

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

Instituto de Biociências

Programa de Pós Graduação em Genética e Biologia Molecular

Estudo do sucesso reprodutivo, dos padrões de cruzamento e do fluxo de pólen em *Aechmea winkleri*, uma espécie endêmica do sul do Brasil

Miriam Valli Büttow

Orientadora: Prof^ª. Dr^ª Fernanda Bered

Porto Alegre, Abril de 2012

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endêmica do sul do Brasil**

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Orientadora: Prof^ª. Dr^ª Fernanda Bered

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**Estudo do sucesso reprodutivo, dos padrões de cruzamento e do fluxo de pólen
em *Aechmea winkleri*, uma espécie endêmica do sul do Brasil**

Resumo

Os padrões de fertilidade e biologia reprodutiva e suas conseqüências no sistema de cruzamento de plantas ainda são pouco compreendidos em espécies não modelo. Todos estes fatores têm aplicação direta para o estudo de conservação das espécies. *Aechmea winkleri* é uma espécie preferencialmente saxícola, endêmica de uma região muito pequena no sul do Brasil. É conhecida apenas uma população desta espécie, a qual sofre com a fragmentação de habitat resultante da conversão de mata atlântica em áreas urbanas e de produção agrícola. Esta fragmentação ameaça a sobrevivência e evolução da espécie. Neste trabalho, foram explorados os fatores genéticos e ecológicos que influenciam o sucesso reprodutivo e a persistência desta espécie na natureza. No **Capítulo II** é apresentado um estudo sobre a proporção de investimento em reprodução clonal e sexual, e foram avaliados componentes de *fitness* e sucesso reprodutivo. Também foi realizado um experimento de polinização controlada para verificar a ocorrência de limitação de pólen. Os resultados mostraram uma média de 16 *ramets* por *genet* e menos de 10% da população com estruturas reprodutivas. Em média, 80,96% (SE \pm 2,74) das plantas frutificaram e observou-se uma taxa de partenocarpia de 33,18% (SE \pm 2,43). Os experimentos de suplementação de pólen não mostraram diferenças entre polinização cruzada manual e polinização aberta, sugerindo que não há limitação de pólen nesta espécie. No **Capítulo III**, é apresentado um estudo onde foi caracterizada a biologia reprodutiva da espécie e testado o impacto de subestrutura de

população nos padrões de sistema de cruzamento e fluxo de pólen. O principal polinizador observado nesta espécie foi a mamangava, *Bombus morio*, e os experimentos de polinização manual mostraram que a espécie *A.winleri* é auto-incompatível. Para a análise molecular (microssatélites) do sistema de cruzamento foram coletadas 59 plantas mãe e em média 14 sementes para cada uma, totalizando 893 indivíduos coletados nas áreas de maior densidade populacional. Baseado em uma análise Bayesiana a posterior, foram identificadas duas subpopulações para esta espécie (Aw1 e Aw2). As estimativas de taxas de fecundação cruzada não diferiram de 100%. Foi observada estruturação de pólen entre as plantas maternas ($\Phi_{FT} = 0,25$), correspondendo a dois doadores efetivos de pólen ($N_{ep} \approx 2$). A distância média de dispersão foi baixa nas duas subpopulações, porém foi mais reduzida na subpopulação Aw2 ($\delta = 21.8$ m). Os resultados mostraram que a reprodução clonal parece não afetar negativamente o *fitness*, mantendo o tamanho da população suficientemente elevado. As distâncias curtas de polinização, no entanto, podem ser em consequência da fragmentação do habitat e do comportamento do polinizador. Assim, nós sugerimos que as estratégias de conservação devem se concentrar na área de ocorrência da espécie e em plantas adultas. Nossos resultados também sustentam o uso de espécies não arbóreas como um modelo adequado para estudos de fluxo de pólen.

Abstract

Fertility and reproductive biology patterns and its consequences for mating systems are still poorly understood in non-model species. These aspects have direct application in the study of species evolution and conservation. *Aechmea winkleri* is a mainly saxicolous species, endemic to a very small region in southern Brazil. There is only one known population of this species, which suffers from habitat fragmentation resulting from the conversion of forest in urban and agricultural areas. Fragmentation is a major threat to species survival and evolution. In this study, we explored the genetic and ecological factors that influence the reproductive success and persistence of this species in nature. In **Chapter II** we studied the proportion of investment in sexual and clonal reproduction, and we measured fitness components and reproductive success. We also verified the occurrence of pollen limitation with a controlled pollination experiment. Results showed an average of 16 ramets by genet and less than 10% of the population presented reproductive structures. Fruit set was on average 80.96% (SE \pm 2.74) and the parthenocarpy rate was 33.18% (SE \pm 2.43). Pollen supplementation experiments showed no differences between manual cross-pollination and open pollination, suggesting that there is no pollen limitation in this species. In **Chapter III**, we characterized the reproductive biology and tested the impact of population substructure in mating system and pollen flow patterns. The main pollinator observed in this species was the bumblebee, *Bombus morio*, and hand pollination experiments showed a self-incompatibility system. For the mating system analysis using microsatellites, we sampled 59 mother plants and an average of 14 seeds for each, totaling 893 individuals collected in the high density areas. Based on a Bayesian assignment, two subpopulations were identified for this species (Aw1 and Aw2). Estimates of outcrossing rates did not differ from 100%. Pollen pool structure was observed between

maternal plants ($\Phi_{FT} = 0.25$), corresponding to two effective pollen donors ($N_{ep} \approx 2$). The average dispersal distance was low in both subpopulations, but lower in the subpopulation Aw2 ($\delta = 21.8$ m). Our results showed that clonal reproduction does not seem to negatively affect fitness, keeping population size large enough. The short pollination distances, however, are in consequence of habitat fragmentation and pollinator behavior. Thus, we suggest that conservation strategies should focus on the species' occurrence area and in adult plants. Our results also support the use of non-arboreal species as a suitable model for pollen flow studies.

Capítulo I

Introdução geral

Introdução geral

1. A família Bromeliaceae

A família Bromeliaceae Juss é uma das famílias de angiospermas neotropicais mais diversa ecológica e morfologicamente (Benzing 2000; Givnish et al. 2011). Sua distribuição geográfica vai do norte da Patagônia, Argentina (latitude 44°S) até os estados de Virgínia, Texas e Califórnia nos Estados Unidos da América (latitude 37°N). É uma família reconhecida por sua recente radiação adaptativa, possuindo diferentes hábitos e variando entre terrestres, rupícolas e epífitas. São encontradas desde o nível do mar até altitudes de 4000 m, em regiões desérticas e úmidas, em solos sujeitos a inundações regulares e em lugares com baixa ou alta luminosidade. Elas podem florescer tanto em condições de muito calor, em rochas e dunas, como em temperaturas próximas de 0° C. As folhas, por exemplo, podem ser dispostas em forma de roseta, possibilitando o acúmulo de água, ou carnosas, servindo como órgãos de reserva. Também podem apresentar numerosas populações de importante papel nos ecossistemas em que ocorrem, servindo de alimento, de abrigo e de local de reprodução para muitas outras espécies (Benzing 2000).

Entre as espécies dessa família, o abacaxi (*Ananas comosus* (L.) Merr.) é o representante de maior importância econômica, sendo a terceira fruta tropical mais produzida no mundo (FAOSTAT 2005). Além disso, espécies da família Bromeliaceae são utilizadas como ornamentais, sendo apreciadas no mundo todo, principalmente na Europa, Estados Unidos e Japão, onde sua produção movimentou uma economia considerável (Paula & Silva 2000). Ainda, a *Bromelia antiacantha* Bertol., conhecida como bananinha-do-mato é utilizada por algumas comunidades na produção de xaropes

caseiros (Zanella et al. 2011).

A família Bromeliaceae apresenta cerca de 3140 espécies neotropicais, com apenas uma espécie no oeste da África (Smith & Downs 1974; 1977; 1979; Luther et al. 2008). Tradicionalmente, essa família tem sido dividida em três subfamílias: Bromelioideae (~650 spp.), Pitcairnioideae (~890 spp.), e Tillandsioideae (~1000 spp.), baseado em Smith & Downs (1979). Entretanto, estudos filogenéticos recentes vêm sugerindo uma classificação em oito subfamílias (Givnish et al. 2007; 2011). A nova classificação separa a parafilética Pitcairnioideae em seis subfamílias e propõe o seguinte relacionamento entre elas: (Brocchinioideae, (Lindmanioideae, (Tillandsioideae, (Hechtioideae, (Navioideae, (Pitcairnioideae, (Puyoideae, Bromelioideae)))))).

São considerados três centros de diversidade para Bromeliaceae: do norte dos Andes até o México e as Antilhas, o Planalto das Guianas e o leste do Brasil (Smith & Downs 1974). O leste brasileiro, representado pela Mata Atlântica, se destaca por apresentar um grande número de espécies endêmicas (Leme & Marigo 1993).

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 (Coffani-Nunes 2002). Entretanto, a coleta predatória e a perda de habitats devido à ação antrópica contribuem para o aumento do número de plantas vulneráveis, ameaçadas de extinção ou mesmo em extinção (Bered et al. 2008).

Apesar de um crescente aumento nos estudos em espécies desta família e de sua importância ecológica e econômica, a bibliografia científica ainda é consideravelmente 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 também pouco conhecidos.

1.1 O gênero *Aechmea* e subgênero *Ortgiesia*

O gênero *Aechmea* Ruiz & Pav. (do grego: ponta de lança) ocorre desde o México e Antilhas até o Uruguai e Norte da Argentina (Reitz 1983; Benzing 2000). Apresenta cerca de 240 espécies, sendo o gênero mais amplo e diverso da subfamília Bromelioideae (Faria et al. 2004; Luther 2008). Cerca de 70% das espécies do gênero estão distribuídas no Brasil (Smith & Downs, 1979) sendo a Mata Atlântica o centro de diversidade para o grupo (Smith 1934). A enorme diversidade estrutural e morfológica somada à pouca compreensão da delimitação natural dessas espécies, e, portanto, dos subgêneros, faz de *Aechmea* um dos maiores desafios taxonômicos atuais em Bromeliaceae (Leme et al. 2010). Estudos envolvendo revisão de alguns subgêneros de *Aechmea* já foram realizados, como para *Chevaliera* Gaudich. ex Beer (Canela et al. 2003; Sousa et al. 2005; 2008), *Lamprococcus* Beer (Aoyama & Sajo 2003), *Macrochordion* de Vriese (Faria et al. 2010) e *Pothuava* Gaudich. (Wendt 1997), porém para os subgêneros *Aechmea*, *Platyaechmea* (Baker) L.B.Sm. & W.J.Kress, e *Podaechmea* (Mez) L.B.Sm. & W.J.Kress, ainda não há trabalhos realizados. Para o subgênero *Ortgiesia* (Regel) Mez, somente um trabalho envolvendo duas espécies do grupo foi realizado (Goetze 2010).

O subgênero *Ortgiesia* tem distribuição geográfica restrita ao Brasil, Argentina, Paraguai e Uruguai. A maioria das espécies é exclusivamente brasileira, ocorrendo do Sul da Bahia até o Rio Grande do Sul. *Aechmea calyculata* (E. Morren) Baker ocorre também na Argentina e *A. recurvata* (Klotzsch) L.B. Sm. ocorre até o Uruguai e no Leste do Paraguai (Smith & Downs 1979). Atualmente existem controvérsias sobre quais espécies realmente pertencem ao subgênero *Ortgiesia*, sendo a listagem publicada por Luther (2008), a mais aceita. Assim, o subgênero *Ortgiesia* apresenta 22 espécies,

sendo que seis são endêmicas de Santa Catarina e ou do Rio Grande do Sul (*A. blumenavii* Reitz, *A. calyculata*, *A. comata* Baker, *A. kertesziae* Reitz, *A. pimentii-velosoi* Reitz e *A. winkleri* Reitz). O alvo do presente estudo é *A. winkleri*, uma espécie de distribuição geográfica extremamente restrita e ameaçada de extinção.

1.2 A espécie em estudo: *Aechmea winkleri*

Aechmea winkleri pertence à subfamília Bromelioideae, subgênero *Ortigiesia*. É endêmica da região central do estado do Rio Grande do Sul (Smith & Downs 1979), especificamente do município de Santa Cruz do Sul. É uma planta perene de vida curta, florescendo apenas uma vez durante o ciclo de vida. Em compensação, produz muitos *ramets* (rosetas) antes e após a floração. Considerada pequena, possui folhas verdes de margem serrilhada com espinhos de 0,5 mm de comprimento. As inflorescências são bipinadas na metade basal e simples para o ápice. As flores são sésseis de 16 mm de comprimento; as sépalas vermelhas, fortemente assimétricas, as pétalas amarelas e o ovário subcilíndrico vermelho com 5 mm de comprimento (Smith & Downs 1979). Possui hábito de “tanque”, sendo capaz de armazenar água e muitas espécies associadas. Possui preferência pelo hábito saxícola, podendo ser também terrestre ou epífita. Nos locais onde ocorre, essa espécie destaca-se no subbosque devido à grande quantidade de plantas crescendo em alta densidade (Winkler 1980; 1982).

Atualmente, as plantas situam-se em uma área de relativa preservação ambiental, no chamado “Cinturão Verde”. Esta área possui um total de 465 ha, e perímetro de 32 Km que circundam o norte e o leste da zona urbana de Santa Cruz do Sul, interior do estado do Rio Grande do Sul. A fim de conservar a biodiversidade no local, em 1994 foram criadas leis pela Prefeitura Municipal que limitam a ocupação e a construção civil dentro do Cinturão Verde (Müller 2009). A vegetação desta área é classificada como Mata Atlântica *latu sensu* (floresta semi decidual: Instituto Brasileiro de Geografia e

Estatística [IBGE] 1986, Oliveira-Filho & Fontes 2000), caracterizada pela presença de espécies como *Euterpe edulis* Mart., *Jacaranda micrantha* Cham., e *Cordia trichotoma* (Vell.) Arráb. ex Steud. A altitude varia de 50 a 150 m e o clima é subtropical úmido, com temperaturas abaixo de 15 °C no inverno e acima de 20 °C no verão (Alves & Colischonn 2001).

Dentre as seis espécies do subgênero *Ortgiesia* que são endêmicas do Sul do Brasil, *A. winkleri* é a única que está em uma região não litorânea (Mata Atlântica *latu sensu*), e é a que apresenta distribuição mais restrita, com somente uma população conhecida. O relacionamento evolutivo entre *A. winkleri* e as outras espécies ainda não é bem compreendido. Recentemente, Goetze (2010) descreveu alguns aspectos relativos à morfologia e à diversidade genética desta espécie, além de ter apresentado um estudo sobre o relacionamento entre *A. winkleri* e *A. caudata* Lindm. Nestes estudos, foram encontrados padrões de diversidade genética semelhantes a outras espécies de bromélias já estudadas e baixa estruturação populacional. Também foi revelado que as duas espécies possuem compartilhamento de haplótipos, porém mais estudos são necessários para o completo entendimento do relacionamento entre estes *taxa*.

2. Estudos de fertilidade e viabilidade em populações de plantas

O valor adaptativo, ou *fitness*, é a capacidade de um indivíduo de gerar descendentes. Por definição, o indivíduo com maior valor adaptativo em uma população é aquele que produz o maior número de progênie. Logo, *fitness* é uma medida relativa; indivíduos mais adaptados produzem mais descendentes em relação aos menos adaptados e, assim, quanto maior o *fitness* de um indivíduo, maior será a sua contribuição para as gerações futuras (Lienert 2004). Como não é possível avaliar

diretamente o *fitness* de uma planta, são usados componentes do *fitness* como, por exemplo, número e tamanho de sementes produzidas por uma planta. Estimar a viabilidade de populações inteiras é ainda mais difícil. Uma aproximação desta medida pode ser feita pela densidade de plântulas, indivíduos jovens, adultos e reprodutivos. De qualquer modo, esta estrutura pode ser devida tanto a componentes genéticos como não genéticos (Lienert 2004). Apesar de mais de três quartos de todas as angiospermas serem hermafroditas, por muito tempo as medidas de *fitness* em plantas basearam-se somente na fertilidade feminina. Isto se deve parcialmente à facilidade de se fazer uma avaliação aproximada da fertilidade feminina, por exemplo, através de contagem de sementes, enquanto a contribuição masculina na produção de sementes é uma análise mais complexa (Morgan & Schoen 1997).

