	57
Arthropods‡‡	95	61 ± 3	63	65	62 ± 3	65
Other invertebrates§§	19	72 ± 7	88	9	77 ± 7	83
Fungi¶¶	31	36 ± 7	20	0	—	—
Taxonomic range						
Between species/within genus	301	73 ± 1	82	175	65 ± 2	67
Between genera/within family	190	60 ± 2	59	96	43 ± 3	42
Between families/within order	92	33 ± 3	25	38	28 ± 6	0
Between orders/within class	21	6 ± 2	0	0	—	—
Mating system						
Primarily selfing	14	54 ± 9	55	5	69 ± 11	76
Mixed	162	65 ± 2	77	67	51 ± 3	49
Outcrossing	405	59 ± 2	62	238	54 ± 2	60
Unknown	30	60 ± 5	59	1	—	—
Geographical distribution						
Tropical	88	59 ± 3	56	39	41 ± 5	33
Tropical-temperate	143	62 ± 3	64	87	57 ± 3	63
Temperate	243	59 ± 2	62	112	58 ± 3	63
Temperate-polar	36	56 ± 5	61	22	56 ± 6	58
Tropical-temperate-polar	19	44 ± 7	46	9	25 ± 11	17
Unknown	82	70 ± 3	79	42	55 ± 4	48
Distribution range						
Narrowly endemic	42	69 ± 5	79	25	59 ± 6	64
Regional	96	58 ± 3	57	44	55 ± 5	56
Widespread	250	56 ± 2	58	123	50 ± 3	55
Unknown	223	65 ± 2	74	119	56 ± 3	57
Generation time						
Annual/semelparous	64	66 ± 3	67	31	63 ± 4	63
Perennial/iteroparous	436	59 ± 2	62	213	53 ± 2	57
Unknown	112	64 ± 3	74	67	53 ± 4	56

*Conifers: Chagne *et al*. 2004. †Monocots: Blum *et al*. 2004; Flanagan *et al*. 2006; Tostain *et al*. 2006. ‡Eudicots: Steinkellner *et al*. (1997); White & Powell (1997); Lanaud *et al*. (1999); Combes *et al*. 2000; Squirrell & Wolff 2001; Hale *et al*. 2002; Escribano *et al*. 2004; Jones *et al*. 2004; Morillo *et al*. 2004; Topinka *et al*. 2004; Perez *et al*. 2006; Porter *et al*. 2006; Terui *et al*. 2006; Salywon & Dierig 2006. §Mammals: Gemmell *et al*. (1997); Ortega *et al*. 2002; Williamson *et al*. 2002; Gaur *et al*. 2003; Dawson *et al*. 2004; Maudet *et al*. 2004; Gunn *et al*. 2005. ¶Birds: Richardson *et al*. 2000; Chbel *et al*. 2002; Martinez-Cruz *et al*. 2002; Maak *et al*. 2003; Dawson *et al*. 2005; Mcrae *et al*. 2005; Rubenstein 2005. **Reptiles: Sinclair *et al*. 2006. ††Fishes: Cairney *et al*. 2000; Iyengar *et al*. 2000; Farias *et al*. 2003; Keeney & Heist 2003; Rodriguez *et al*. 2003; Yue *et al*. 2003; Lippe *et al*. 2004; Rogers *et al*. 2004; Coulibaly *et al*. 2005; Feulner *et al*. 2005; Holmen *et al*. 2005; Perry *et al*. 2005; Vasemagi *et al*. 2005; Ovenden *et al*. 2006; Tonnis 2006. ‡‡Arthropods: Belfiore & May 2000; Mohra *et al*. 2000; Zhu *et al*. 2000; Daly *et al*. 2002; Flanagan *et al*. 2002; Huttunen & Schotterer 2002; Dawson *et al*. 2003; Funk *et al*. 2006; Mavarez & Gonzalez 2006; Schug *et al*. 2004; Shearman *et al*. 2006; Smith *et al*. 2005. §§Other invertebrates: Eackles & King 2002; McMullin *et al*. 2004. ¶¶Fungi: Slippers *et al*. 2004; Wadud *et al*. 2006. ***Successful marker amplification in individual studies was inferred by comparison to expected fragment size and/or direct sequencing of amplified fragments. †††Sample sizes (*n*) refer to the number of cross-species encounters assessed for marker amplification and, among those that amplified, for marker polymorphism. Sample sizes are generally smaller for marker polymorphism because they represent a subset of the samples assessed for amplification, and because not all reviewed studies tested for polymorphism. SE, standard error.

Table 3 ANOVA of the effects of taxonomic group, taxonomic range of marker assay, and interaction between these factors on the percentage of markers amplified (A) and the percentage of markers polymorphic (B) in cross-species amplification tests

A. Percentage markers amplified

	General linear model					Mixed model†	
Source of variation*	d.f.	SS	MS	*F*	*P* value	*F*	*P* value
Taxonomic group	9	126.41	14.05	5.89	**0.000**	13.07	**0.000**
Taxonomic range of marker assay	3	375.40	125.13	52.45	**0.000**	49.60	**0.000**
Taxonomic group × range of assay	11	58.33	5.30	2.22	**0.012**		
Error	580	1383.83	2.39				

B. Percentage markers polymorphic

	General linear model					Mixed model†	
Source of variation*	d.f.	SS	MS	*F*	*P* value	*F*	*P* value
Taxonomic group	7	97.77	13.97	5.71	**0.000**	5.63	**0.000**
Taxonomic range of marker assay	2	111.37	55.69	22.77	**0.000**	19.44	**0.000**
Taxonomic group × range of assay	8	53.13	6.64	2.72	**0.007**		
Error	291	711.55	2.45				

The residuals of both models were weighted by the number of markers assayed in each study. Significant factors at the 0.05 level are indicated with bold type. *For key to taxonomic predictor variables see Table 2. †In mixed models, taxonomic predictor variables were nested within the random factor 'microsatellite source species' and significance was tested using maximum-likelihood estimation. d.f.: degrees of freedom; SS: sums of squares; MS: mean squares.

genera in terms of 'per cent markers amplified' (Fig. 1A). The drop in transfer success to the next level (between genera/within family) was steeper for invertebrates and birds compared to reptiles and mammals, but in birds, an appreciable percentage of markers amplified successfully even between different families (Fig. 1A). The observed patterns are largely consistent with earlier observations by Schlötterer *et al.* (1991), Primmer *et al.* (1996, 2005), Rosetto (2001), and Primmer & Merilä (2002) on specific groups of taxa although, to our knowledge, cross-transferability has never been compared across taxa and kingdoms on this scale. A conspicuous pattern in plants is the greatly reduced chance of successful amplification in monocots compared to eudicots (Fig. 1A). Although observations for fungi were available only for the extremes of the taxonomic range of marker transfer, success rates appear to be intermediate and within the range of other groups of taxa (Fig. 1A). When transfer success was scored as 'per cent polymorphic markers', then birds, reptiles, mammals, fishes, and invertebrates other than arthropods fared best within and between genera (Fig. 1B). Plants clearly fared worse than animals, again with greater transfer success in eudicots than in monocots.