É esperado que o tamanho populacional de espécies vegetais afete sua demografia e diversidade genética e diversos estudos indicam uma correlação positiva entre tamanho populacional e sucesso reprodutivo (Ågren 2008). Muitos fatores, conjunta ou isoladamente, produzem esta correlação: Primeiro, populações pequenas de plantas polinizadas por animais são, provavelmente, menos atraentes para os polinizadores do que populações grandes. Como consequência, a visitação pode ser diminuída e pode haver menor produção de sementes devido à limitação de pólen. Em segundo lugar, os processos estocásticos em plantas auto-incompatíveis podem resultar em perda de cruzamentos compatíveis, o que pode ser também limitante na produção de sementes para uma determinada fração da população. Em terceiro lugar, o endocruzamento pode ser mais frequente em populações pequenas e, neste caso, a depressão endogâmica pode reduzir o *fitness* da progênie em níveis maiores do que os observados em populações grandes (Ågren et al. 2008). Como consequência da endogamia, muitos estudos mostram que plantas endogâmicas produzem menos flores,

frutos e sementes, e sementes com menor taxa de germinação. A depressão por endogamia também pode ser expressa por menor tamanho de planta, menor tamanho de folha e menos biomassa (Lienert 2004; Sampaio et al. 2012). Além disso, a diminuição das interações mutualísticas e da variabilidade genética pode resultar na diminuição das taxas vitais e, conseqüentemente, em menores taxas de crescimento populacional, com um aumento do risco de extinção em populações pequenas (Kolb et al. 2010). Estas correlações positivas entre tamanho populacional, diversidade genética e *fitness* são ainda mais acentuadas em espécies raras e auto-incompatíveis, sugerindo que estas espécies seriam as mais sensíveis aos efeitos da fragmentação de habitats (Leimu et al. 2006).

Frequentemente, a fertilidade das populações, refletida pela produção de flores, frutos e sementes, é afetada pelo tamanho populacional e pela fragmentação do habitat, sendo a reprodução um dos primeiros processos biológicos afetados. Um efeito negativo da redução no tamanho das populações na reprodução vem sendo documentado em alguns trabalhos (Kennington 1997; Yakimowski & Eckert 2007; Sebbenn et al. 2011). Por outro lado, outros estudos mostram que nem sempre populações pequenas e ou isoladas possuem redução na diversidade genética e ou em componentes do *fitness*. O lado positivo destes resultados é que mesmo plantas isoladas e populações pequenas contribuem para a manutenção da espécie e devem ser consideradas quando da elaboração de estratégias de conservação (Lammi et al. 1999; Lander et al. 2010).

Em Bromeliaceae, não existem muitos estudos envolvendo a fertilidade de populações ou espécies. Um estudo detalhado foi realizado com *Vriesea gigantea* (Mart. ex Schult. f.) Mez, revelando alta produção de flores, frutos e sementes, porém limitação de pólen e pólen de baixa viabilidade foram encontrados em algumas populações (Paggi et al. 2007; Palma-Silva et al. 2008).

3. Reprodução clonal

A reprodução clonal é uma forma de crescimento que resulta na produção de indivíduos geneticamente iguais, capazes de crescimento e reprodução independentes. Cerca de 70% das plantas apresentam alguma forma de crescimento clonal (Klimes et al. 1997), deste modo, este tipo de reprodução parece ser vantajoso para a competição e colonização de novos habitats (Price 1999). Mesmo assim, a grande maioria das plantas de reprodução clonal se reproduz também por meio sexual. A hipótese mais aceita para a manutenção da reprodução sexual em espécies clonais é a de que esta permite a criação e manutenção da diversidade genética necessária para a adaptação a mudanças de condições ambientais (Charpentier 2002).

A reprodução clonal pode ter várias consequências na função e manutenção da reprodução sexual. Por exemplo, pode existir uma compensação fisiológica da alocação de recursos entre reprodução sexual e vegetativa. Além disso, o crescimento clonal pode interferir nos padrões de polinização e, por consequência, no sistema de cruzamento. Como o crescimento clonal afeta severamente a distribuição espacial e temporal de flores, é esperado que um crescimento clonal intensivo influencie os padrões de dispersão de pólen e a oportunidade de fecundação cruzada, afetando assim, a evolução do sistema de cruzamento em plantas (Barrett 1998, 2010; Charpentier 2002). A multiplicação e o florescimento simultâneo de ramos reprodutivos de um mesmo *genet* podem resultar em uma taxa significativa de geitonogamia (transferência de pólen de uma flor para outra dentro de um mesmo *genet*). Ainda, em espécies onde os *ramets* são ligados por entrenós curtos e os *genets* são espacialmente agrupados, o fluxo de pólen entre genótipos distintos pode ficar limitado (Jacquemyn & Honnay 2008). Conseqüentemente, a multiplicação clonal pode gerar alguma subdivisão na população (Loveless & Hamrick 1984).

A contribuição relativa do crescimento clonal em uma população ou espécie pode ser bem variável. Em um estudo com *Geum reptans* Linn. (Weppler et al. 2006), os autores demonstraram que tanto a reprodução clonal quanto a sexual são necessárias para o crescimento da população, e que a reprodução sexual não se restringe à manutenção da diversidade genética. Em Bromeliaceae, a reprodução clonal é muito comum, sendo reportada com detalhe em algumas espécies. Em *Aechmea magdalenae* (André) André ex Baker, por exemplo, Villegas (2001) encontrou grande variação sazonal na produção de *ramets*, com um aumento da reprodução clonal na transição da estação seca para úmida. *A. nudicaulis* (L.) Griseb., por sua vez, permaneceu com uma taxa de crescimento estável entre as estações e diferentes microhabitats (Sampaio et al. 2005).

Por fim, recentes estudos sustentam a hipótese de que quando as condições para reprodução sexual não são favoráveis, a reprodução clonal fornece garantia de reprodução (Vallejo-Marín & O'Brien 2007), contribuindo também para a manutenção das populações de plantas.

4. Biologia reprodutiva: aspectos ecológicos e genéticos

4.1 Aspectos ecológicos:

A biologia da polinização é o estudo dos mecanismos pelos quais a planta doa e recebe pólen. Por serem sésseis, as plantas necessitam de um agente que transfira o pólen, e esta “necessidade” acabou originando diversas adaptações florais (Barrett 1998; Cheptou et al. 2002). Devido à grande diversidade reprodutiva das angiospermas, este tema de estudo recebeu muita atenção de naturalistas e leigos ao longo do tempo (Ayasse & Arroyo 2011). Além disso, o entendimento da reprodução das plantas é de

imensa importância prática para a biotecnologia, para a conservação da biodiversidade e para o controle de espécies invasivas (Barrett 2010).

A família Bromeliaceae está entre as famílias de plantas com maior diversidade de modos de polinização (Benzing 2000). Existem espécies polinizadas por beija-flores (ornitófilas), insetos (entomófilas) e por morcegos (quiropterófilas) (Kessler 2002; Canela & Sazima 2003; Araújo et al. 2004; Schmid et al. 2011). As flores possuem grande diversidade de cores, formatos e odores, geralmente relacionados com o modo de polinização (Martinelli 1994; Benzing 2000). Apesar de a maioria das bromélias serem polinizadas por vertebrados, principalmente morcegos e beija-flores, insetos como abelhas, mamangavas e borboletas podem ser importantes agentes polinizadores em algumas espécies (Krömer et al. 2008; Kamke et al. 2011).

As bromélias possuem características florais como hercogamia e dicogamia que previnem a auto-polinização e facilitam a fecundação cruzada via animais (Martinelli 1994; Benzing 2000). Observações de morfologia floral e experimentos de polinização manual mostraram que grande parte da família possui um sistema misto de cruzamento e são auto-compatíveis (Matallana et al. 2010), no entanto, a auto-incompatibilidade também está presente em alguns grupos (Canela & Sazima 2003; Vosgueritchian & Buzato 2006; Kamke et al. 2011). A subfamília Tillandsioideae possui uma alta frequência de auto-compatibilidade e autogamia, em vários gêneros como *Alcantarea*, *Guzmania*, *Racinea*, *Tillandsia*, *Vriesea*, and *Werauhia* (Martinelli 1994; Benzing 2000; Lasso & Ackerman, 2004; Paggi et al. 2007; Matallana et al. 2010).

Uma descrição mais completa com todas as informações já descritas sobre o sistema de cruzamento e polinizadores do gênero *Aechmea* encontra-se na Tabela 1. Para as poucas espécies do gênero em que já foi feito estudo de sistema de cruzamento,

é interessante notar que existem tanto espécies auto-compatíveis quanto auto-incompatíveis. Existe também variação dentro da mesma espécie, por exemplo, em *A. distichantha* Lem., uma população foi descrita como auto-compatível (Sazima et al. 1999) e outra como auto-incompatível (Scrok & Varassin 2011). Quanto aos polinizadores, os beija-flores predominam, mas também há registros de abelhas, mamangavas, borboletas, vespas e mariposas.

Os estudos de como os fatores ecológicos influenciam o sistema de cruzamento foram iniciados por Darwin (1862), que propôs a hipótese de garantia de reprodução para a evolução da auto-fecundação em plantas. Como a fecundação cruzada exige um agente externo, como o vento ou um polinizador, a probabilidade de cruzamento está diretamente relacionada à possibilidade de transferência de pólen (Eckert et al. 2010). Os padrões resultantes da dispersão de pólen, geralmente refletem o comportamento do polinizador, e podem não otimizar a qualidade ou quantidade de cruzamentos. Polinizadores territorialistas, por exemplo, tipicamente movem-se em curtas distâncias entre flores, visitando flores próximas, seja dentro de uma mesma planta, seja em plantas vizinhas. Este comportamento trás importantes implicações para o cruzamento em plantas. Voos curtos podem limitar a dispersão mediada por pólen, influenciando a estrutura genética das populações, o tamanho de vizinhança e a frequência de cruzamentos entre indivíduos relacionados (revisado por Mitchell et al. 2009)

4.2 Aspectos genéticos: sistema de cruzamento e fluxo de pólen

Estimativas precisas do sistema de cruzamento em plantas podem ser obtidas através de marcadores moleculares, principalmente isoenzimas e microssatélites. Porém, os marcadores microssatélites, ou SSR (*simple sequence repeats*) são os que possuem as características mais desejáveis para este tipo de estudo: são distribuídos ao acaso, ocorrem em grande quantidade no genoma dos eucariotos, são codominantes, são fáceis

de detectar via reação em cadeia da polimerase (PCR), e possuem alta diversidade alélica (Selkoe & Toonen 2006; Ashley 2010). Embora os microssatélites tenham se tornado um instrumento popular em estudos de diversidade genética, sua utilidade pode ser limitada devido ao alto custo e tempo para o desenvolvimento de *primers* espécie-específicos. Uma alternativa para este problema é a utilização de *primers* heterólogos que possuam boa amplificação na espécie de interesse (Barbará et al. 2007).

Os métodos de estimativa direta da taxa de cruzamento são baseados no modelo misto de cruzamento, o qual assume que todo indivíduo de uma população produz uma porção s de suas sementes por auto-fecundação e uma porção $t = 1-s$ por fecundação cruzada, através de pólen de diferentes plantas, dispersas aleatoriamente na população (Ritland & Jain 1981; Ritland 1990; 2002). Este modelo também permite estimar a porção de indivíduos de uma determinada progênie proveniente de cruzamento entre indivíduos aparentados. Se este cruzamento ocorrer, a taxa de auto-fecundação unilocus - s_s deve ser mais alta que a taxa multilocus - s_m , e esta diferença fornece uma estimativa mínima para a aparente auto-fecundação devida ao cruzamento entre aparentados (*biparental inbreeding*). A razão para essa diferença esperada entre s_s e s_m é que quanto maior o número de loci utilizados, menor é a probabilidade de confusão entre uma progênie auto-fecundada ou proveniente de cruzamento entre indivíduos relacionados (Griffin & Eckert 2003). Este modelo permite ainda estimar variação nas taxas de cruzamento entre famílias e a correlação de paternidade, que é a probabilidade de que dois indivíduos de uma mesma progênie sejam irmãos completos (Ritland & Jain 1981; Ritland 2002).

Na última década, as espécies arbóreas foram as mais contempladas nos estudos de sistema de cruzamento, e dentre estas, são poucos os exemplos de espécies neotropicais: *Caryocar brasiliense* Camb. (Collevatti et al. 2010), *Senna multijuga*

(Rich.) Irwin & Barneby (Ribeiro & Lovato 2004) e *Tabebuia roseo-alba* (Ridl.) Sand. (Feres et al. 2011). Em Bromeliaceae, Paggi (2009) estudou o sistema de cruzamento de *Vriesea gigantea*, encontrando um sistema misto, com altas taxas de auto-fecundação.

A dispersão de genes é outro fator chave para a manutenção e distribuição da variação genética. Em particular, o fluxo gênico através do pólen vem sendo destacado como o fator principal para a distribuição da diversidade genética dentro e entre populações (Hamrick & Godt 1996). Muito do recente interesse dos biólogos evolutivos e conservacionistas no estudo do fluxo gênico deve-se à crescente preocupação com o impacto da fragmentação dos habitats e das populações e com a capacidade da espécie em manter os níveis naturais de diversidade genética (Austerlitz et al. 2004).

Estudos de diversidade genética fornecem uma estimativa do fluxo gênico efetivo a longo prazo, mas não são eficientes para revelar a contribuição de processos demográficos no fluxo gênico (Austerlitz & Smouse 2001a). Portanto, o conhecimento das taxas e padrões de fluxo gênico em tempo real é essencial na previsão dos efeitos do distúrbio antrópico nas espécies (Smouse & Sork 2004).

Nas últimas décadas, foram desenvolvidos métodos que pudessem estimar o fluxo de pólen entre as plantas de uma determinada população (Austerlitz & Smouse 2001b; Smouse et al. 2001). O método TWOGENER analisa a estrutura genética do *pool* de pólen amostrado nas plantas individuais relativos ao *pool* de pólen global (Smouse et al. 2001) baseado na análise molecular da variância (AMOVA- Excoffier et al. 1992). Além disso, esse método permite estimar a distância média de polinização e o tamanho efetivo de vizinhança. Entre as vantagens deste método, está a combinação da simplicidade de amostragem de um estudo de genética de populações com os aspectos

de dedução entre plantas maternas e progênie dos estudos de paternidade (Smouse et al. 2001).

A grande maioria dos estudos que utilizam o método TWOGENER até então estão restritos a árvores e arbustos (Hardy et al. 2004; Fernández-Manjarrés et al. 2006; Gonzales et al. 2006; Bittencourt & Sebbenn 2007; Llorens et al. 2012). Até o momento, o único trabalho com uma espécie de Bromeliaceae epífita é o de Paggi (2009). Neste estudo foi encontrada alta estruturação do *pool* de pólen e um baixo número de doadores de pólen em *Vriesea gigantea*.

Objetivos

Este estudo tem como objetivo geral investigar a fertilidade, sucesso reprodutivo e aspectos genéticos e ecológicos da biologia reprodutiva de *Aechmea winkleri*, visando contribuir para o desenvolvimento de estratégias para a conservação e manejo da única população conhecida desta espécie. Os resultados e ações de conservação também serão importantes para as espécies relacionadas.

Objetivos específicos

- Verificar o investimento de *A. winkleri* em reprodução clonal e sexual e estimar o tamanho populacional;
- Estimar o *fitness* da espécie baseado na produção de flores, frutos e sementes, altura de planta e inflorescência, taxas de germinação de sementes, viabilidade de pólen e semente e verificar a existência de limitação de pólen;
- Estudar a biologia reprodutiva e as características ecológicas da polinização desta espécie;
- Testar a influência de subestrutura populacional nos padrões de sistema de cruzamento e estimar a estrutura genética do *pool* de pólen de *A. winkleri*;
- Contribuir para estratégias de conservação da espécie.

Tabela 1 Taxa de *Aechmea* Ruiz & Pav. (Bromeliaceae: Bromelioideae), e possíveis polinizadores e sistema de cruzamento, como citado pelos autores.

Espécie	Sistema de cruzamento	Polinizador	Referência
<i>A. araneosa</i>	AC		Matallana et al. (2010)
		borboleta, beija-flor	Varassin & Sazima (2000)
<i>A. beeriana</i>	AI	beija-flor	Nara & Webber (2002)
<i>A. bromeliifolia</i>	-	beija-flor	Knuth (1904) <i>apud</i> Martinelli (1994) *
		<i>Chlorostilbon lucidus</i>	Machado et al. (2007)
		beija-flor	Varassin & Sazima (2000)
<i>A. capixabae</i>	AC		Matallana et al. (2010)
<i>A. caudata</i>	AI	<i>Bombus morio</i>	Kamke et al. (2011)
		beija-flor	Ruschi (1949) <i>apud</i> Martinelli (1994) *
<i>A. coelestis</i>	-	beija-flor	Ruschi (1949) <i>apud</i> Martinelli (1994) *
<i>A. comata</i> (= <i>A. lindenii</i>)	AC	beija-flor	Matallana et al. (2010)
		abelhas	Lenzi et al. (2006)
		borboletas	
<i>A. constantinii</i>	-	beija-flor, abelhas	Rios et al. (2010)

		borboleta	
<i>A. cyllindratta</i>	-	abelhas	Kaehler et al. (2005)
<i>A. distichantha</i>	AC	beija-flor	Scrok & Varassin (2011)
	AI	borboleta	Sazima et al. (1999)
<i>A. distichantha</i> <i>var. distichantha</i>	AI (preliminar)	beija-flor	Martinelli (1994) *
<i>A. fasciata var. fasciata</i>	AI (preliminar)	beija-flor	Martinelli (1994) *
<i>A. gamosepala</i>		<i>Bombus morio</i>	Araújo et al. (2004)
<i>A. lamarchei</i>	AC		(Matallana et al. 2010)
		beija-flor	Ruschi (1949) <i>apud</i> Martinelli (1994) *
<i>A. ligulata</i>	-	beija-flor	Ruschi (1949) <i>apud</i> Martinelli (1994) *
<i>A. macrochlamys</i>	AC		Matallana et al. (2010)
<i>A. magdalenae</i>	-	<i>Phaetornis superciliosis</i>	Murawski & Hamrick (1990)
		beija-flor	Stiles (1978) <i>apud</i> Martinelli (1994) *
<i>A. mariae-reginae</i>	-	beija-flor	Stiles (1978); Stiles & Freeman (1993) <i>apud</i> Martinelli (1994) *
<i>A. mexicana</i>	-	beija-flor	Stiles (1978) <i>apud</i> Martinelli (1994) *
<i>A. mutica</i>	AC		Matallana et al. (2010)

		beija-flor	Varassin & Sazima (2000)
<i>A. nudicaulis</i>	AI	beija-flor	Matallana et al. (2010)
		abelha	Schmid et al. (2011)
		borboleta	Varassin & Sazima (2000)
<i>A. nudicaulis var. aureo-rosea</i>	-	beija-flor, abelhas	Ule (1898) <i>apud</i> Martinelli (1994) *
<i>A. organensis</i>	-	beija-flor	Rushi (1949) <i>apud</i> Martinelli (1994) *
<i>A. pectinata</i>	AI	beija-flor	Canela & M Sazima (2003) Matallana et al. (2010)
<i>A. pineliana</i>	AI		Matallana et al. (2010)
		beija-flor	Varassin & Sazima (2000) Ule (1896); Ruschi (1949) <i>apud</i> Martinelli (1994) *
<i>A. pubescens</i>	-	beija-flor	Stiles (1978) <i>apud</i> Martinelli (1994) *
<i>A. purpureo-rosea</i>	-	abelhas	Benzing (1980) <i>apud</i> Martinelli (1994) *
<i>A. ramosa</i>	-	beija-flor	Ruschi (1949) <i>apud</i> Martinelli (1994) *
<i>A. recurvata var ortgiesii</i>	-	beija-flor	Ule (1898) <i>apud</i> Martinelli (1994) *
<i>A. sphaerocephala</i>	-	vespa (<i>Ageronia</i>), mariposas, abelhas	Knuth (1904) <i>apud</i> Martinelli (1994) * Ruschi (1949) <i>apud</i>

		beija-flor	Martinelli (1994) *
<i>A. triangularis</i>	-	borboleta	Varassin & Sazima (2000)
		beija-flor	
<i>A. triticina</i>	-	beija-flor	Ruschi (1949) <i>apud</i> Martinelli (1994) *
<i>A. victoriana</i>	-	beija-flor	Varassin & Sazima (2000)
<i>A. winkleri</i>	AI	<i>Bombus morio</i>	Capítulo III da presente tese

Esta tabela registra todas as indicações encontradas na literatura relacionadas ao sistema de cruzamento, polinizadores, visitantes e síndromes de polinização de espécies de *Aechmea*. Todos os estudos são baseados em detalhadas observações e experimentos de campo. Dados derivados de observações superficiais de visitantes florais, ou baseadas na síndrome de polinização, são marcados com (*). AI – auto-incompatível, AC – auto-compatível.

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Capítulo II

Clonal reproduction and high fertility in *Aechmea winkleri* (Bromeliaceae), a narrow endemic species from Southern Brazil

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1 **Clonal reproduction and high fertility in *Aechmea winkleri* (Bromeliaceae), a**
2 **narrow endemic species from Southern Brazil**

3

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13

14 **Abstract**

15 Reproduction investment and plant fertility are the central subject of many questions in
16 plant evolutionary and conservation biology. Clonal and sexual reproduction has direct
17 consequences for the population structure. In this study we aim to investigate the
18 investment in sexual and clonal reproduction, and fitness components of *Aechmea*
19 *winkleri*. We accessed the proportion of clonal and sexual investment by counting the
20 number of ramets and genets in sampling plots. Measures of plant fitness and
21 reproductive success were taken, and fertility was accessed considering plant and
22 inflorescence size, flower production, fruit and seed set, seed viability and germination
23 rate. Open-pollination and pollen supplementation studies were used to estimate pollen
24 limitation in natural population of the narrow endemic bromeliad *Aechmea winkleri*.
25 The results showed high investment in clonal reproduction, with an average of 16,0

26 ramets per genet and less than 10% of flowering individuals in the populations. Mean
27 plant and inflorescence height were 55.2 cm (SE±0.937) and 45.2 cm (SE±1.054),
28 respectively. Fruit set was in average 80.96 (SE±2.74) and we observed a parthenocarpy
29 rate of 33.2 (SE±2.43). Pollen supplementation experiments showed no differences
30 between manual cross-pollination and open-pollination, suggesting no pollen limitation
31 for this species. Clonality seems to not negatively affect fitness traits, maintaining the
32 population size sufficiently high. We suggest that conservation strategies should focus
33 on the species occurrence area and on adult plants.

34

35 *Key words:* endangered species; *in situ* conservation; pollen supplementation;
36 reproductive success.

37

38 The very restricted geographic distribution of some species has long fascinated
39 ecologists and evolutionary biologists. In the few studies of this issue, narrow endemic
40 species have been reported to have lower colonization ability than their widespread
41 congeners (Lavergne et al. 2004). Nevertheless, endemism and rarity phenomena result
42 from a diverse array of evolutionary scenarios and interactions, making it difficult to
43 identify the effect of evolutionary patterns on reproductive strategies (Castro et al.
44 2008).

45 Clonal propagation is extremely widespread and common in angiosperms (Lloyd
46 1980; Silvertown 2008; Vallejo-Marín et al. 2010) and most perennial flowering plants
47 combine sexual reproduction with some form of clonal reproduction (Eckert 2000;
48 Silvertown 2008). Plants with both reproductive strategies are better adapted to
49 maintain a stable population and to colonize new habitats (Liu et al. 2010). The relative
50 importance of sexual and clonal recruitment varies in response to diverse biotical and

51 abiotical conditions, and their contribution to population growth rate is expected to
52 differ (Villegas 2001; Ticktin 2003; Weppler et al. 2006; Kolb et al. 2010). Indeed, our
53 knowledge about the role of sexual vs. clonal recruitment in the maintenance and
54 growth of plant population is still quite limited (Fischer & Kleunen 2002; Winkler
55 2002; Clark-Tapia et al. 2005). In general, long-term persistence of clonal plants
56 depends on a balance between the survival of genets through clonal growth after
57 population establishment and recruitment through sexual reproduction (Jacquemyn et al.
58 2006). In clonal populations, the frequency of ramet production by different genets may
59 result in the dominance of competitive genotypes which can eventually lead to a loss of
60 genetic variability. Therefore, clonality can have a significant effect on the fitness of
61 individual ramets, by affecting the probability of setting fruits and seeds (Clark-Tapia et
62 al. 2005; 2006; Dorken & Drunen 2010; Carrillo-Angeles et al. 2011).

63 The relationships among plant population size, fitness and genetic diversity are
64 of fundamental importance in plant ecology, evolution and conservation (Oostermeijer
65 2003; Frankham 2010). Small populations are more likely to suffer the negative genetic
66 consequences of increasing inbreeding depression and reduced genetic variation. Also,
67 ecological mechanisms such as pollinator limitation, may further reduce fitness in small
68 populations (Ellstrand & Elam 1993; Fernández-Manjarrés et al. 2006; Jacquemyn et al.
69 2006; Leimu et al. 2006; Parra-Tabla et al. 2011). In this view, flower and fruit
70 production are shaped by interactions with both the abiotic and biotic environment.
71 Flower production is often correlated strongly with plant size, which generally increases
72 with resource availability and decreases with population density. Thus, flower, fruit and
73 seed production can be influenced by a variety of environmental factors and population
74 characteristics (Ågren et al. 2008; Dauber et al. 2010).

75 Although many plant populations are naturally isolated and small, populations of
76 numerous plant species have become more isolated and further decreased in size due to
77 the recent anthropogenic fragmentation of habitats (Ellstrand & Elam 1993; Hmeljevski
78 et al. 2011; Sebbenn et al. 2011). Urban areas for example, are considered as the most
79 pervasively changed landscapes. Plant communities within urban areas occur in small
80 fragmented and more or less isolated patches. The persistence of species within such
81 patches depends, at least in part, on the effective functioning of pollination for plant
82 reproduction, a process that has been shown to be vulnerable to population
83 fragmentation in a variety of plant species (Eckert et al. 2010; Hennig & Ghazoul 2011).
84 It is clear that habitat fragmentation may alter life-history traits that are important in
85 governing the reproductive success of plant populations and dispersal of seeds.
86 However, to what extent this is already happening at a large scale is not clear, because
87 empirical evidence for habitat fragmentation-driven selection remains rather scarce
88 (Jacquemyn et al. 2006; 2012; Bittencourt & Sebbenn 2009).

89 The Bromeliaceae is a Neotropical family with nearly 3000 species, with only
90 one species in western Africa (Smith & Downs 1974; 1977; 1979; Givnish et al. 2011).
91 *Aechmea winkleri* Reitz is a narrow endemic species, with only one known population
92 (Goetze 2010), within the urban area of a small city in Rio Grande do Sul State,
93 southern Brazil. This species has been subjected to habitat loss because forest regions
94 are becoming urban and agricultural areas, threatening its survival and evolution. The
95 growing real estate market and the consequent anthropic contact results in a decrease of
96 population size, either due to habitat fragmentation, or due to illegal extraction. At the
97 present moment there is no information about reproductive success aspects in *A.*
98 *winkleri*.

99 Here, we aim to contribute to discuss the following main question: How can one
100 narrow endemic species, be fertile, reproduce and survive since its occurrence is
101 restricted to an urban area? To help to shed light on these matter, we evaluated the
102 reproductive success of the only one known population of *A. winkleri* located in
103 southern Brazil considering: (1) the investment in clonal and sexual reproduction and
104 the estimated population size; (2) the fitness based on the production of flowers, fruit
105 set, the height of plants and inflorescences, rates of seed germination, seeds and pollen
106 viability; (3) the occurrence of pollen limitation. The results obtained will be essential
107 to give a diagnosis on the reproductive success of this narrow species, and should be
108 taken into account in conservation actions for *A. winkleri*.

109

110 **Material and Methods**

111 **Study species:** *Aechmea winkleri* is a bromeliad species which belongs to
112 subgenus *Ortgiesia* and Bromelioideae subfamily. It is preferentially saxicolous but also
113 terrestrial or epiphytic, with preferences for moderately shade environments, growing
114 densely aggregated and standing out in understory (Winkler 1980; 1982) (Figure 1).
115 Like many other Bromeliaceae species, *A. winkleri* exhibits a semelparous life cycle,
116 which means it breeds only once in life before die. This small bromeliad forms a well
117 shaped rosette with green leaves that in the direct sunlight are tinged with purple at the
118 tips. The bright red flower stem has a branched inflorescence at the base and flowers
119 with yellow petals. *A. winkleri* is endemic of the central part of Rio Grande do Sul State
120 (Smith & Downs 1979). The species occurs in a very restricted (4.65 km²) and
121 particular region, the transition area between Atlantic Rain forest and Pampa biomes, in
122 southern Brazil. The reminiscent forest areas suffer from habitat fragmentation resulted
123 from conversion of forests in urban and agricultural regions, and as recurrently in the

124 Bromeliaceae family, *A. winkleri* is listed as an endangered species by the Official List
125 of the Endangered Brazilian Flora Species (MMA 2008).

126 **Study site:** The study was conducted in an area called “Cinturão Verde” (“green
127 belt” – environmental preservation area) in a small municipality (29°41 S and 52°26’ W)
128 in Rio Grande do Sul State, southern Brazil. With a total area of 465 hectares, mostly
129 privates, the “Cinturão Verde” surrounds the urban area of Santa Cruz do Sul city and
130 present a perimeter of 32 linear km (Figure 2). It was created in 1994 by and classified
131 as special zone, with strict rules for occupancy aiming the area conservation (Müller
132 2009). “Cinturão Verde” is classified as *latu sensu* Brazilian Atlantic rainforest (semi-
133 deciduous forest: Instituto Brasileiro de Geografia e Estatística [IBGE] 1986, Oliveira-
134 Filho & Fontes 2000). Nowadays this region is suffering with illegal occupancy and
135 deforestation due to construction of residential condominiums. Therefore, we selected
136 four areas (replications) where plants occur in abundance: Bairro Universitário (BU),
137 Clube União (UN), Parque da Gruta (PG) and Pinus Parque (PP) (Figure 2). The plants
138 in PG area are located on the rocks of a waterfall, making it risky and impractical to
139 install regular plots at this site, for this reason some experiments were not conducted in
140 this area (Figure 2). According to previous studies, *A. winkleri* composes a single
141 population, occurring in four major locations, with high gene flow among the plants
142 from these areas (Goetze 2010).