Can organismal or geographical attributes of each taxon predict cross-species transfer success? As visible from the ANOVA shown in Table 4, taxonomic range and taxonomic group clearly explained a greater proportion of the variation than any other factor. Nevertheless, we found a significant effect of mating system and generation time on the per cent of amplified markers (Table 4), with lower amplification success in primarily selfing and short-lived (annual or semelparous) species in our data set (Fig. 2A, D). Lower success rates in selfing species may be explained by a greater likelihood of selfers to accumulate mutations because of smaller effective population size (N_e) (Higgins & Lynch 2001; Lynch & Conery 2003). This may involve transposition, chromosomal rearrangements, local insertion/deletions, or point mutations, all of which could affect conservation of the markers. We note that modes and rates of genome turnover in flowering plants appear to be strikingly different from those observed in animals (Lim *et al.* 2007). Still, our result must be interpreted with care as sample sizes for this particular comparison were unbalanced (Table 2; Table 4). Lower amplification success in annual or semelparous species may be explained by the direct effect of generation time or by indirect effects of differences in metabolic rates (Gillooly *et al.* 2005). We also found a significant negative effect of genome size (*C* value) on cross-species amplification success (Fig. 3). Thus, genome size may not only affect the amplification of microsatellite markers in the source taxon (Garner 2002), but also the success of marker transfer between species. The great variation in amplification success at intermediate genome size ratios in the middle of the graph shown in Fig. 3 is primarily due to fishes (Salmonidae, Carcharhinidae, Clariidae, Scophthalmidae) and reflects large differences in the taxonomic range of cross-species marker transfer in this group.

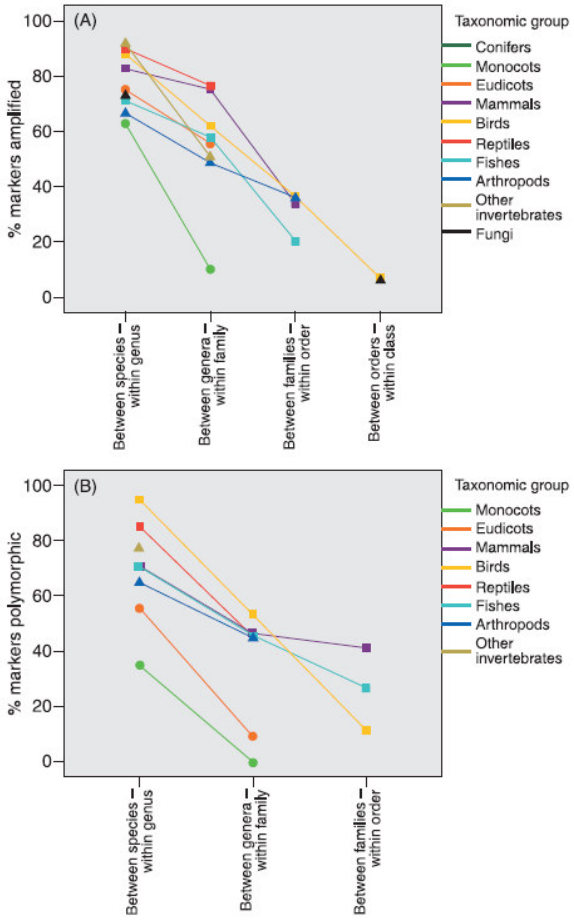

Fig. 1 Mean values for the percentage of markers amplified (A) and, among those that amplified, percentage of markers polymorphic (B) across different taxonomic groups and taxonomic ranges of molecular marker assays for a total of 611 cross-species encounters from 64 studies. The taxonomic groups considered in the analysis are indicated by different colours. In addition, the following symbols help distinguish between major groups of taxa: filled circles for plants, filled rectangles for vertebrates, and filled triangles for invertebrates and fungi. The only mean value available for conifers ('between species-within genus' category) is not visible as it overlaps exactly with the values for fishes and fungi.

All our observations on organismal/geographical variables are for transfer success in terms of marker amplification — no significant effect on polymorphism was found. This suggests that differences in polymorphism upon cross-species transfer are either due to factors not included in our review, or to multiple factors with individual effects too small to be detected here. Reduced marker polymorphism upon cross-species transfer has often been attributed to 'ascertainment bias' whereby a microsatellite chosen to be maximally long in the source species is then likely to be shorter in a new target species (Ellegren *et al.* 1995). It is thought that ascertainment operates in part via a restriction in microsatellite length, such that occasional deletions or internal point mutations lead to shorter and less polymorphic loci upon cross-species transfer (Vowles & Amos 2006). In a previous study of molecular properties recorded for a large number of microsatellites cross-amplified in birds, only the repeat number in the source species had a significant effect on cross-species polymorphism success (Primmer *et al.* 2005).

Conclusions

Our results indicate that cross-species transferability of microsatellite markers is unevenly distributed across taxa (Fig. 1A, B). High amplification success within and between genera in many groups of animals and plants indicates a great potential to use microsatellites and their flanking regions as a source of single- or low-copy nuclear sequences, as suggested by Zhang & Hewitt (2003). Of course, the likelihood of orthology vs. paralogy of cross-amplified loci will have to be evaluated on a case-by-case basis, e.g. based on the evolutionary information inherent in the growing number of complete genomic sequences available (Vision *et al.* 2000; Lynch & Conery 2003; Tuskan *et al.* 2006).

Variation between taxa is even greater when cross-species transfer success is evaluated in terms of marker polymorphism, the ultimate criterion for the direct use of microsatellite length polymorphisms as markers (Fig. 1B). In effect, tests for cross-species transferability of polymorphic markers can be expected to yield returns in most groups of animals within and between genera and even across different families in some cases (> 40% transfer success in mammals, > 25% in fishes, and > 10% in birds at this level; Fig. 1B). By contrast, transferability of polymorphic markers in plants is likely to be successful mainly within genera (success rate close to 60% in eudicots and close to 40% in the reviewed monocots). Between genera, transfer rates are approximately 10% for eudicots, and students of monocots such as orchids or grasses are very unlikely to get away without isolating novel markers from the genomes of new target genera. An exception, in our experience, are large adaptive radiations with low levels of DNA sequence divergence such as Bromeliaceae, where polymorphic markers are readily transferred between species of the same subfamily and beyond (Palma-Silva *et al.* 2006; Barbará *et al.* 2007).