143 **Reproduction investment and population size:** This study was accomplished
144 in 2009 and 2010 in three out of the four areas of occurrence of *A. winkleri*, during the
145 flowering period. For data collection, one permanent plot of 10 x 20 m was placed in
146 each area where the density of plants was high (Figure 2). One main census per year
147 was conducted during the flowering season, in which the following data were collect
148 inside all plots: (1) number of terrestrial and epiphytes genets; (2) number of ramets in

149 each genet; and (3) counting of flowering ramets per genet. The ramet population was
150 structured into four categories of number of ramets per genet: 1-10, 11-20, 21-40 and
151 40-80, with the percentage of genets and the fraction of reproductive ramets included in
152 each category. The definition of Harper et al. (1977) for ramets and genets was
153 followed, so new individuals formed by clonal propagation are referred to as ramets,
154 and all ramets produced by a genotype are collectively referred to as a genet. Given
155 that ramets usually do not separate from the mother plant, we might consider each group
156 of plants as a genet and the rosettes that are part of a group as ramets of the same genet.
157 Although genetic analysis was not the aim of this work and we didn't genotyped each
158 group, we assumed that each one was a genet. This assumption is possible based on
159 genetic diversity results obtained by Goetze (2010) and because the specific site where
160 these plants grow is formed by fragments of rocks that slid from the hills, forming
161 distinct groups of plants in each rock. In the patches formed by densely aggregated
162 rosettes, ramets typically remained connected to their mother plants. The proportion of
163 ramets / genet was considered as the rate of investment in clonal reproduction, while the
164 proportion of vegetative / reproductive individuals was considered as the rate of
165 investment in sexual reproduction. We obtained the plants density for each area from
166 the number of ramets counting.

167 **Fitness traits:** To verify aspects of fitness we measured plant and inflorescence
168 height (cm) in the four sampled areas in 2009 (Figure 2). As we did not find any
169 differences among areas (see Results and Table 2), we choose two out of four groups to
170 verify the reproductive success based on flower, fruit and seed set. Then, field studies
171 were carried out in 2009 and 2010 during the reproductive period of the plants (March
172 to August) in BU and UN (Figure 2). Reproductive success was evaluated through
173 flower production, and fruit set with the following measures: (1) the reproductive

174 potential (total number of flowers per plant); (2) fruit set (i.e., the fraction of flowers
175 developing into a mature fruit) as % fruits =total number of fruits per plant/total number
176 of flowers per plant x 100 (according to Burne et al. 2003). Comparisons within areas
177 among years were performed by *T* test (5%) (SPSS 19.0, IBM, New York, NY, USA).

178 *Seed germination rate and viability:* To analyze seed viability, 15 plants of *A.*
179 *winkleri* were randomly sampled in each group (Figure 2), except for PP where 16
180 plants were sampled, totaling 61 plants. One to three fruits (mean of 43.51 seeds/fruit)
181 were used per plant. Seeds were disinfected and placed in petri dishes with a culture
182 medium, containing ½ MS salts (Murashige & Skoog, 1962), B5 vitamins (Gamborg et
183 al. 1968), 3% sucrose, and 0.3% Phytigel (Sigma, Saint Louis, MO, USA), pH 6.4. The
184 dishes were incubated in a climate-control chamber with relative humidity near 100%
185 and photoperiod of 16 h light at 25°C and 8 h dark at 22°C. Germination was monitored
186 daily for 30 days. The germination rates were determined as the proportion of seeds that
187 had germinated, by counting the number of seedlings. Comparisons among groups were
188 made by a one-way ANOVA (SPSS 19.0, IBM, New York, NY, USA).

189 *Pollen viability:* In 2009, young floral buds were collected, fixed in 3:1 ethanol :
190 glacial acetic acid for 24 h at room temperature. After fixation, the buds were
191 transferred to 70% alcohol and stored in a freezer at -20°C.. Squash preparations were
192 stained following Alexander's (1980) method, in which empty unviable pollen grains
193 stain green and full viable pollen grains stain purple. One flower per individual was
194 analyzed. Samples of 1500 pollen grains per flower (500 pollen grains per anther in
195 three anthers), totalizing 63,000 pollen grains from 42 individuals of BU area were
196 analyzed (Figure 2). Slides were examined and documented using a Zeiss Axioplan
197 Universal photomicroscope (Carl Zeiss, New York, NY, USA).

198 *Pollen supplementation:* Field studies were conducted during the plant
199 reproductive cycle, flowering in March and fruiting in August 2009. To determine
200 whether fruit production in *A. winkleri* is limited by reception of pollen, we performed a
201 supplemental pollination experiment in plants from BU area (Figure 2). The pollination
202 treatments were accomplished as follows: (1) open-pollination – three to five flowers
203 per plant, from five plants, which opened during the dawn and were available to any
204 visitor, were tagged; (2) manual cross-pollination – one to three flowers per plant from
205 five plants were emasculated at pre-anthesis and were hand- pollinated by rubbing
206 anthers with fresh pollen from a different genet in the stigma and then bagged. The
207 results (i.e., aborted or developing fruit) of the pollination treatments were recorded six
208 months later. Mature fruits were collected and the number of seeds per fruit was
209 counted. To evaluate whether pollen supplementation affected seed set, the number of
210 seeds from hand-pollinated and naturally pollinated fruits were compared. Comparisons
211 between treatments were made with *T* test (5%) (SPSS 19.0, IBM, New York, NY,
212 USA).

213

214 **Results**

215 **Reproduction investment and population size**

216 In the sampled areas the mean number of ramets was much higher than the mean
217 number of genets, showing high investment in clonal reproduction (Table 1).

218 Considering all areas and years, the mean number of ramets per genets, was 16.0.

219 Nevertheless, plants from BU presented in average 26.6 ramets in each genet (Table 1).

220 In Figure 3 the genet size were categorized by number of ramets in each genet and the
221 proportion of reproductive individuals was estimated in each class considering all

222 sampled areas and two years of observation (2009 and 2010). The investment in sexual

223 reproduction was estimated based on the proportion of reproductive individuals in each
224 genet (cluster). In average, about 40% of genets have 11 to 20 ramets, and all classes
225 show less than 10% of reproductive individuals (Figure 3).

226 We sampled three out the four high density occurrence areas of *A. winkleri* and it
227 was estimated in average 1660 plants in 200m² (Table 1). Our results project a density
228 of 8.3 plants per m².

229 **Fitness traits**

230 Considering fitness traits, the overall means of plant and inflorescence height
231 was 55.22 cm (SE ± 0.973) and 45.17 cm (SE ± 1.054) respectively, with no significant
232 differences ($P > 0.05$) among the four areas analyzed in 2009 (Table 2). The mean
233 number of flowers per plant was 114.87 (SE ± 5.27), without significant differences
234 between the sampled areas ($P > 0.05$, Table 3). The mean number of flowers that set
235 fruits, with or without seeds, was 91.91 (SE ± 4.94), which corresponds to a fruit set
236 mean of 80.96%. Within the total fruits produced by each plant, there are a proportion
237 of fruits that do not set seeds, having a reddish color. These fruits are probably formed
238 by parthenocarpy and occurred at a mean rate of 33.18% (SE ± 2.43, Table 3). Fruits
239 that set seeds turn on from green to dark purple when ripe. We identified a significant
240 decrease in fruit set rate from 2009 to 2010 in BU and in the percentage of fruits with
241 seeds (Table 3). At the same year, there was a significant increase in parthenocarpy rate
242 in plants from this sampled area.

243 *Seed germination and pollen viability*: After 7 days in culture, 76.63 % (SE ±
244 3.66) of the seeds, in average, had germinated and in 30 days of evaluation 82.03%
245 (SE± 3.20) had germinated in average. We did not found significant differences among
246 the sampled area concerning seed germination. The pollen viability rate was considered
247 high, since 90.60% (SE ± 3.40) of 63,000 pollen grains were viable.

248 *Pollen supplementation experiment*: The experiment of pollen supplementation
249 showed a similar value for both treatments, manual cross-pollination had a mean of
250 105.11 seeds per fruit (SE \pm 10.3), while the open-pollination produced a mean of
251 101.92 (SE \pm 7.92) seeds per fruit. There was no statistical significance between
252 treatments ($P=0.19$).

253

254 **Discussion**

255 **Reproduction investment and population size**

256 *Aechmea winkleri* invests in both clonal and sexual reproduction since for each
257 genet we count 16.0 ramets in average (Table 1). We also showed that the sexual
258 investment was relatively low, because the rate of reproductive plants in each class of
259 genets was less than 10% in average (Figure 2). In Bromeliaceae, clonal reproduction
260 has been reported in a range wide variety of species from almost all subfamilies
261 (Porembski et al. 1998; Sarthou et al. 2001; Villegas 2001; Kessler 2002; Wendt et al.
262 2002; Canela & Sazima 2003; Ticktin 2003; Gonzalez-Astorga 2004; Mondragón et al.
263 2004; Ordano & Ornelas 2004; Cascante-Marín et al. 2006; Cavallari et al. 2006; Lenzi
264 et al. 2006; Vosgueritchian & Buzato 2006; de Sousa et al. 2007; Rocha-Pessoa &
265 Rocha 2008; Barbará et al. 2009; Cavallero et al. 2009; Paggi et al. 2010; Wang et al.
266 2010; Domingues et al. 2011; Hmeljevski et al. 2011; Kamke et al. 2011; Schmid et al.
267 2011; Zanella et al. 2011), with a wide variation in number of ramets per genet. Even
268 though the effect of clonal expansion on the reproductive sexual success of clonal
269 species is not new (Harper 1977; Fischer & Kleunen 2002; Clark-Tapia et al. 2005),
270 there is still little empirical evidence of what role does an increase in clonality play on
271 reproductive success (Winkler 2002; Vallejo-Marín & O'Brien 2007; Scobie & Wilcock
272 2009; Dorken & Drunen 2010; Carrillo-Angeles et al. 2011). Two main consequences

273 of clonal growth are possible: in one hand, clonal spread combined with reduced sexual
274 recruitment will yield a population structure in which nearby ramets are often members
275 of the same genotype, resulting in geitonogamous self-fertilization and consequent
276 incompatibility or inbreeding depression. On the other hand, by increasing longevity in
277 some genotypes, clonality can amplify generation time. This, in turn, may result in the
278 increase of effective population size due to enhance in time of overlapping generations,
279 and the subsequent inhibition of population subdivision (Izquierdo & Piñero 1998;
280 Jacquemyn & Honnay 2008). This is important because the proportion of sexual and
281 vegetative reproduction is one of the main factors influencing persistence and dispersion
282 of plant species. Experiments in reproductive biology and mating system will help to
283 elucidate the direction in which this species is heading. Though, the low F_{ST} values
284 obtained by Goetze (2010), favor the second hypothesis.

285 Considering the sampled areas, BU revealed a great investment in clonal
286 reproduction (26.56 ramets per genet in average), almost twice as much that UN site and
287 almost four times more than PP locality (Table 1). Also, we found that most genets are
288 formed by 11 to 40 ramets (Figure 3). Our results agree with the ones found in *Aechmea*
289 *pectinata* Baker, where the assemblages are composed of 10-14 individuals (Canela &
290 Sazima 2003).

291 We estimated a density of 8.3 plants per m^2 in the high density occurrence sites
292 of *A. winkleri*. Considering that we have four high density areas of ca. $800m^2$, we could
293 extrapolate this value in a $3200 m^2$ area. As a result, we estimate an amount of 26,560
294 plants, i. e. ramets, present in the high density areas. In the same way, we found 0.6
295 genets per m^2 , totaling 1920 genets. Determining the total number of plants of one
296 threaten species is of fundamental importance, and combing this data with reproduction

297 investment, success and genetic diversity will help to propose management actions and
298 conservation (Angeloni et al. 2011).

299 **Fitness**

300 Considering fitness measures, we did not find differences among sampled areas
301 (Table 2). This may be due to the closeness of the four study sites and to the similar
302 environmental conditions. In *A. winkleri*, flower production has small variation, while
303 significant decreased in fruit set rate and % of fruits with seeds were observed between
304 years of the site BU (Table 3). Even so, the mean fruit set of 80.96 (\pm SE 2.73) in *A.*
305 *winkleri* (Table 3) is high when compared to another bromeliad species such as *Vriesea*
306 *gigantea* Gaug. (mean fruit set lower than 50%), despite of its wide range geographic
307 distribution (Paggi et al. 2007). Another important trait in *A. winkleri* is the production
308 of fruits without seeds (mean of 33.18% - Table 3), probably through parthenocarpy.
309 These fruits remain with a reddish color, while the fertilized ones became dark purple
310 when ripe. Parthenocarpy has also been reported for *Aechmea lindenii* (E. Morren)
311 Baker, but in higher levels (52.95%), associated with low pollinator visit frequency and
312 an adaptation expression of dispersion and reduction in predation of fruits and seeds
313 (Lenzi et al. 2006). In zoochoric fruits, higher fruit production, even through
314 parthenocarpy, can operate with an increase in plant capacity to attract dispersers
315 (Fuentes 1995).

316 Concerning the other fitness traits evaluated, seeds were moderately viable from
317 all analyzed populations, presenting a germination rate of 82.03%. In *V. gigantea* the
318 mean germination rate was 94% (Paggi et al. 2007), and Downs (1963) reported similar
319 results for other bromeliads species. Even with high germination rates in controlled
320 conditions, seedlings recruitment in the field is almost absent (personal observation).
321 This may be of crucial importance for persistence of population. Germination and

322 seedling establishment tend to be the most vulnerable stages in the life cycle of plants
323 because seedling mortality is often high. Low recruitment was observed in different
324 conditions for other four bromeliad species (Cascante-Marín et al. 2008), and for *A.*
325 *bracteata* (SW.) Griseb. with germination less than 1% in the field (Goode & Allen
326 2009). However, since sexual reproduction and seedling recruitment are accompanied
327 by genetic recombination and due to the emergence of new genotypes, even low levels
328 of seedling recruitment may positively affect the amount of clonal diversity within
329 populations (Jacquemyn et al. 2006). In addition to the reproductive success and traits
330 of fitness, the occurrence of pollen limitation was also assessed. Based on pollen
331 supplementation experiments and pollen viability, *A. winkleri* do not present pollen
332 limitation (see Results), showing that the pollinator is effective in the population. In five
333 species studied by Winter et al. (2008), pollen limitation was the most probable cause
334 for decreased plant fitness in small and/or isolated populations. Also, in species with a
335 naturally patchy distribution, habitat quality, population size and density are the main
336 factors influencing reproductive output (Ågren et al. 2008). Since the magnitude of
337 pollen limitation observed in natural populations depends on historical constraints and
338 contemporary ecological factors (Knight et al. 2005; Aizen & Harder 2007), the absence
339 of pollen limitation in this area may mean that the pollinator service has not been
340 affected yet by habitat fragmentation and urbanization. An important remaining issue,
341 which will help to determine the population reproductive success, is the number of
342 pollen donors and the genetic diversity of mother plants and progenies. Leimu et al.
343 (2006), in a meta-analysis study, clearly demonstrated that population size, plant fitness
344 and genetic diversity are generally related. Despite of its narrow distribution, *A. winkleri*
345 presents a relatively high population size, no evidences for pollen limitation (this study)
346 and high genetic diversity (Goetze 2010).

347

348 **Concluding remarks**

349 The results described here are the first reported on population size, reproduction
350 investment, plant fitness e reproductive success for *A. winkleri*. A special concern about
351 *A. winkleri*, is its restrict geographic distribution and occurrence next and even inside an
352 urban area, subject to increasing anthropogenic pressure. Our results show that the
353 species presents both asexual and sexual reproduction. Despite the high clonal
354 investment, sexual reproduction is also effective, since *A. winkleri* produces high
355 amounts of fruits and seeds and there is no pollen limitation. Moreover, the clonality
356 seems to not negatively affect the fitness traits, maintaining the population size
357 sufficiently high. The evident disturbance and fragmentation in the occurrence area of
358 *A. winkleri* does not seem to have affected the species reproductive success. However,
359 genetic data are necessary to increment the conclusions.