Despite encouraging aspects, it is clear that the potential for cross-species transfer of microsatellites is more limited than molecular ecologists would wish for. Although EST-derived microsatellites may be conserved over larger evolutionary distances, their transfer beyond the genus level often appears to be limited too (Pashley *et al.* 2006; Bouck & Vision 2007). Also, molecular ecology studies increasingly aim at comparing genetic, demographic, behavioural, and breeding system parameters among related species or multiple species co-occurring in the same community. This

Table 4 ANOVA of the effects of taxonomic group, taxonomic range of marker assay, mating system, geographical distribution, distribution range, and generation time, on the proportion of markers amplified across species

Source of variation*	General linear model d.f.	SS	MS	*F*	*P* value
Taxonomic group†	7	151.02	21.57	7.97	**0.000**
Taxonomic range of marker assay	3	279.67	93.22	34.42	**0.000**
Mating system	2	25.05	12.53	4.63	**0.011**
Geographical distribution	4	10.23	2.56	0.95	0.438
Distribution range	2	4.10	2.05	0.76	0.470
Generation time	1	17.01	17.01	6.28	**0.013**
Error	298	807.04	2.71		

The residuals were weighted by the number of markers assayed in each study. Percentage of variation explained by the entire model (R^2) = 36%. Interaction effects were not tested. Significant main effects at the 0.05 level are indicated with bold type. *For factor levels of predictor variables see Table 2. †Reptiles and fungi were excluded from this analysis because of missing data for some of the variables. d.f.: degrees of freedom; SS: sums of squares; MS: mean squares.

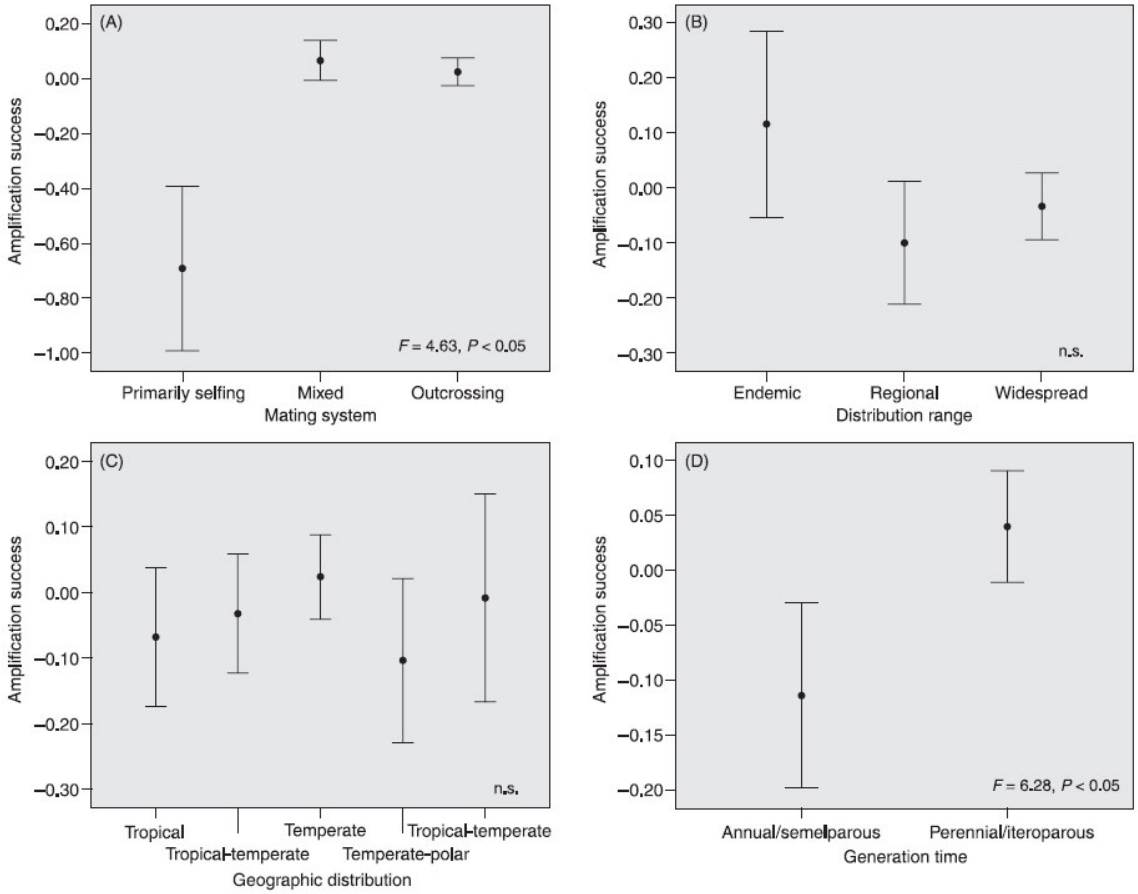

Fig. 2 Effects of four different organismal or geographical factors on the proportion of markers amplified (means ± SE), adjusted for differences in the taxonomic range of cross-species transfer. *X*-axes, from top to bottom panel: mating system (primarily selfing, mixed, or outcrossing), geographical distribution (tropical, tropical-temperate, temperate, temperate-polar, or tropical-temperate-polar), distribution range (narrowly endemic regional), or widespread and generation time (annual/semelparous, or perennial/iteroparous). *Y*-axes: amplification success adjusted for differences in the taxonomic range of cross-species transfer.

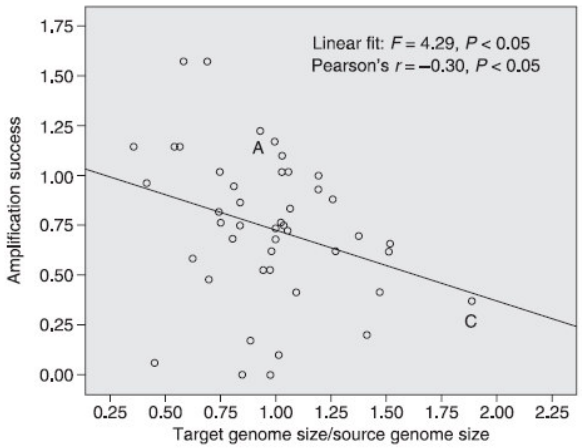

Fig. 3 The relationship between amplification success and the ratio of genome size (*C* value) between target and source species in 45 cases for which genome size estimates were available for both target and source, including plants, fishes, one bird and one insect. Amplification success decreases with increasing genome size in the target species relative to the source. *X*-axis: genome size ratio. *Y*-axes: arcsine-transformed percentages of amplified markers. 'A' and 'C' denote the only available entries for birds (Anatidae) and insects (Culicidae), respectively.

raises issues of interspecific differences in mutation rates, constraints on microsatellite evolution, and homoplasy (Estoup & Cornuet 1999; Amos 1999). Several alternative sources of nuclear markers which may be more easily transferable are currently under development. These include EST-derived single nucleotide polymorphisms and exon-primed, intron spanning markers (Bouck & Vision 2007), single-copy nuclear polymorphisms identified from whole genome sequences or genomic libraries (Zhang & Hewitt 2003), and nuclear DNA 'barcodes' potentially applicable across entire kingdoms (Chase *et al*. 2005). These developments, in combination with the increasing efficiency and decreasing costs of DNA sequencing, raise the hope that molecular ecologists will soon have an upgraded 'toolbox' of transferable markers from which to choose.

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Anexo III

Isolation and characterization of microsatellite loci in *Pitcairnia albiflos* (Bromeliaceae), an endemic bromeliad from the Atlantic Rainforest, and cross-amplification in other species

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PERMANENT GENETIC RESOURCES

Isolation and characterization of microsatellite loci in *Pitcairnia albiflos* (Bromeliaceae), an endemic bromeliad from the Atlantic rain forest, and cross-amplification in other species

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Abstract

Eight microsatellite markers were isolated from *Pitcairnia albiflos*, an endangered endemic bromeliad species restricted to 'inselberg' rock outcrops in the state of Rio de Janeiro, Brazil. The number of alleles observed for each locus ranged from two to 12. Average observed and expected heterozygosities were 0.408 and 0.663, respectively. A cross-amplification test in 16 taxa suggests that the markers will be useful in numerous related bromeliad species. The loci will be used to study genetic structure and reproductive biology in fragmented inselberg populations and the origin and maintenance of barriers to gene flow between sympatric *Pitcairnia* species.

Keywords: Atlantic rain forest, Bromeliaceae, cross-amplification, microsatellites, *Pitcairnia*

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Pitcairnia albiflos is a rare and threatened plant species that occurs on several 'inselberg' rock outcrops in Rio de Janeiro state, Brazil. Analysis of seed production in controlled crosses suggests that it is self-compatible with adaptations for avoiding self-pollination under favourable pollination conditions (Wendt *et al*. 2001). Natural interspecific hybridization between *P. albiflos* and a sympatric congener species has been documented (Wendt *et al*. 2000). Inselbergs in tropical rain forests are characterized by endemism and habitat fragmentation. Patterns of genetic diversity and gene flow have been evaluated in only a few inselberg-adapted species of bromeliads (Sarthou *et al*. 2001; Barbará *et al*. 2007). The aim of this study was to develop a set of polymorphic microsatellite [SSR (simple sequence repeat)] markers for *P. albiflos*.

Total genomic DNA was extracted from leaves of *P. albiflos* following the protocol of Doyle & Doyle (1990). Marker isolation involved construction of a genomic library enriched for $(CT)_n$ and $(GT)_n$ repeats. The methodology was based on biotinylated oligonucleotide sequences bound to streptavidin-coated magnetic particles as described by Kijas *et al*. (1994) with modifications by Billote *et al*. (1999). Microsatellite-enriched DNA fragments were ligated into pGEM-T Easy vector (Promega) as described by the supplier and used to transform XL1-Blue competent cells (*Escherichia coli*, Stratagene). A total of 96 recombinant colonies were obtained and 48 were sequenced using the BigDye terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems) on the ABI PRISM 377 DNA Sequencer (Applied Biosystems). For 37 clones containing SSR motifs, forward and reverse sequences were aligned in SEQUENCHER version 4.1.2 (Gene Codes), and primers were designed for 15 loci using the PRIMER 3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

For each SSR, the forward primers were synthesized with a 19-bp M13 tail (5′-CACGACGTTGTAAAACGAC-3′) following the method of Schuelke (2000), which involved three primers: a forward SSR-specific primer with the M13

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Table 1 Characteristics of microsatellite loci from *Pitcairnia albiflos*, including locus name, primer sequences, repeat type, no. of alleles, allele size range, observed (H_O) and expected (H_E) heterozygosities, and tests for departure from Hardy–Weinberg equilibrium (HWE)

Locus	Primer sequences*	Repeat motif	No. of alleles	Size range (bp)	H_O	H_E	GenBank Accession no.
PaA05	F: ACCGGGTTCAGGGAAAATAC R: TTGAGGCTAAGAGCGAGGAG	$(TTC)_{10}NN(CT)_{17}$	8	228–258	0.450	0.539	EU293085
PaA09	F: AGAAGAGAACCCACCCCAAG R: GTGTTCCGCGACACTACAAA	$(CT)_{25}$	9	191–213	0.545	0.801†	EU293086
PaA10	F: AACCATTGACATCCGCTGTT R: CTTCGGAAGCTCCTCTGGAT	$(ATG)_{10}$	2	146–149	0.318	0.459	EU293087
PaB11	F: AGAGGCTGAGAGAGGTAAACCA R: CGAGCCCTCTTTCTGAACC	$(AG)_9$	3	159–171	0.364	0.635†	EU293088
PaB12	F: CCCGAGGGACATTCTCTCTT R: CATGGCGCAGTAGTGTTTTC	$(CT)_{19}NN(CT)_4NN(CT)_4NN(TG)_7(TC)_5$	12	219–259	0.636	0.869†	EU293089
PaC05	F: TCGATGTCGACGGTAGTGAG R: TCCTCTCGCTTTGATTCACC	$(AG)_{18}NN(GA)_7$	3	149–153	0.524	0.638	EU293090
PaD07	F: TCCATGTGCCTCATCATAGC R: TGCCCACAAAGCATATCAGT	$(TG)_{10}$	3	233–239	0.095	0.623†	EU293091
PaZ01	F: TGACCAGATAGCACCATCCA R: TTGAGTGTTGGAGCCCACTT	$(AG)_{20}$	7	185–199	0.333	0.735†	EU293092
Mean			5.9		0.408	0.663	

*All forward primers were M13-tailed at the 5′ end. Significant departures from HWE: †$P < 0.001$.

tail at its 5′ end, a reverse locus-specific primer, and a universal M13 primer labelled with one of two fluorescent dyes, FAM or JOE (Applied Biosystems). All polymerase chain reaction (PCR) amplifications were performed in a PE Applied Biosystems 9700 thermocycler using a 'touch-down' cycling programme and the protocols described by Palma-Silva *et al.* (2007). Microsatellite alleles were resolved on a 3100 DNA Analyser (Applied Biosystems) and were sized against ROX molecular size standard using GENESCAN 3.7 and GENOTYPER 3.7 software (Applied Biosystems).