360 We suggest that the conservation efforts should focus primarily on habitat
361 conservation and management strategies should focus on the protection of adult plants
362 from accidental destruction. Also, further studies on plant longevity, mortality rate,
363 recruitment of new individuals and mating system will be essential to evaluate the *A.*
364 *winkleri* population dynamics.

365

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372

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607 tetraploid species *Bromelia antiacantha* (Bromeliaceae). *American Journal of*
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609

610 **List of tables**

611 Table 1 Means of genets and ramets and proportion of ramets for each genet of
612 *Aechmea winkleri*. Means were calculated concerning the three sampled areas and based
613 on two years (2009 and 2010) of demographic count in plots of 10 x 20 m.

Locality (plot 10 x 20m)	Mean number of genets	Mean number of ramets	Proportion (ramet/genet)
BU	91.50	2430	26.6
PP	193.00	1430	7.4
UN	80.00	1120	14.0
Mean	121.50	1660	16.0

614 For locality abbreviations see Material and Methods.

615

616 Table 2 Fitness: means of plant and inflorescence height of *Aechmea winkleri* in
 617 southern Brazil. Values shown are means \pm SE.

Locality	n	Plant height (cm) \pm SE	Inflorescence height (cm) \pm SE
BU	20	57.40 \pm 2.018	47.15 \pm 1.841
UN	20	52.30 \pm 1.753	43.65 \pm 1.387
PG	7	56.00 \pm 2.127	39.86 \pm 4.097
PP	20	55.35 \pm 1.375	45.90 \pm 2.205
Mean (Total)	(67)	55.22 \pm 0.937	45.17 \pm 1.054

618 For locality abbreviations see Material and Methods.

619 ANOVA analysis showed no statistical differences between sites for plant ($P=0.240$)
 620 and inflorescence height ($P=0.255$).

621 n: sample size.

622

623 Table 3 Reproductive success: means \pm SE of flower production, rate of fruit set and
 624 parthenocarpy in two years and two sampled area of *Aechmea winkleri*.

Locality	Year	n	Number of flowers	Fruit set rate (%)	Fruits with seeds (%)	Parthenocarpy rate (%)
BU	2009	15	120.47 \pm 7.16	90.95 \pm 2.80*	71.12 \pm 3.71*	28.88 \pm 3.72*
	2010	10	104.50 \pm 13.57	73.32 \pm 8.35*	50.49 \pm 4.94*	49.51 \pm 4.94*
UN	2009	10	110.30 \pm 11.27	82.87 \pm 5.01	72.21 \pm 4.07	27.78 \pm 4.07
	2010	10	120.80 \pm 11.96	71.64 \pm 4.24	71.30 \pm 4.27	28.70 \pm 4.27
Mean			114.87 \pm 5.27	80.96 \pm 2.74	62.4 \pm 4.51	33.18 \pm 2.43

625 Comparisons were made within years among populations and within populations among
 626 years.

627 * Significant differences observed only within sites among years, *T* test (5%), *P* < 0.005.

628 For locality abbreviations see Material and Methods.

629 n: sample size

630 **Figure legends**

631 Figure 1. Clumped distribution and flowering individuals of *Aechmea winkleri* in Santa
632 Cruz do Sul, Rio Grande do Sul State, Brazil.

633

634 Figure 2. Map showing the study region and table indicating the area sampled for each
635 approach. Symbols indicate the four sampled areas of *Aechmea winkleri*. Mean distance
636 between the sites are 2.5 Km.

637

638 Figure 3. Rate of investment in sexual reproduction in *Aechmea winkleri*. Genet size
639 was determined considering the number of ramets and was categorized in four classes.
640 Reproductive ramets within each size class are shown in gray. Means of two years of
641 observation (2009 and 2010) and sampled areas.

642

643 **Figure 1**

644

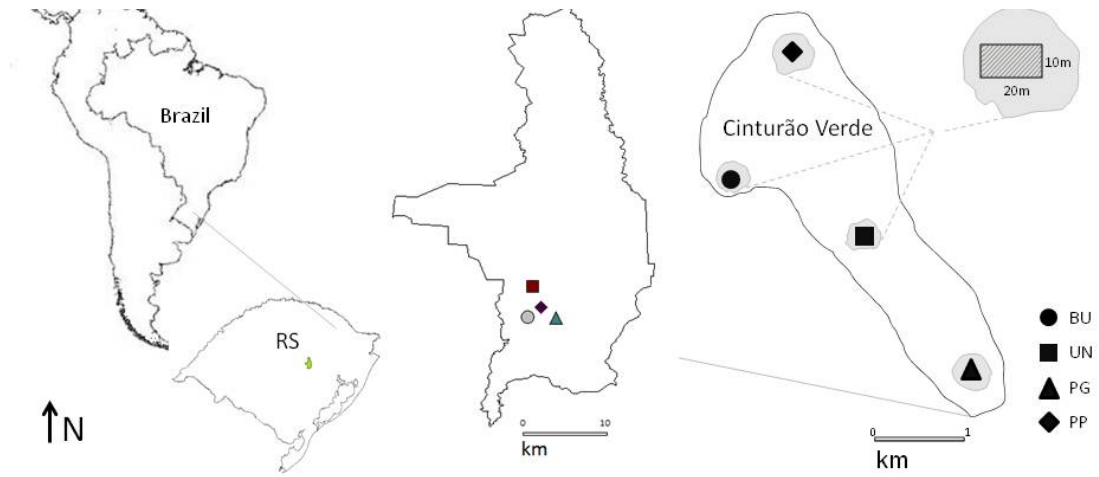


645

646

647 **Figure 2**

648



Study sites	Reproductive investment and plant counting*	Plant and inflorescence height	Number of flowers, fruits and seed set	Seeds germination and viability	Pollen viability and limitation
BU	X	X	X	X	X
UN	X	X	X	X	
PG		X		X	
PP	X	X		X	

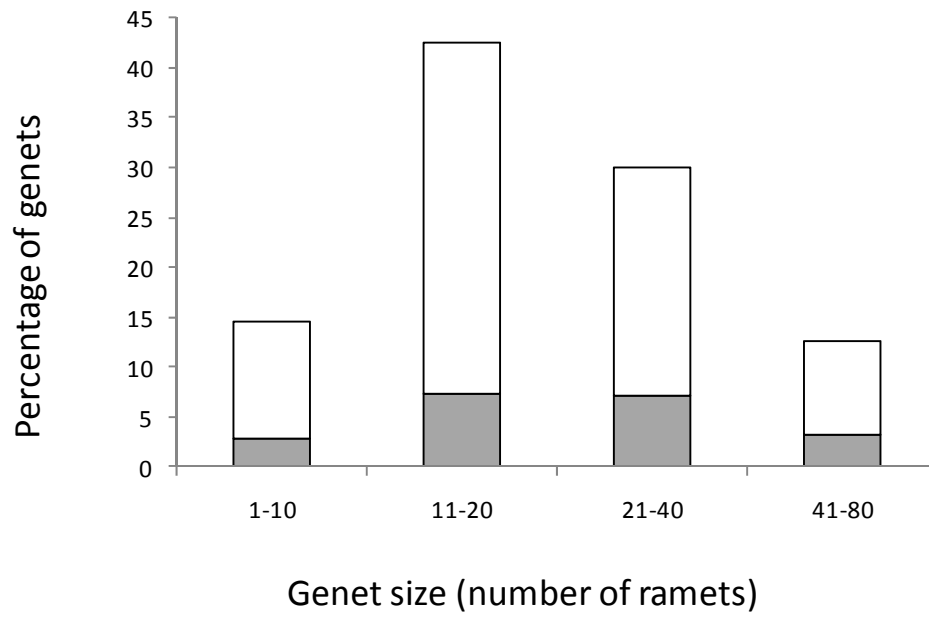
*Plant counting was performed in 20X10 m plot.

649

650

651 **Figure 3**

652



653

Capítulo III

Short distance pollen dispersal and high outcrossing rate in a narrow endemic bromeliad species from Southern Brazil

Artigo a ser submetido para *American Journal of Botany*

1 **Short distance pollen dispersal and high outcrossing rate in a narrow endemic**
2 **bromeliad species from Southern Brazil**

3

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11

12 **Abstract**

13 Restricted gene dispersal resulting from limited pollen migration has been considered
14 the main reason for spatial genetic structuring in plant populations. In this study we aim
15 to characterize the reproductive biology of a narrow endemic species and test for the
16 impact of substructure population in the mating system and pollen flow parameters. We
17 found strong evidence of self-incompatibility since there was no pollen tube growth and
18 seed set after manual self-pollination. The main pollinator seems to be the bumblebees,
19 due to its intense activity in the study areas. Subpopulations inferred through spatially
20 Bayesian assignment showed no differences in the mating system, but strongly marked
21 differences in the pollen flow. Microsatellites markers-based estimates of outcrossing
22 rate were consistently high over the entire populations and subpopulations, and not
23 significantly different from 100%. Strong pollen pool structure was observed across
24 maternal plants ($\Phi_{FT} = 0.25$), corresponding to two effective pollen donors ($N_{ep} \approx 2$).
25 The average dispersal distance was extremely reduced in subpopulation Aw2 ($\delta = 21.8$

26 m), with an average dispersal distance of 3.8 m. These results showed that *Aechmea*
27 *winkleri* could be particularly susceptible to habitat fragmentation and loss. Our results
28 also support the use of non-arboreal plants as a suitable model for pollen flow studies.

29

30 *Key words:* *Aechmea winkleri*, Bromeliaceae, hand-pollination, gene flow, genetic
31 diversity, mating system, pollen pool structure

32

33 A plain picture of the evolutionary dynamics of plant populations requires an
34 understanding of mating system and patterns of pollen flow. Mating system, a major life
35 history trait, affects numerous population and species level processes (Barrett 1998;
36 Goodwillie et al. 2005; 2010; Barrett 2010). In its turn, mating systems and pollen flow
37 can be influenced by both ecological and genetic factors (Barrett 1998; Cheptou 2007;
38 Eckert et al. 2010).

39 Gene flow by pollen is determined mainly by the reproductive system features
40 such as self-incompatibility mechanisms, selective abortion of seeds and fruits, and by
41 ecological factors such as flowering phenology and population spatial distribution and
42 density which consecutively, affect pollinator behavior (Mitchell et al. 2009; Hennig &
43 Ghazoul 2011). In mass-flowering plants, with high synchrony of flowering of
44 neighboring plants, pollen dispersal distance may be shorter due to high proportion of
45 pollination between neighbors (Collevatti et al. 2010; Eckert et al. 2010). Also, pollen
46 dispersal often reflect pollinator foraging behavior, for example, foraging pollinators
47 typically move short distances between flowers, often visiting neighboring plants and
48 several flowers in sequence (Mitchell et al. 2009).

49 Pollen flow between habitat patches may help maintain genetic diversity and
50 diminish the negative effects of fragmentation by increasing heterozygosity, alleviating
51 inbreeding, and counteracting genetic drift (Ward et al. 2005). Therefore, knowledge of
52 contemporary pollen- mediated gene flow is important for conserving plant populations,
53 particularly plant populations exposed to anthropogenic pressures (Sork et al. 2002;
54 Wang et al. 2010). Some authors have suggested that fragment or population shape may
55 influence pollinator behavior and patterns of mating and pollen dispersal but this
56 remains largely untested (Hamrick & Godt 1996; Llorens et al. 2012).

57 Over the last decades, the development of variance- component or two-
58 generation methods (Smouse et al. 2001; Robledo-Arnuncio et al. 2006), in which
59 maternal plants and progeny are subsampled, has made the estimation of pollen
60 movement possible even in large populations. Numerous recent studies have been
61 published characterizing the mating systems, variances of reproductive success and
62 gene flow at the instantaneous time scale, named contemporary approaches (Austerlitz
63 et al. 2004; Bacles et al. 2005; Klein et al. 2011). However, up to now these methods
64 have been applied mostly to long-lived woody perennials (Sork et al. 2002; De-Lucas et
65 al. 2008; Larsen & Kjær 2008; Bittencourt & Sebbenn 2009; Sebbenn et al. 2011); the
66 analyses of mating patterns and pollen movement in dense populations of herb species
67 remains extremely limited (Hufford et al. 2011). Despite the potential impacts of cryptic
68 population structure on inference (Austerlitz & Smouse 2001; Dyer et al. 2004), this
69 factor has generally been ignored in analyses of mating system. Nonetheless, understory
70 herbs, such as some bromeliad species are suitable systems to explore questions of
71 pollen movement in non-arboreal species.

72 Bromeliaceae is among the plant families with the highest diversity of
73 pollination modes (ornithophily, chiropterophily, entomophily, mixed/unspecific) across

74 its geographic distribution (Kessler & Krömer 2000; Canela & Sazima 2005; Wendt et
75 al. 2008). Mixed pollination by hummingbird, butterflies and bees were observed for
76 several *Aechmea* spp. (Wendt et al. 2008). The breeding system for the genus has been
77 reported in detail for a few species, with records of self-compatibility (Lenzi et al. 2006;
78 Matallana et al. 2010; Scrok & Varassin 2011) and self-incompatibility (Sazima et al.
79 1999; Nara & Webber, 2002; Canela & Sazima 2003; Matallana et al. 2010; Kamke et
80 al. 2011). Additional reports on mating system of bromeliads are based on inferences
81 from population genetics data (González-Astorga 2004; Barbará et al. 2007; 2009;
82 Palma-Silva et al. 2009). The first detailed description of a bromeliad mating system,
83 including outcrossing rates, progenies analysis and pollen pool structure was carried out
84 by Paggi (2009) with *Vriesea gigantea* Gaud.

85 *Aechmea winkleri* Reitz is a very particular species from one of the most
86 representative genera of Bromeliaceae family. This species belongs to subgenus
87 *Ortgiesia* which comprises a group of species of difficult taxonomic delimitation which
88 occurs in the Atlantic Forest *stricto sensu*, in the coastal region of southern Brazil
89 (Smith & Downs 1979). Interestingly, *A. winkleri* presents an extremely restricted
90 geographic distribution in an inland region of the Atlantic Forest (*latu sensu*). In this
91 study, we integrated ecological and genetic approaches in an attempt to elucidate how
92 these factors can influence reproduction in this narrow endemic species. Specifically,
93 we aim to: (1) characterize the reproductive biology and pollination biology; (2)
94 estimate mating system parameters; (3) verify the influence of population substructure
95 in the mating patterns; (4) estimate the pollen pool genetic structure and others
96 population dynamics features, such as the effective number of pollen donors per
97 maternal plant, the effective size of pollination neighborhoods and distances over which
98 pollen dispersal occurs. The results obtained here will be useful in understanding the

99 behavior of pollen flow in non-arboreal plants, and in determining species' conservation
100 actions.