A total of 22 individuals from a single population of *P. albiflos* (Rio de Janeiro, RJ, Brazil) were used to evaluate SSR polymorphism. MSANALYSER 4.00 software was used to calculate observed and expected heterozygosities and GENEPOP 3.4 (Raymond & Rosset 1995) was used to test for departure from Hardy–Weinberg equilibrium and linkage equilibrium. Eight SSRs from *P. albiflos* were polymorphic, with number of alleles per locus ranging from two to 12 with an average of 5.9 alleles per locus. The observed heterozygosity for the polymorphic loci ranged between 0.095 and 0.636 with an average of 0.408 (Table 1). Five loci showed a significant departure from Hardy–Weinberg equilibrium due to heterozygote deficiency and 12 out of 28 pairwise locus comparisons showed significant linkage equilibrium ($P < 0.05$), consistent with inbreeding and/or Wahlund effects due to the self-compatible mating system of this species. Although null alleles cannot be ruled out, the MICRO-CHECKER software (van Oosterhout *et al.* 2004) found no evidence for scoring error due to 'stuttering' or 'large allele dropout'.

All variable markers were tested for cross-amplification in individuals of 16 species belonging to three subfamilies of Bromeliaceae: Pitcairnioideae (six species), Bromelioideae (eight species) and Tillandsioideae (two species) (Table 2). As expected (Barbará *et al.* 2007), the markers were most effectively transferred to species belonging to the same subfamily (Pitcairnioideae). One locus amplified in all species from the subfamily Pitcairnioideae (PaB12). Four loci (PaA10, PaC05, PaD07 and PaZ01) successfully amplified among almost all species from all three subfamilies (Table 2), thus indicating a high potential for use in comparative or phylogenetic studies. The newly developed primers will be used to test long-standing hypotheses regarding the origin and maintenance of barriers to gene flow between sympatric *Pitcairnia* species. Moreover, the microsatellite loci will be invaluable tools for conservation genetics surveys in many other bromeliad taxa, for example, for assessing the effect of landscape fragmentation on historical and contemporary gene flow, for species delimitation, and for studies on the origin and breakdown of reproductive barriers among closely related populations and species of the adaptive radiation Bromeliaceae.

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Table 2 Cross-amplification of eigth microsatellite markers isolated from *Pitcairnia albiflos* across all three subfamilies of Bromeliaceae

Species (sample size)	Subfamily	PaA05	PaA09	PaA10	PaB11	PaB12	PaC05	PaD07	PaZ01
Pitcairnia fuertesii (1)	Pitcairnioideae	+	–	+	–	+	+	+	–
Pitcairnia sodiroi (1)	Pitcairnioideae	+	+	+	–	+	+	+	+
Pitcairnia staminea (2)	Pitcairnioideae	+	+	–	+	+	+	+	+
Puya raimondii (1)	Pitcairnioideae	–	–	+	–	W	+	+	+
Deuterocohnia lorentziana (1)	Pitcairnioideae	–	–	+	–	+	+	+	–
Dyckia dystachya (1)	Pitcairnioideae	–	–	+	–	+	+	+	+
Aechmea recurvata (1)	Bromelioideae	–	–	+	–	–	W	–	W
Aechmea winkleri (1)	Bromelioideae	–	–	+	–	–	W	–	+
Ananas frizmuelleri (1)	Bromelioideae	–	–	+	–	–	+	W	+
Bromelia antiacantha (2)	Bromelioideae	W	–	+	–	+	+	+	+
Edmundoa lindenii (1)	Bromelioideae	–	–	+	–	–	+	+	+
Fosterella micrantha (1)	Bromelioideae	–	–	–	–	–	+	+	–
Fosterella rojasii (1)	Bromelioideae	–	–	–	–	–	–	+	W
Orthophytum disjunctum (1)	Bromelioideae	–	–	+	–	–	+	+	+
Tillandsia tricolor (1)	Tillandsioideae	–	–	+	–	–	+	+	+
Alcantarea imperialis (1)	Tillandsioideae	–	–	+	–	–	–	+	+

+, successful amplification with either a single band or two bands visualized; W, weak amplification; —, unsuccessful amplification.

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Anexo IV

Bromélias, beleza exótica do novo mundo

Capítulo do Livro Publicado pela EMBRAPA "Origem e Evolução de Plantas Cultivadas"

Bromélias: a beleza exótica do Novo Mundo

Fernanda Bered, Eliane Kaltchuk dos Santos, Clarisse Palma da Silva e Gecele Matos Paggi

Introdução

Há muito tempo as bromélias vêm sendo utilizadas pelos povos nativos das Américas, estando fortemente presente em suas culturas. Atualmente, mais de 90 espécies são utilizadas, no mundo todo, para diversos fins: fibras, forragem, alimentação humana, rituais místicos, combustíveis, ornamentais, entre outros (Bennet et al. 1999)**.** Dentre estas categorias de usos, podemos destacar a importância das bromélias como plantas ornamentais de grande valor comercial, como alimentação humana, e medicinal. Nestas categorias, os gêneros mais utilizados são: *Aechmea, Alcantharea, Ananas, Bromelia, Billbergia, Catopsis, Guzmania, Neoregelia, Nidularium, Pitcairnia, Pseudoananas, Puya, Tillandsia* e *Vriesea.*

O abacaxi, *Ananas comosus* (L.) Merr. é uma das frutas tropicais mais amplamente cultivadas no mundo. Além de ser muito apreciado pelo seu saboroso fruto, o abacaxi possui propriedades medicinais devido à presença da bromelina, uma enzima com propriedades proteolíticas semelhantes à papaína. Atualmente, a bromelina é utilizada em medicamentos com ação antiinflamatória e analgésica (Bennett, 2000).

Bromeliaceae é a maior família de plantas típicas do Novo Mundo. A distribuição geográfica apresenta como limite norte de ocorrência os estados da Virgínia, Texas e Califórnia, nos Estados Unidos (latitude 37°N), e como limite sul o norte da Patagônia, na Argentina (latitude 44°S). A única exceção é *Pitcairnia feliciana* (A. Chev.) Harms & Mildbr. que está localizada no Oeste da África, na região da Guiné (Porembsky & Barthllot, 1999). Possivelmente, a origem e a dispersão das primeiras espécies tenham ocorrido em época próxima à separação do supercontinente Gondwana, o que explicaria a

presença de uma espécie primitiva remanescente na África. Evidências como características do pólen e macrofósseis indicam que Liliopsida (classe na qual as bromélias estão inseridas) evoluiu no meio do Cretáceo, embora acredite-se que as Bromélias tenham surgido a partir da metade do Terciário – entre 40 e 65 milhões de anos atrás – (Benzing, 2000). Provavelmente o surgimento da família é fenômeno relativamente recente, devendo ter ocorrido no próprio Novo Mundo, como sugere sua distribuição continental restrita (Smith, 1934)

A família Bromeliaceae é composta por espécies neotropicais, que sofreram uma extensa radiação adaptativa, ocupando ambientes extremos e com hábitos que variam de terrestres a epífitos. Essas plantas podem ser encontradas desde o nível do mar a altitudes superiores a 4000 metros, em regiões desérticas, úmidas e também em solos sujeitos a inundações periódicas, em locais com muito ou pouca luminosidade. Vivem muito bem sobre areias e rochas escaldantes e resistem a temperaturas próximas a 0°C (Benzing, 2000).