101

102 **Material and Methods**

103 **Study species and study site**

104 The bromeliad *Aechmea winkleri* is a narrowly distributed species from southern
105 Brazil (Smith & Downs 1979). The only one known population of this species occurs in
106 an area called “Cinturão Verde”, in the municipality of Santa Cruz do Sul, South Brazil.
107 This area has 465 ha surrounding the urban area of the city on its north and east sides
108 and is considered as Atlantic rain forest *lato sensu* (semi-deciduous forest: Instituto
109 Brasileiro de Geografia e Estatística [IBGE] 1986, Oliveira-Filho & Fontes 2000). It
110 was created in 1994 and classified as a special zone, with strict rules for occupancy to
111 conserve the area (Müller 2009). The extreme narrow distribution and occurrence in a
112 vicinity of an urban area are the main factors threatening the survival and evolution of
113 *A. winkleri* and other species on the region. It is listed as endangered on the Official List
114 of Endangered Brazilian Flora Species (Ministério do Meio Ambiente [MMA] 2008),
115 and is restricted to isolated patches surrounded by intensively managed matrixes,
116 especially urban areas. These patches have high density of individuals, which can be
117 partly attributed to clonal reproduction, since most genets present from 10 to 20 ramets.
118 Typical inflorescences (40-50 cm height) are upright panicles and include ~ 115 yellow
119 flowers and flowering mainly takes place between April and early June (Büttow et al. –
120 unpublished data). Sampling were made in the four areas where plants occur in great
121 density: BU - Bairro Universitário (29°41'31”S, 52°26'11.8”W), UN - Clube União
122 (29°41'47.4”S, 52°25'16.9”W), PG - Parque da Gruta (29°42'37.5”S, 52°24'30.5”W)

123 and PP - Pinus Parque (29°40'61''S, 52°25'46.7''W) (Figure 1). Geographical distances
124 between these areas ranged from 1 to 5 km with an average of 2.5 km.

125 **Reproductive biology**

126 *Hand pollination experiments*

127 To investigate the effect of the pollen source on fruit production and seed set, the
128 following treatments were applied: (1) outcrossing: emasculated flowers were pollinated
129 with a fresh pollen mixture collected from distinct plants and bagged ($n=12$); (2) open-
130 pollination (control): flowers were tagged and not manipulated ($n=37$); (3) selfing:
131 flowers were pollinated with their own pollen and bagged ($n=13$); (4) autonomous
132 selfing: flowers were bagged with a paper bag to exclude insect visits ($n=11$); (5)
133 agamospermy: emasculated flowers were bagged ($n=27$). After six months, fruit set and
134 number of seeds per fruit were recorded. Data were analyzed using a Kruskal-Wallis
135 procedure followed by Tukey test at 5% probability in the SPSS 19.0 (IBM, New York,
136 NY, USA).

137 The self-incompatibility system was investigated in order to determine at which
138 level the inhibition of self-pollination occurs. The outcross and selfing treatments
139 described above were repeated and 24h, 48h and 72h after pollination, flowers were
140 collected and fixed in 3 : 1 (ethanol : glacial acetic acid) to allow further pistils
141 observation using a fluorescence microscopy and aniline blue staining (Olympus
142 microscope, Zeiss Company, Germany). Five to ten pistils per sample per treatment
143 were prepared for examination, totalizing 48 pistils (Table 2) softened with NaOH 9N
144 (37°C, 20 minutes). The proportion of penetrated ovules in self- and cross- pollinated
145 pistils was accessed by scraping out ovules and scoring for the presence of pollen tube
146 at the micropyle. Comparisons of the proportion of penetrated ovules within hours were

147 made by a non-parametric Kruskal-Wallis test (5%) in self- and cross-pollinated pistils
148 were tested with a Mann-Whitney test of means (5%) (SPSS 19.0, IBM, New York,
149 NY, USA).

150 *Pollination biology*

151 Observations of the flower biology and visitor behavior were made during the
152 flowering season of May 2009. The following sequence of floral parameters was noted:
153 anthesis and corolla senescence ($n= 10$ individuals), and number of open flowers per
154 inflorescence by day ($n= 38$ inflorescences). The nectar volume and sugar concentration
155 at anthesis were analyzed during the flowering season of May 2009, in 42 flowers.
156 Nectar was collected with a micropipettes in a random sample of flowers protected
157 from visitors with a paper bag, before anthesis. Nectar sugar concentration was
158 measured with a pocket refractometer. The data collected in °BRIX were transformed in
159 sugar % with a conversion table (Bellingham and Stanley, Ltd, England). Flower
160 visitors were observed directly or through binoculars for records about behavior along
161 the inflorescences and pollen deposition place, on 20 individuals from ~ 06h00 to 18h00
162 over three days. Some floral visitors were photographed or captured and identified by
163 specialists or with a field guide book (Mata et al. 2006).

164 **Mating system analysis**

165 *Plant material, DNA extraction, polymerase chain reaction and genotyping*

166 We collected leaf samples from 14 -16 mother plants (MP) in each area (total:
167 59 MP) and stored it in liquid nitrogen. Sampled MP were at least 5 m apart from each
168 other in order to avoid clone individuals. The geographic coordinates (GPS) of the
169 sampled MP were registered. From each MP we also collected two to five mature
170 fruits. Seeds were sterilized and placed in petri dishes containing $\frac{1}{2}$ MS medium

171 (Murashige & Skoog 1962). The petri dishes were incubated in a climate-control
172 chamber (Digmec – YTWC-1) with relative humidity near 100 % and photoperiod of 16
173 h light at 25 °C and 8 h dark at 22 °C for six months. Five to 30 seeds from each fruit
174 were grow up in order to obtain the seedlings. For mating system analysis we used in
175 average 14 seedlings for each MP, totaling 893 individuals (59 MP and 834 seedlings).
176 Genomic DNA was extracted from MP and seedlings, using a cetyl trimethylammonium
177 bromide protocol based on the method of Doyle & Doyle (1990). Five microsatellite
178 loci previously characterized for other bromeliad species were successfully amplified in
179 *A. winkleri* and employed in this study (Palma-Silva et al. 2007 - loci VgC01; Paggi et
180 al. 2008 - loci PaA10, PaC05, PaD07, PaZ01). One loci isolated for *A. winkleri* (Goetze
181 2010 – loci Aw03) was also utilized (Supplemental Table 1). The six nuclear
182 microsatellites were polymerase chain reaction (PCR)-amplified following methods
183 described by Pinheiro et al. (2008) with a three-primer protocol including unlabelled
184 M13-tagged forward and unlabelled/untagged reverse primers for each marker, and a
185 third ‘universal’ M13-primer labelled with one of the fluorescent dyes, 6-FAM or PET
186 or VIC, according to Schuelke (2000). All PCR amplifications were performed in a PE
187 Applied Biosystems 9700 thermocycler. Microsatellite alleles were resolved on an
188 Applied Biosystems 3730xl Automatic DNA Sequencer by Macrogen (Korea) making
189 use of the different fluorescent dyes for multiplexing, and were precisely sized against
190 ROX (Applied Biosystems) molecular size standard using Peak Scanner Software v1.0.
191 Genotyping errors attributed to stutter, short allele dominance, and null alleles were
192 checked using MICROCHECKER software (Van Oosterhout et al. 2006).

193 *Mating system, genetic diversity and subpopulation structure*

194 The multilocus outcrossing rate (t_m), the average single locus outcrossing rate (t_s) and
195 the biparental inbreeding rate ($t_m - t_s$) were calculated using Newton–Raphson

196 likelihood estimation in the program MLTR V3.4 (Ritland 2002). Standard errors were
197 estimated based on 1000 bootstraps between individuals within a progeny array. The
198 model assumes that: (a) each mating represents a random event of an outcross or a self-
199 fertilization, with probabilities equal to t and $(1 - t)$, respectively; (b) no selection and no
200 mutation following fertilization may occur; (3) there is no assortative mating or
201 variability in pollen pool frequencies (Ritland & Jain 1981). With version 3.4 of MLTR
202 (Ritland 2002) some departures from these assumptions could be accommodated and be
203 treated as additional facets of the mating system. We tested the significance level of t_m
204 by a one-tailed Student's T -test based on the null hypothesis that $t_m = 1$ (SPSS 19.0,
205 IBM, New York, NY, USA). MLTR also estimates the correlated mating parameters:
206 the correlation of paternity (r_p), which is the probability that the two siblings are
207 outcrossed full-sibs, the effective number of pollen donors ($1/r_{p(m)}$), and full ($t_m * r_{p(m)}$)
208 and half sibs [$t_m (1-r_p)$] estimates (Ritland & Jain 1981; Ritland 2002). Genetic diversity
209 in parent and offspring generations was estimated using maternal genotypes and all the
210 individuals from each progeny array. The program GENALEX 6 (Peakall & Smouse
211 2006) was used to calculate observed (H_O) and expected (H_E) heterozygosities, the
212 number of alleles per locus (A) and the fixation index (F). We tested the P value for F
213 significance with exact test SPSS 19.0 (IBM, New York, NY, USA).

214 To explore the potential interaction of genetic structure and mating system in the
215 *A. winkleri* population, spatially explicit Bayesian assignment was carried out with
216 maternal genotypes using the software GENELAND (Guillot et al. 2005; 2008;
217 François et al. 2006). The spatial prior implemented in GENELAND particularly
218 improves assignment in instances where cryptic subpopulations are dense and modestly
219 diverged. The number of subpopulations in the analysis, K , was allowed to vary
220 between 1 and 10 and treated as a simulated variable in a correlated alleles model

221 during Markov Chain Monte Carlo (MCMC) analysis. The MCMC chain was run for
222 10^5 iterations, saving 1% of iterations and discarding the first 200 as burn-in. Ten
223 independent runs were conducted to confirm the consistency of results.

224 *Pollen pool genetic structure*

225 Following Smouse et al. (2001) we made a TWOGENER analysis on the
226 progeny arrays of *A. winkleri*. The principle of this method is to estimate Φ_{FT} , the
227 differentiation of allelic frequencies among the pollen pools sampled by several mother
228 plants in the population. The relation between Φ_{FT} and dispersal distance has been
229 shown for given dispersal curves (Austerlitz & Smouse 2001), allowing the
230 development of several estimates of pollen dispersal (Austerlitz & Smouse 2002;
231 Austerlitz et al. 2004). A general estimate is based on the global Φ_{FT} measured on all
232 the sampled seedlings. This provides an estimate of the pollen dispersal distance (δ)
233 assuming a given dispersal curve. We tested the normal dispersal functions and used a
234 proportion of the known adult density as entry into the estimation. The effective
235 neighborhood pollination area (A_{ep}), as well as the radius, and the effective number of
236 pollen donors N_{ep} can also be inferred from the TWOGENER estimation (Austerlitz &
237 Smouse 2001; Fernández-Manjarrés et al. 2006). Computations were performed using
238 the POLDISP 1.0 software (Robledo-Arnuncio et al. 2007).

239

240 **Results**

241 **Reproductive biology**

242 *Hand pollination experiments*

243 The results of hand- pollination experiments are presented in Table 1. Fruits with
244 seeds were only produced in the outcrossing and open-pollination treatments and there
245 were no significant differences between them. Selfing and autonomous selfing
246 treatments resulted in non fertile fruits, i.e., fruits without seeds, produced by
247 parthenocarpy. Non fertile fruits are small and reddish (Figure 2 D), while fertilized
248 fruits were bigger and varied from green to dark purple when ripe, (Figure 2 G and I).
249 Therefore, we considered the seed set as a measure of successful pollination. The
250 observation of pollen tube development as a function of the pollen source revealed that,
251 in self-pollination treatment, the pollen grains were able to germinate, but it failed to
252 pass the stigmatic papillae (Figure 2 B and C). Unlike, in outcrossing pollination, pollen
253 tube development along the style was observed in almost all individuals (Figure 2 E). In
254 the self-pollination treatment, none ovule was penetrated after 48 and 72h, with no
255 significant differences comparing with the 2% of ovules penetrated in 24h. In contrast,
256 around 75% of ovules had been penetrated after 72 hours in the cross-pollination
257 treatment (Table 2, Figure 2 F). Together, these results reveal that *A. winkleri* is a self-
258 incompatible species and depends on pollen transfer vectors to effective pollination.

259 *Floral biology: anthesis, nectar and floral visitors*

260 Flowers of *A. winkleri* are hermaphrodite, tube shaped, robust, sepals are orange-
261 reddish and petals are of intense yellow color. They do not have scent or nectar guides.
262 Flower visitors and pollinators are rewarded with pollen and nectar. Anthesis of *A.*
263 *winkleri* is diurnal, beginning at 05h00 and is characterized by little separation of the
264 petals tips (Figure 2 A). The stigma slightly exceeds the anthers and is receptive
265 throughout the flower life. The pollen is available since the first hour of anthesis. Nectar
266 accumulates at the base of the corolla tube. Flowers remain open for approximately 13
267 hours. We recorded a mean of 3.55 (SE \pm 0.25) flowers open by day, by inflorescence.

268 Nectar volume at anthesis was in average 9.23 μL (SE \pm 0.74) and sugar concentration
269 was of 22.54% (SE \pm 2.53). The main floral visitors were bumblebees (*Bombus morio*)
270 and *Heliconius* spp. Our observations suggest that the most likely effective pollinators
271 are the bumblebees, since they are the most frequent visitor, carry pollen grains in their
272 body and touch the flower reproductive structures (Figure 2 H). Also, they have
273 relatively fast foraging habit (they stay about eight seconds in each inflorescence, while
274 butterflies stay about 20 seconds in the same inflorescence). During our field
275 observations, only one hummingbird species was observed (*Chlorostilbum lucidus*)
276 visiting *A. winkleri*. Visits of this hummingbird were seen each day in the morning, with
277 only one inflorescence being accessed.

278 **Mating system analysis**

279 *Identification of subpopulation structure and genetic diversity analysis*

280 Spatially explicit Bayesian assignment revealed two cryptic subpopulations
281 within *A. winkleri* (Aw) with fully consistent results across all runs. The number of
282 populations along the Markov chain was plotted, and the density was always
283 substantially greater for $K = 2$ than any other number of subpopulations (data not
284 shown). The run with the highest likelihood value was chosen for assignment of
285 sampling sites to the two subpopulations (Aw1 and Aw2). However, the assignment of
286 sampling sites was nearly identical across each run. Subpopulation Aw1 contain all
287 sampled individuals from UN, PG and PP areas and one individual from BU, and
288 subpopulation Aw2 contain the remaining 14 individuals from BU area (Figure 1) and
289 Supplemental Table 2). Pairwise F_{ST} between the maternal subpopulations (Aw1 and
290 Aw2) was calculated as 0.009 (data not shown).

291 Genetic diversity estimates in both the maternal and progeny generations in the
292 entire *A. winkleri* (Aw) population were quite similar. However, the average number of
293 alleles per locus was higher in the progeny than in the mother plants (5.667 and 4.667,
294 respectively) and the observed heterozygosity was higher in maternal than in progeny
295 generation of Aw (0.647 and 0.593 respectively: Table 3). Negative fixation indexes
296 showed an excess of heterozygotes in the population.

297 *Mating system parameters*

298 The outcrossing rate (t_m) based on the progeny arrays was consistently high for
299 the population (Aw) and subpopulations Aw1 and Aw2: 95.3%, 95.1% and 95.5%,
300 respectively (Table 4) and not significantly different from 100% ($P < 0.001$). The
301 correlation of paternity was 0.815 (SD ± 0.084) indicating that certain adults in the
302 population performed better than others. The estimate of biparental inbreeding ($t_m - t_s$)
303 for Aw2 was significantly greater than zero, indicating the presence of a moderate
304 proportion of related mating ($t_m - t_s = 0.102$; Table 4). Effective number of pollen donors
305 N_{ep} was estimated in 1.23 (Table 4).

306 *Pollen pool genetic structure and pollen dispersal*

307 Results from TWOGENER analyses represent a significant departure from the
308 null hypothesis of random pollen movement ($P < 0.001$), in both the entire population
309 Aw and in the subpopulations Aw1 and Aw2. The Φ_{FT} values showed small variation,
310 and considering the entire population it was of 0.2562 (Table 5), indicating that spatially
311 separated maternal plants presented strongly structured pollen pools. Smouse et al.
312 (2001) recommended sampling $K = [1 / \Phi_{FT}]$ for within family replication ($K =$ number
313 of seeds sampled per maternal plant). Our results suggest that $K = 4$ is sufficient to have
314 a good estimative of the mean level of differentiation among maternal plants in Aw

315 population and in the two groups Aw1 and Aw2. Since in average our $K = 14.14$, our
316 data provide a robust foundation for the Φ_{FT} estimates.