As bromélias são plantas perenes, herbáceas, com rosetas foliares basais, raramente arbustivas ou arborescentes. Os caules são rizomas, prostrados ou estolões curtos ou longos, dos quais se elevam as rosetas. As margens das folhas podem ser lisas ou portar espinhos. As folhas lanceoladas inserem-se em espiral no caule formando uma roseta basal (cisterna). Quanto mais alargada a bainha foliar que forma a cisterna, mais a planta se torna capacitada a acumular água (Judd et al., 1999; Leme, 1984). Na cisterna, conhecida como tanque, cuja capacidade às vezes ultrapassa um litro, também são acumulados restos orgânicos de animais e vegetais, assim como uma série de microorganismos que auxiliam na decomposição da matéria orgânica. As cisternas também são utilizadas como fonte de recurso (abrigo, alimento, sítios reprodutivo...) para muitas populações naturais de espécies associadas, grupo conhecido como fitotelmata (ex: anfíbios, aranhas, insetos, crustáceos, etc.), entretanto as mesmas não estão presentes em todas as bromélias.

Taxonomia e evolução

Atualmente, são conhecidas cerca de 2750 espécies (Benzing 2000), distribuídas em aproximadamente 60 gêneros, sendo quase 50% deste total encontrado no Brasil. Tradicionalmente, a família Bromeliaceae, está dividida em três subfamílias: Bromelioideae (~650 spp.), Pitcairnioideae (~890 spp.) e Tillandsioideae (~1000 spp.), com base na análise de caracteres morfológicos: flores, frutos, sementes e posição do ovário em relação ao perianto (Smith & Downs, 1974, 1977 e 1979).

Segundo Smith (1934), as bromélias teriam se originado na América do Sul, no Planalto das Guianas. As subfamílias Pitcairnioideae e Tillandsioideae, que possuem grande número de gêneros e espécies, teriam como centro de diversidade a região dos Andes, enquanto Bromelioideae o leste do Brasil.

A falta de evidências fósseis é um dos principais pontos que torna difícil a reconstituição da filogenia da família Bromeliaceae. No entanto, não existem muitas dúvidas em relação à sua monofilia (Gilmartin & Brown, 1987; Terry et al. 1997a, b; Crayn, 2004). Por outro lado, muitas são as especulações quanto à relação de Bromeliaceae com outras famílias. Da mesma forma, as relações entre as subfamílias e entre os gêneros têm sido temas muito polêmicos (Smith 1934 e Brown 2000).

Gilmartin e Brown (1987) foram os primeiros a estudar as relações filogenéticas entre as três subfamílias de Bromeliaceae, através de características morfológicas. Estes autores concluíram que Bromelioideae e Tillandsioideae são táxons irmãos e que a subfamília Pitcairnioideae possui a posição mais ancestral na família.

A partir da década de 90 as relações evolutivas em Bromeliaceae começaram a ser traçadas com base em dados de seqüências de DNA. No entanto, os estudos de sistemática molecular em Bromeliaceae (Ranker et al., 1990; Clark & Clegg, 1990; Givinish et al, 1990a, b) não se adequaram à filogenia sugerida pelos dados morfológicos de Gilmartin & Brown (1987) e as relações entre as subfamílias não encontraram um consenso (Brown, 2000). A discrepância entre o grau de divergência molecular e morfológica nas bromélias é bem ilustrada pela subfamília Tillandsioideae, que, geneticamente,

mostrou ser altamente similar (<1,8% de divergência genética), mas morfologicamente e ecologicamente é muito diversa (Horres et al. 2000). Trabalhos mais recentes ainda discutem as relações e monofilia das subfamílias e de seus gêneros (Terry et al. 1997a, b; Horres et al. 2000; Faria et al. 2004; Barfuss et al. 2005).

Sistema de Cruzamento

Apesar de ser uma família de grande valor econômico, em Bromeliaceae, poucos estudos sobre o sistema de cruzamento têm sido publicados. A fecundação cruzada é o sistema de cruzamento mais comumente encontrado entre as espécies de bromélias. No entanto, estudos da morfologia floral e experimentos de polinização confirmaram a existência de diversos sistemas de cruzamento. Tais estudos também demonstraram que aspectos da ecologia e história natural estão relacionados ao tipo de sistema de cruzamento predominante nas espécies (Martinelli, 1994; Benzing, 2000).

Algumas características adicionais, as quais influenciam as proporções da progênie que são de autofecundação ou de fecundação cruzada, podem ser encontradas em muitas espécies. Dicogamia (maturação assincrônica dos órgãos sexuais) e heterostilia (diferença espacial entre os órgãos sexuais, que limita a autopolinização) promovem a alogamia em muitas espécies autocompatíveis, e diminuem a obstrução do estigma em espécies autoincompatíveis (Benzing, 2000). Protoginia (amadurecimento do gineceu primeiramente) ocorre em quase 150 espécies do gênero *Tillandsia* subgênero *Tillandsia* (Gardner, 1982). Protandria (amadurecimento do androceu primeiramente) foi observado em 17 espécies de *Vriesea* polinizadas por pássaros e morcegos (Martinelli, 1994).

A cleistogamia foi relatada principalmente para espécies de *Tillandsia* (Gardner, 1982, 1986; Gilmartin & Brown, 1985) e para *Aechmea bracteata* (Sw.) Griseb. (Benzing, 1980), tendo sido considerada um sistema derivado em Bromeliaceae por Brown & Gilmartin (1989).

Qualquer que seja a condição fundamental para a família, a fecundação cruzada é reforçada pela autoincompatibilidade amplamente encontrada nas

subfamílias Bromelioideae e Tillandsioideae e, embora não confirmado, provavelmente em Pitcairnioideae também (Benzing, 2000). A autoincompatibilidade em Bromeliaceae foi geneticamente confirmada para *Ananas comosus*, *A. ananassoides* (Baker) L.B. Smith e *A. bracteatus* Lindl. Schult. & Schult.f. (Brewbacker e Gorrez, 1987). Por outro lado, Paggi et al. (2006) relataram a ocorrência de autocompatibilidade e de sistema misto de cruzamento, com elevada taxa de autofecundação em *Vriesea gigantea* Gaud. A dioicia ocorre em todas as subfamílias, mas em poucas espécies. (Benzing, 2000). A grande maioria dos gêneros exibe hermafroditismo e alguns, como o gênero *Catopsis*, exibem espécies dióicas e hermafroditas.

Citogenética e citotaxonomia da Família Bromeliaceae

A família Bromeliaceae é citogeneticamente muito homogênea, com pequena variação no número básico e no nível de ploidia. Apesar desta família apresentar uma marcante diversidade morfológica e adaptação aos mais diversos ecossistemas, há uma relativa conservação quanto ao número cromossômico, com prevalência de 2n=50 cromossomos, sendo x=25 o número básico, com exceção de *Cryptanthus* x=17 (Marchant, 1967; Brown *et al.,* 1997; Cotias-de-Oliveira *et al.,* 2000; Ceita, 2002; Palma-Silva *et al.,* 2004 e Gitaí *et al.*,2005)

A evolução cariotípica da família ainda não se encontra totalmente esclarecida. O pequeno tamanho dos cromossomos, aneuploidias e raças poliplóides fenotipicamente indiferenciadas dificultam a tentativa de reconstruir o cariótipo original da família e identificar a possível incidência de evolução reticulada (Benzing et al. 2000).

Os primeiros trabalhos propuseram x=8 como número básico para a família, inspirado nas primeiras contagens cromossômicas para Bromeliaceae (Billings, 1904 *apud* Weiss, 1965; Lindschau, 1933 *apud* Weiss, 1965; Gauthé, 1965; Weiss, 1965; Sharma & Ghosh,1971; McWillams, 1974).

Brown & Gilmartin (1989), usando evidências filogenéticas obtidas a partir de dados morfológicos (Gilmartin & Brown, 1987), propuseram um modelo de evolução cariotípica onde Bromeliaceae e Velloziaceae (x=8)

seriam táxons irmãos. Esses autores analisaram mais de 80 espécies pertencentes às três subfamílias. Segundo eles, o número básico x=25 teria se originado da hibridação entre paleodiplóides, com números básicos x=8 e x=9, produzindo um paleotetraplóide (n=17). Uma nova hibridação entre o paleodiplóide (x=8) e a linhagem paleotetraplóide (x=17) teria produzido o hexaplóide (n=25). Essa teoria foi corroborada por Gitaí *et al*. (2005) que observaram a ocorrência de dois pares de cromossomos com satélites em espécies diplóides, através da coloração com nitrato de prata.

A poliploidia do número básico x=25 tem sido raramente encontrada na família Bromeliaceae, ocorrendo na maior parte das vezes na subfamília Bromelioideae: *Bromelia* (duas espécies), *Nidularium* (uma sp.), *Pseudananas* (uma sp.), *Orthophytum* (três sp.), *Deinacanthon* (uma sp.) e *Ananas* (uma sp.). Em Pitcairnioideae são relatados poliplóides nos gêneros *Dyckia* (três sp.), *Deuterocohnia* (uma sp.), *Pitcairnia* (uma sp.) e *Fosterella* (uma sp.) (Brown & Gilmartin, 1986; Cotias-de-Oliveira *et al*., 2000; Gitaí *et al*., 2005). Em Tillandsioideae, poliplóides são relatados para o gênero *Guzmania* (uma sp.) e para várias espécies do gênero *Tillandsia* pertencentes ao sub-gênero *Diaphoranthema* (Brown & Gilmartin, 1986).

A bimodalidade cariotípica em Bromeliaceae foi observada inicialmente por Marchant (1967). Certas espécies apresentaram variabilidade quanto ao tamanho dos cromossomos em um mesmo cariótipo, havendo em algumas delas uma clara bimodalidade. Cotias-de-Oliveira *et al.* (2000) estudaram espécies da subfamília Bromelioideae e Pitcairnioideae, em algumas dessas espécies foram observados cromossomos com tamanhos diferentes, mas não foi observada uma clara expressão de bimodalidade. Já Ceita (2002) não encontrou bimodalidade cariotípica em nenhum dos seis gêneros da subfamília Bromelioideae analisados. Palma-Silva (2003), ao estudar espécies de Tillandsioideae observou uma nítida assimetria no tamanho dos cromossomos.

Gitaí *et al.* (2005) verificaram que indivíduos morfologicamente indistingüíveis de *Deuterocohnia lorentziana* (Mez) M.A. Spencer & L.B. Sm (Pitcairnioideae) apresentaram diferentes níveis de ploidia (2n=50 e 2n=150) e cromossomos com diferenças de tamanho e morfologia. Indivíduos diplóides

apresentaram cromossomos maiores (1,14 - 2,29 μm) com tendência à bimodalidade (19 pares grandes e 6 pares pequenos) enquanto os tetraplóides apresentavam cromossomos de menor tamanho (0,5 – 1,94 μm) com somente dois dos pares maiores.

Tais resultados sugerem a eliminação de seqüências do genoma durante o processo de poliploidização ao longo da evolução da família Bromeliaceae. Assim, em Bromeliaceae a fonte de variabilidade cromossômica seria dada pelo desenvolvimento de conjuntos bimodais e pela reorganização do padrão cariotípico. A evolução cromossômica freqüentemente se processa a partir de conjuntos relativamente uniformes, todos com cromossomos metacêntricos e de tamanhos iguais, para conjuntos não uniformes (bimodais) contendo cromossomos de tamanhos desiguais (Marchant, 1967; McWilliams, 1974).

Análises meióticas são raras em Bromeliaceae. Palma-Silva et al. (2004) realizaram o primeiro estudo do comportamento meiótico em indivíduos silvestres de bromélias, tendo observado alta regularidade meiótica em nove espécies do gênero *Vriesea* e duas do gênero *Aechmea.*

História antiga

Antes de Colombo fazer sua segunda viagem para as Américas, as bromélias eram desconhecidas pelos europeus. A maioria dos exploradores daquela época estava mais interessada em descobrir novas rotas comerciais e adquirir objetos de valor, e não novas espécies. Embora muitas vezes a exploração portuguesa e espanhola seja associada com a idéia de subjugação religiosa dos nativos e um implacável interesse por metais e gemas preciosos, novas plantas comestíveis também foram itens importantes para favorecer ou recompensar a volta para casa (Benzing, 1980).

Não surpreende o fato de que, dentre todas as bromélias que Colombo e sua tripulação provavelmente encontraram nas Índias Ocidentais, o abacaxi foi a única que realmente lhes chamou a atenção. Eles descreveram o fruto como “maior que um melão, cheiroso e de sabor muito adocicado” (Benzing, 1980).