317 The two subpopulations differed primarily in their estimates of average pollen
318 dispersal distance (δ), with a longer estimate of mean pollen dispersal distances in Aw1
319 (49.8 m) than in Aw2 (21.8 m). Subpopulation Aw2 also showed the lowest effective
320 neighborhood pollination area ($A_{ep} = 3.5$ m, Table 5).

321

322 **Discussion**

323 In this study we attempt to integrate ecological and genetic approaches to have a
324 better understanding of the reproductive biology, the mating system and pollen flow in a
325 perennial, clonal and narrow endemic bromeliad species subject to anthropic pressures.
326 We recorded the reproductive biology, the pollination process, the outcrossing rates and
327 the pollen pool genetic structure. Also, we have employed the two-generation method
328 for an herbaceous species, which has a patchy distribution and a meter-scale average
329 density larger than that seen in hectares of many other species evaluated in the
330 literature. Our analyses have provided an opportunity to test the applicability of two-
331 generation procedures to dense, clonal and non- arboreal plant species, and access the
332 effects of cryptic subpopulation structure on mating system and pollen flow.

333 *Reproductive and pollination biology*

334 Results from hand-pollination experiments showed that *A. winkleri* requires
335 pollinator visits in order to set seed and that this species is unable to produce seeds
336 through selfing and/or agamospermy (Table 1). Additional experiments of pollen tube
337 growth after manual self-pollination showed that germinated pollen grain cannot cross

338 the style and reach the micropyle. These data strongly support the existence of self-
339 incompatibly system, which prevents self-pollen to fertilize ovules and produce seeds.
340 As a result, self-incompatibility system will promote outcrossing and possibly avoid
341 inbreeding depression (Porcher & Lande 2005; Wright et al. 2008) in the study species.
342 In the genus *Aechmea*, self-incompatibility has been found in several species, such as *A.*
343 *fasciata* (Lindl.) Baker (Martinelli 1994), *A. beeriana* Smith & Spenc.(Nara & Webber
344 2002), *A. pectinata* Baker (Canela & Sazima 2003), *A. nudicaulis* (L.) Griseb.
345 (Matallana et al. 2010), and *A. caudata* Lindm. (Kamke et al. 2011). Interestingly, in *A.*
346 *distichantha* (L.) Griseb. there are reports of self-incompatibility (Bianchi et al. 2000)
347 and self-compatibility (Scrok & Varassin 2011), showing that different populations of
348 the same species may vary concerning breeding systems. Krömer et al. (2008) showed
349 that trochilophilous bromeliad species have in average a sugar concentration of 20.1%
350 (SD \pm 3.9) and, although we have not evaluated nectar features across all the anthesis
351 period, sugar concentration in *A. winkleri* were compatible with hummingbird-
352 pollinated flowers (22.54%, SE \pm 2.53). In our study area, hummingbirds were not
353 frequently seen. In its turn, bumblebees (*Bombus morio*) activity is so high that the
354 contribution of hummingbirds to pollination in *A. winkleri* is of minor significance. In
355 the typical melittophilous *A. gamosepala* A. Cast., *B. morio* is the exclusive pollinator
356 (Araújo et al. 2004). Conversely, mixed pollination by hummingbirds, butterflies and
357 bees was found by Canela & Sazima (2003) for *A. pectinata*. Also, according to Kamke
358 et al. (2011), pollination tests showed that no seeds developed after hummingbird visits
359 in a southern population of *A. caudata*, while *B. morio* was highly effective in
360 promoting seed set, and no pollen limitation was observed. Indeed, for a variety of
361 ornithophilous species, insects (mainly bees and butterflies) may act as effective
362 pollinators in the absence of pollination by birds (England et al. 2001; Wilson et al.

363 2008; Kamke et al. 2011). Bumblebees usually visited all open flowers in an
364 inflorescence, and visited all nearby inflorescences after move to other directions. Due
365 to the pollinator behavior, and in the absence of a self-incompatibility system, this
366 species would be prone to geitonogamous selfing. However, *A. winkleri* seems to avoid
367 selfing since it is self-incompatible. On the other hand, due to the high density in which
368 plants occur (Büttow et al. – unpublished data) and pollinator behavior, pollination may
369 occur preferentially between close neighbors.

370 *Cryptic population substructure revealed*

371 The results obtained by Goetze (2010) together with our estimates on the genetic
372 diversity of mother plants showed that *A. winkleri* composes a single population [i.e:
373 low $F_{ST} = 0.03$ (Goetze 2010) and this study $F_{ST} = 0.009$] despite the relative distance
374 among the four main areas where plants grow more densely aggregated (see sampling
375 areas in Figure 1). We tested for subpopulation structure based on a recently developed
376 Bayesian algorithm with the software GENELAND, which is useful in the many
377 different situations where low differentiation is encountered (Guillot et al. 2005; Guillot
378 2008). This analysis revealed two subpopulations with clear differences especially in
379 pollen flow parameters, which would not be detected with the population in its entirety.
380 Therefore, the subpopulations revealed using GENELAND should be more likely than
381 subpopulations defined a priori based on visual analysis of the population.

382 *Mating system parameters and pollen flow*

383 The mating system analysis clearly indicates that the species is highly
384 outcrossing even though low number of pollen donors per mother tree was observed.
385 Subpopulations Aw1 and Aw2 were quite similar in terms of mating system parameters:
386 outcrossing, bi-parental and correlated paternity rates were nearly indistinguishable

387 between the two subpopulations, and the biparental inbreeding rate was only slightly
388 elevated in the denser Aw2 subpopulation (Table 4). On the other hand, substantial
389 differences were revealed in the two-generation analysis of pollen flow, with shorter-
390 distance dispersal occurring in Aw2 (Table 5). High outcrossing rates result from self-
391 incompatibility and our estimation in *A. winkleri* ($t_m = 0.95$) is similar to those found in
392 other self-incompatible species (Ward et al. 2005; Friedman & Barrett 2008). However,
393 another factors such as population density, pollinator abundance and composition have
394 a concomitant impact on outcrossing rate and pollen-mediated dispersal at a landscape
395 scale (Franceschinelli & Bawa 2000; Dick et al. 2003; Degen et al. 2004; Eckert et al.
396 2010). Some studies reported a positive correlation between outcrossing rate and
397 density of flowering individuals, especially for tree species (Ward et al. 2005; Friedman
398 & Barrett 2008). Conversely, low outcrossing rates were reported in a low-density
399 population of *Cavanillesia platanifolia* (Carl) Sigism. ($t_m = 0.213$) compared to a high-
400 density population ($t_m = 0.661$) (Murawski & Hamrick 1992). Self-incompatible species
401 are particularly susceptible to effective population decrease because compatible
402 partners are necessary for the production of fertile fruits (Charlesworth & Charlesworth
403 1987). The low, but significant biparental inbreeding rate suggests that subpopulation
404 Aw2 could be subjected to these pressures.

405 The analysis of pollen movement dynamics revealed that the genetic
406 composition of pollen pools was similar among subpopulations and in the entire
407 population Aw ($\Phi_{FT} = 0.2562$; Table 5). The effective pollination neighborhood (A_{ep})
408 around a maternal plant is evidently less than 20 m², suggesting that maternal plants are
409 preferentially pollinated by pollen drawn from near neighbors, less than 50 m away
410 (Table 5). For the subpopulation Aw2, the distances are even lower, of only $A_{ep} = 3.8$
411 m² and average dispersal distance $\delta = 21.8$ m (Table 5). Our findings demonstrate that

412 pollen movement within each subpopulation is restricted, as showed by the average
413 distance of pollen dispersal do not reaching the average distance between sampling
414 areas (2.5 Km – see Material and Methods).

415 Estimate of effective number of pollen donors N_{ep} ($1/r_{p(m)}$) based on Ritland's
416 method (2002) was 1.23 (Table 4) and were lower than calculations following Austerlitz
417 & Smouse (2001, 2002) ($1/2\Phi_{FT}$), consistent with previous observations indicating that
418 $r_{p(m)}$ underestimates correlated paternity at the family level (Hardy et al. 2004) (Tables 4
419 and 5). Small amounts of pollen have probably come from longer distances, but with
420 our observed density of flowering individuals (Büttow et al. – unpublished data), most
421 pollen is drawn from very few flowering individuals and within a short distance of the
422 maternal parent. This reinforces the possibility of mating among related individuals as
423 sow in subpopulation Aw2. The estimated effective number of pollen donors was also
424 consistent with previous mating system studies of herbs (Gonzales et al. 2006 and
425 references therein). Furthermore, the low values observed for effective pollen donors
426 ($N_{ep} \approx 2$, Table 5) suggest that a majority of seeds within each mother plant are sired by
427 two pollen donors, with a few progeny having different fathers, due either to pollen
428 carryover or multiple pollinator visits. Again, this is a function of self-incompatibility in
429 this species as the clonal neighbors cannot serve as pollen donors to ramets of their own
430 clonal clump. As suggested by Sork et al. (2002), genetic incompatibility or
431 phenological variation among individuals inflate Φ_{FT} , thus reducing local N_{ep} . For a
432 given value of Φ_{FT} , either genetic incompatibility or phenological variation violates the
433 assumptions of pollen distance model. The important point is that genetic
434 incompatibility systems and phenological variation reduce the available pollen pools for
435 any particular female and exacerbate the tendency for different females to sample

436 different sets of males, resulting in higher values of Φ_{FT} and lower values of N_{ep} (Sork
437 et al. 2002).

438 **Conservation implications**

439 In the present study we found that *A. winkleri* has a strict outcrossing mating
440 system, with high correlated paternity and low number of pollen donors. Besides, the
441 short effective dispersal distance suggests that increase in habitat fragmentation may be
442 relevant for subpopulations isolation. In addition, the pollinator behavior, and the low
443 effective number of pollen donors suggest that the subpopulations may be vulnerable to
444 increased breeding among related individuals, possibly with decrease in fitness in
445 consequence of inbreeding depression (Culley et al. 1999). Therefore, the patterns found
446 in this study, particularly the ones related to pollen flow may be constraining to the
447 maintenance of genetic connectivity at the landscape scale, especially in the scenario of
448 continuous habitat fragmentation. We suggest that the entire occurrence area of *A.*
449 *winkleri* should be conserved, defining the “Cinturão Verde” as a conservation unity,
450 which will benefit not only our study species, but also all other species in the same area.
451 Furthermore, this area should be preserved in order to serve as a connection between
452 another Atlantic Forest fragments.

453

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460

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671 outcrossing and selfing in plants. *International Journal of Plant Sciences*. 169(1)
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673 **List of Tables**

674 Table 1 Hand-pollination experiments with five controlled treatments.

Treatment	Number of flowers treated	Fruits setting seeds		Seed set mean \pm SE
		n	%	
Outcrossing	12	12	100	105.11 \pm 10.32 a
Open pollination	37	37	100	101.92 \pm 7.92 a
Selfing	13	0	0	0 b
Autonomous selfing	11	0	0	0 b
Agamospermy	27	0	0	0 b

675 Different letters reveal significant differences at $P < 0.001$ by Tukey test at 5%.

676 Means with the same letters in the rows are not significantly different by the Kruskal-Wallis Test (5%).

677

678 Table 2 Self-incompatibility analysis: Percentage of penetrated ovules after 24, 48, and 72 h of MSP (manual self-pollination) and MCP (manual
 679 cross-pollination) in *Aechmea winkleri*.

	Selfing		Outcrossing	
	n	Mean % (SE)	n	Mean % (SE)
24 h	9	2.63 (1.75) a	9	35.03 (10.79) a
48 h	9	0 a	10	62.91 (8.25) a
72 h	5	0 a	6	74.37 (4.87) a
Mean		1.03 ± 0.72 B		55.63 ± 6.01 A

680 Means with the same lower-case letters in the rows are not significantly different by the Kruskal-Wallis Test (5%) and means with the same
 681 capital letters in the lines are not different by Mann Whitney Test (5%), $P < 0.005$.

682

683 Table 3 Genetic diversity of maternal and progeny generations in the Aw population and the Aw1 and Aw2 subpopulations.

	<i>n</i>	<i>A</i>	<i>H_O</i>	<i>H_E</i>	<i>F</i>
Maternal Aw	59	4.667	0.647	0.500	-0.259
Maternal: Aw1 subpopulation	45	3.833	0.611	0.469	-0.238
Maternal: Aw 2 subpopulation	14	3.833	0.762	0.575	-0.323
Progeny	834	5.667	0.593	0.506	-0.126
Progeny: Aw1 subpopulation	629	5.000	0.576	0.479	-0.141
Progeny: Aw2 subpopulation	205	4.500	0.630	0.547	-0.128

684 *n*, number of sampled mother plants and progeny; *A*, average alleles per locus; *H_O*, average observed heterozygosity; *H_E*, average expected

685 heterozygosity; *F*, fixation index = $(H_E - H_O) / H_E$. Exact tests showed no statistical significance ($P > 0.05$).

686

687 Table 4 Mating system parameters for Aw and subpopulations. Bootstrap-based standard deviations are in parentheses.

Parameter	Aw	Aw1	Aw2
Single-locus outcrossing rate, t_s	0.914 (0.029)	0.945 (0.035)	0.853 (0.063)
Multilocus outcrossing rate, t_m	0.953 (0.020)	0.951 (0.027)	0.955 (0.041)
Biparental inbreeding rate, $(t_m - t_s)$	0.039 (0.019)	0.006 (0.018)	0.102 (0.045)
Single-locus correlated paternity, $r_{p(s)}$	0.761 (0.087)	0.710 (0.090)	0.755 (0.160)
Multilocus correlated paternity, $r_{p(m)}$	0.815 (0.084)	0.764 (0.096)	0.770 (0.116)
Effective number of pollen donors, $1/r_{p(m)}$	1.23	1.34	1.32
Full sibs $t_m * r_{p(m)}$	0.777	0.727	0.735
Half sibs $[t_m (1 - r_p)]$	0.176	0.224	0.253

688

689

690 Table 5 Estimates of pollen pool structure parameters based on a normal distribution for Aw population and the Aw1 and Aw2 subpopulations of
 691 *A. winkleri* based on TWOGENER analysis. Density (d) based on ecological observations.