As espécies de bromélias ornamentais permaneceram desconhecidas na Europa até que, em 1776, *Guzmania ligulata* (L.) Mez foi introduzida nesse continente. Depois disso, no século XIX, outras espécies vistosas, como *Billbergia pyramidalis* Lindl., *Billbergia amoena* LOOD. LONDL. e *Aechmea fasciata* Baker, começaram a chegar, em número sempre crescente, na Inglaterra, França, Alemanha e em outros países do oeste da Europa. O cultivo de *Bromelia antiacantha* Bertol. foi iniciado por Bertolini na Itália em 1824. Por volta de 1857, muitas espécies começaram a ser cultivadas em Berlim, o que foi evidenciado pelas numerosas citações de Beer "Die Familie der Bromeliaceen", o primeiro estudo compreendendo todo o grupo. Entre 1865 e 1885 houve um grande interesse na família Bromeliaceae na região da Bélgica central, próximo a Liege, onde Edouard Morren publicou a descrição de várias espécies com elaboradas ilustrações na Belgique Horticole. Outros botânicos também tiveram grande importância no estudo de bromélias ornamentais na última metade do século XIX, como C. Kock na Alemanha, Regel na Rússia, Antonie na Áustria e Lemaire, Linden e André na França. A grande maioria das espécies ornamentais descritas neste período e posteriormente, eram de origem brasileira (Smith, 1955).

O cultivo de bromélias continuou a ganhar adeptos na Europa Central e Ocidental até o início do século XX, quando as condições políticas e econômicas culminaram na Primeira Guerra Mundial, provocando uma falta de estímulo na horticultura em geral. Após 1945, o interesse pelo cultivo de espécies de bromélias voltou a aumentar não só na Europa, mas também nos Estados Unidos, Austrália, Nova Zelândia e em outros países (Benzing, 1980).

A horticultura de bromélias desenvolveu-se mais tarde nos Estados Unidos, entretanto produtores já listavam espécies para venda desde o final do século 19. Na década de 50, o cultivo de bromélias começou a contar com o apoio da Sociedade de Bromélias, muito importante até os dias de hoje. No Brasil, apesar de serem muito apreciadas nos jardins imperiais do Rio de Janeiro desde 1968, o interesse pelo seu cultivo para a comercialização como plantas ornamentais é muito recente.

História recente

Apesar das inúmeras finalidades para as quais as bromélias podem ser utilizadas, as quais já foram descritas neste capítulo, o seu valor ornamental é indiscutível e parece ser o principal potencial econômico das diferentes espécies pertencentes à família Bromeliaceae. No Brasil, a comercialização das bromélias como plantas ornamentais data aproximadamente do início da década de 1990. Neste país, assim como em outros de colonização latina, a valorização de espécies silvestres como ornamentais é pouco tradicional, diferente de países europeus, os quais têm incorporado em sua cultura a apreciação da jardinagem e da integração do homem com a natureza (Tombolato et al., 2004). No Brasil, o precursor da utilização das bromélias em paisagismo foi Burle Marx, na década de 1960, e, desde então, essas plantas foram lentamente conquistando a preferência dos paisagistas e consumidores. Além de agradar o gosto popular, as bromélias resolvem um problema do paisagismo moderno, que é a ocorrência, cada vez maior, de jardins pequenos. Essas plantas são de fácil adaptação e necessitam de pouca terra para sua sobrevivência (Martinelli, 2001; Biodiversity reporting, 2006). Além disso, as bromélias preenchem pré-requisitos básicos quando pensamos em espécies ornamentais, já que apresentam beleza, durabilidade e versatilidade. Um dos aspectos interessantes é que as espécies podem não só apresentar flores vistosas, mas também brácteas e folhagens com grande apelo estético, podendo, ainda, uma planta durar vários meses. Aspectos como estes têm tornado estas plantas cada vez mais apreciadas e, desta forma, sido mais extraídas da natureza (Coffani-Nunes, 2002).

Apesar da crescente demanda na utilização de bromélias como plantas ornamentais, atualmente são poucos os produtores efetivos que atendem ao mercado consumidor, havendo, portanto, uma procura maior do que a oferta. Sendo assim, surge uma lacuna que é preenchida com o comércio ilegal de espécies de bromélias provenientes do extrativismo (Coffani-Nunes, 2002). Em função da clandestinidade da atividade extrativista, a obtenção de dados sobre a comercialização do produto torna-se bastante complexa. Um levantamento pioneiro foi realizado por Coffani-Nunes (2002) com o objetivo de identificar a origem do material comercializado no eixo Rio-São Paulo, entretanto, o autor

concluiu que determinar a origem das plantas pode ser uma tarefa bastante difícil, já que aspectos como o perfil socioeconômico e as características de cada região estão diretamente envolvidos neste processo.

A diferenciação entre plantas produzidas em viveiros daquelas provenientes de extrativismo é bastante difícil, já que depende diretamente da declaração do comerciante. Em geral, o que se observa é que plantas cultivadas apresentam maior qualidade e uniformidade, e aquelas coletadas da floresta, em geral, apresentam folhas danificadas e pouca homogeneidade entre as matrizes. No Rio Grande do Sul não há uma estimativa concreta da proporção de plantas provenientes de extrativismo; entretanto, os depoimentos dos paisagistas nos permitem inferir que grande parte do produto comercializado no estado ainda é proveniente de extrativismo ilegal (Rocha, B.M., 2006 – comunicação pessoal).

Existem muitas espécies ornamentais, ou mesmo com potencial ornamental, ainda sob exploração extrativista, principalmente por falta de pesquisas que definam técnicas de cultivo. Estas espécies são consideradas nativas, ou silvestres, e são definidas como aquelas não manipuladas pelo homem, sendo que diversas espécies da flora brasileira, incluindo diferentes tipos de bromélias, pertencem a este grupo de plantas. A partir da manipulação inicial destas plantas, ainda sem um trabalho de melhoramento, as mesmas passam a ser denominadas semidomesticadas. A espécie é considerada domesticada somente após os processos de melhoramento genético e cultural. Algumas espécies nativas do Brasil foram domesticadas no passado, a exemplo do abacaxi, amendoim e cacau. Mais recentemente, algumas espécies ornamentais vêm sendo exploradas e denominadas como semidomesticadas, tais como orquídeas, petúnias e madressilvas (Tombolato et al., 2004).

Perspectivas

Para que haja um aumento na produção comercial de bromélias, deve haver um investimento expressivo neste setor, entretanto, as práticas culturais adequadas às diferentes espécies só poderão ser aplicadas mediante o

aumento da pesquisa na área. Aspectos básicos como sistema reprodutivo e número cromossômico ainda são desconhecidos para muitas espécies, o que torna inviável a domesticação e melhoramento destas plantas.

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