Population	d (m ²)	Φ_{FT}	N_{ep} ($1/2\Phi_{FT}$)	δ – Average dispersal distance (m)	A_{ep} (m ²)	Radius (m)	Error
Aw	0.08	0.2562	1.95	49.4	19.5	2.5	30.6107
Aw1	0.1	0.2524	1.98	49.8	19.8	2.5	19.2758
Aw2	0.15	0.2652	1.89	21.8	3.8	1.1	1.4193

692

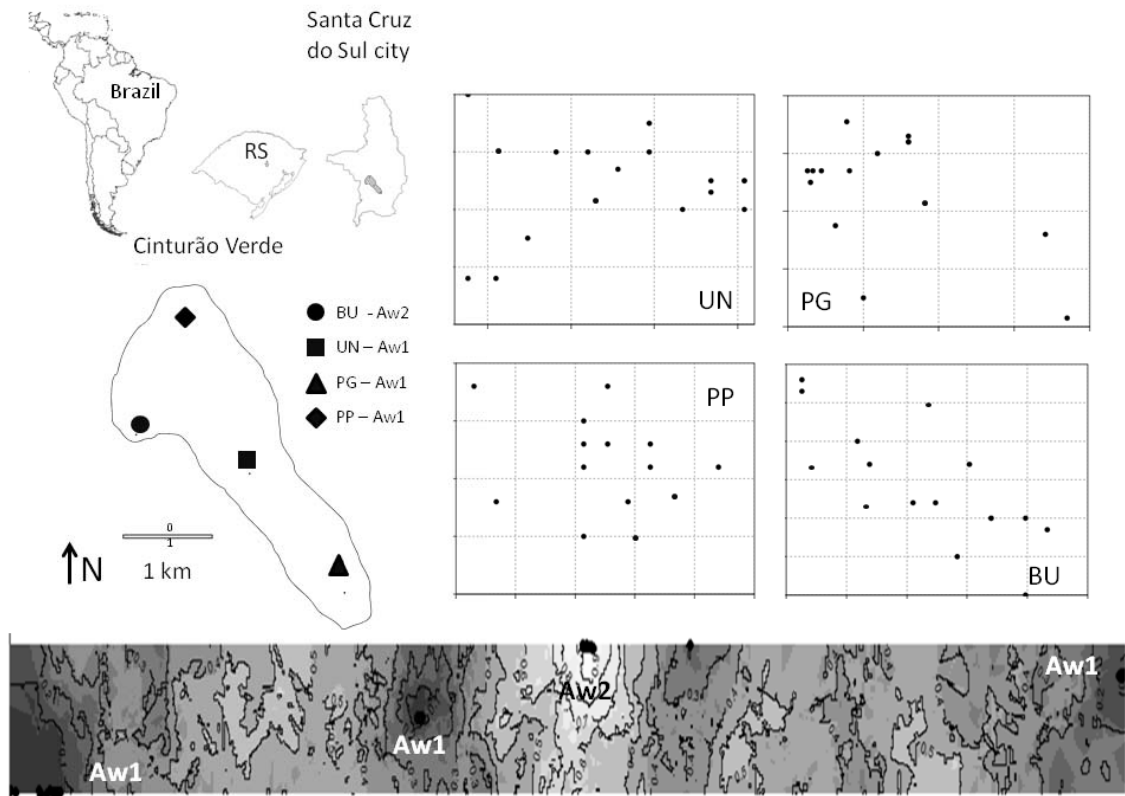
693 **Figure legends**

694 Figure 1 Above: Map showing Rio Grande do Sul state, Santa Cruz do Sul city and the
695 “Cinturão Verde” area. Sample sites BU, UN, PG and PP and the correspondent
696 subpopulation assignment as generated by GENELAND. Mother plants (MP) sampled
697 in each area are plotted in the maps on the right. Below: Heat map from GENELAND
698 analysis of probability of membership in the Aw1 subpopulation. Areas in black have
699 higher probability of membership in Aw1 and contour lines correspond to 0.05%
700 probability.

701

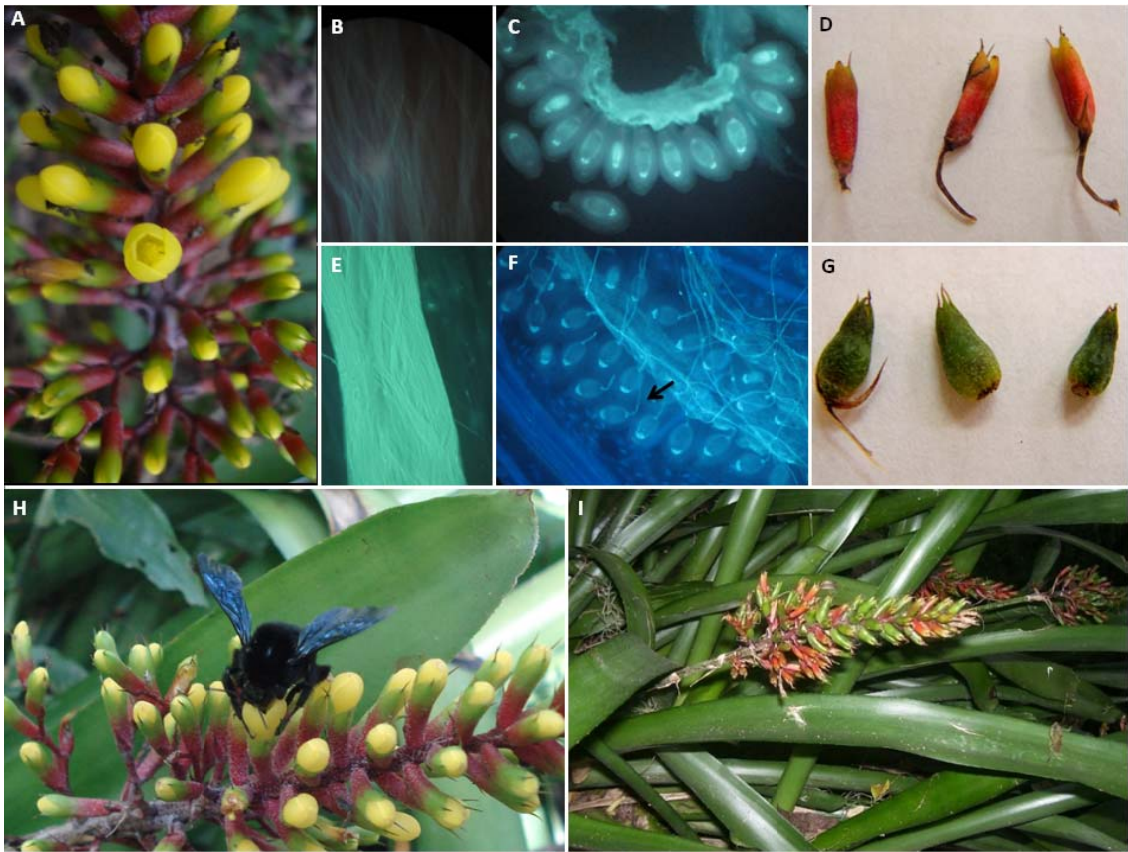
702 Figure 2 Flower, manual pollination treatments, fruit set, pollinators and fruit ripening
703 in *Aechmea winkleri*, southern Brazil. Flower detail, showing narrow petal opening (A);
704 manual self-pollination after 72 hours of treatment, style without pollen tube growth (B)
705 and no penetrated ovules (C); parthenocarpic reddish fruit (D); manual cross-pollination
706 after 72 hours of treatment, style with dense pollen tube growth (E) and penetrated
707 ovules at the micropyle (F – arrow); fertilized fruit (G); an individual of *Bombus morio*
708 feeding on nectar and acting as pollen vector (H); and fruit ripening (I).

710 Figure 1



711

712 Figure 2



713

713 Supplemental Table 1. Genetic variability at six nuclear microsatellite loci in *Aechmea winkleri*, including locus name, number of alleles per
 714 locus (A), number of effective alleles per locus (N_E), observed (H_O) and expected (H_E) heterozygosity, inbreeding coefficient (F_{IS}), overall
 715 inbreeding coefficient (F_{IT}), and fixation index (F_{ST}) for each locus.

	A	N_E	H_O	H_E	F_{IS}	F_{IT}	F_{ST}
VgC01	4.500	2.126	0.659	0.530	-0.244	-0.241	0.002
PaZ01	5.500	2.247	0.618	0.552	-0.118	-0.113	0.005
Aw03	6.000	1.360	0.219	0.265	0.172	0.172	0.000
PaD07	5.500	2.459	0.923	0.593	-0.557	-0.554	0.002
PaC05	7.000	2.036	0.574	0.507	-0.131	-0.129	0.002
PaA10	5.000	2.345	0.729	0.573	-0.272	-0.271	0.001
Mean	5.583	2.096	0.620	0.503	-0.192	-0.189	0.002

-3145.959200	-3564.142496	0.64286	0.35714	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
-3145.940667	-3564.142027	0.64286	0.35714	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
-3145.968467	-3564.144531	0.64286	0.35714	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
-3145.949933	-3564.147035	0.64286	0.35714	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
-3145.961053	-3564.140931	0.64286	0.35714	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
-3149.758533	-3564.376614	0.64286	0.35714	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
-3149.747413	-3564.376614	0.69643	0.30357	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
-3149.749267	-3564.377233	0.69643	0.30357	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
-3149.756680	-3564.377697	0.69643	0.30357	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
-3149.745560	-3564.377697	0.69643	0.30357	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
-3149.743707	-3564.377388	0.69643	0.30357	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
-3149.749267	-3564.377852	0.69643	0.30357	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
-3149.749267	-3564.377388	0.69643	0.30357	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
-3149.749267	-3564.376923	0.69643	0.30357	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
-3149.747413	-3564.377233	0.69643	0.30357	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
-3149.743707	-3564.377078	0.69643	0.30357	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
-3149.738147	-3564.376769	0.69643	0.30357	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
-3149.738147	-3564.376614	0.69643	0.30357	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
-3149.738147	-3564.377543	0.69643	0.30357	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
-3147.271360	-3564.293320	0.69643	0.30357	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Capítulo IV

Considerações finais

Considerações finais

A presente tese está constituída por dois artigos nos quais foram discutidos aspectos genéticos e ecológicos da biologia reprodutiva de *Aechmea winkleri*, uma espécie endêmica, de distribuição geográfica restrita e ameaçada de extinção. Além de possuir uma distribuição restrita, a única população conhecida desta espécie ocorre em uma área de Mata Atlântica secundária fragmentada pela ação humana e que sofre com o extrativismo devido ao apelo ornamental desta bromélia.

Os primeiros estudos de genética de populações desta espécie demonstraram alta diversidade e baixa estruturação genética nesta população (Goetze 2010), porém muitas questões relativas à biologia reprodutiva e ao sistema de cruzamento ainda necessitavam um maior esclarecimento. Os diferentes sistemas de cruzamento e, conseqüentemente, o fluxo gênico, têm um grande efeito na composição genética e na fertilidade de populações naturais. Tais aspectos estão também diretamente relacionados a questões evolutivas e de conservação de espécies. Devido à sua ocorrência em habitats afetados pela ação antrópica, os estudos realizados objetivaram conhecer o quanto o processo de fragmentação pode influenciar no fluxo de pólen, na área de vizinhança efetiva e no número efetivo de doadores de pólen. Neste contexto, o sistema de cruzamento possui aplicação direta para a conservação, devido à sua influência na estruturação da diversidade genética dentro e entre populações, assim como na manutenção da diversidade ao longo do tempo. Todos estes fatores fizeram com que a espécie *A. winkleri* se tornasse alvo dos nossos estudos.

Portanto, com o objetivo de compreender os padrões ecológicos (reprodução clonal e fertilidade) e genéticos (sistema de cruzamento de fluxo de pólen), uma investigação mais aprofundada da biologia reprodutiva foi realizada. O conjunto de dados obtidos nestes trabalhos descreve um panorama inicial que pode contribuir para o

entendimento de questões relacionadas ao impacto da fragmentação no sistema de cruzamento em bromélias, principalmente das espécies endêmicas proximamente relacionadas, como as do subgênero *Ortigiesia*.

Nestes estudos, foi evidenciado um alto investimento em reprodução clonal (Capítulo II), aliado a um sistema de auto-incompatibilidade, relatado no Capítulo III. Estes resultados concordam com o encontrado em um recente estudo que relata uma correlação entre estas duas características em espécies de *Solanum*, o que parece acompanhar a história evolutiva do grupo (Vallejo-Marín & O'Brien 2007). Como reportado nesses capítulos, tanto a reprodução clonal quanto a auto-incompatibilidade ocorrem com certa frequência em Bromeliaceae, no entanto, nenhum estudo demonstrando a co-ocorrência destas características foi feito nesta família. Este deve ser um tema importante de próximas pesquisas em Bromeliaceae, já que, como sugerido por Vallejo-Marín et al. (2010), as consequências do crescimento clonal na evolução de estratégias reprodutivas de plantas pode ser mais significativo do que imaginado anteriormente.

Outro resultado que pode estar relacionado com a reprodução clonal é o índice de fixação (F) negativo encontrado nas subpopulações de *A. winkleri* (Capítulo III). Recentes estudos demonstram que a reprodução clonal pode produzir um excesso de heterozigotos (refletido pelo F negativo) o que pode favorecer a permanência da auto-incompatibilidade (ver Navascués et al. 2010 para maiores detalhes). No entanto, os estudos teóricos demonstram que a reprodução clonal resulta em diferentes consequências para a dinâmica evolutiva da auto-incompatibilidade, levando tanto à sua manutenção, quanto ao seu desaparecimento (Navascués et al. 2010).

O experimento de polinização controlada descrito no Capítulo II demonstrou que esta espécie não sofre com limitação de pólen para a produção de frutos e sementes. Este resultado está diretamente relacionado com a presença e comportamento do polinizador, relatados no Capítulo III. A presença expressiva de mamangavas (*Bombus morio*) nas áreas de alta densidade de plantas mostra que esse inseto assume o papel de polinizador principal nesta espécie, apesar das características ornitófilas apresentadas por *A. winkleri*. Por outro lado, a ausência de beija-flores no local pode ser um indicativo de que a floração de *A. winkleri* não seja suficiente para atrair e manter espécies de beija-flor nestas áreas. Pode ainda, haver um antagonismo entre as mamangavas e beija-flores, sendo que a presença intensa das mamangavas inibe a visitação de beija-flores (Canela & Sazima 2005; Kamke et al. 2011).

Uma das consequências da polinização por mamangavas, que possuem comportamento territorialista, seria taxas de fecundação cruzadas moderadas a baixas, como encontrado em *Vriesea gigantea* (Paggi 2009). Este resultado não foi encontrado no presente trabalho, provavelmente, devido ao sistema de auto-incompatibilidade presente na *A. winkleri*, que impede que o pólen de uma flor fertilize seu próprio óvulo. No entanto, foi detectado cruzamento entre indivíduos aparentados em uma das subpopulações (Aw2, Capítulo III), o que, em conjunto com a menor área efetiva e menor distância de polinização, provavelmente estão contribuindo para criar e acentuar uma estruturação na população de *A. winkleri*.

De maneira geral, em termos de conservação, os resultados encontrados nesta tese são mais animadores do que o esperado para uma espécie de tão restrita distribuição: reprodução clonal, alta produção de flores, frutos e sementes, baixa estruturação, ausência de limitação de pólen e altas taxas de fecundação cruzada. No entanto, quando os dados de fluxo de pólen são analisados, a situação torna-se mais preocupante.

Considerando-se as duas subpopulações, o número efetivo de doadores de pólen é aproximadamente dois ($N_{ep} \approx 2$), o que mostra que poucos indivíduos contribuem com genes para a próxima geração. Da mesma forma, a distância média de polinização, que não ultrapassa a distância média entre áreas de amostragem, o que pode vir a acentuar a tendência de estruturação nas subpopulações.

Os padrões de diversidade observados nesta tese foram formados provavelmente pelo comportamento dos polinizadores associado à fragmentação do habitat. Tanto a atividade do polinizador, restrita a uma pequena área, quanto a perda de conectividade entre as áreas de maior densidade, causada pela fragmentação, podem se tornar barreiras ao fluxo gênico fazendo com que a estruturação entre as subpopulações se intensifique.

Dentre as estratégias de conservação, as mais efetivas são aquelas que envolvem não só a preservação de uma determinada espécie, mas sim o ambiente em que ela ocorre, favorecendo a preservação dos processos ecológicos e evolutivos dos quais aquela espécie faz parte. O estudo da biologia reprodutiva (envolvendo as taxas de fecundação cruzada, investimento reprodutivo, sistema de cruzamento e as interações planta versus polinizadores) é relevante para reforçar a importância da conservação de uma espécie endêmica e ameaçada, da conectividade entre as subpopulações e da preservação do habitat. Uma área de preservação *ex situ*, em um jardim botânico, por exemplo, poderia ser uma alternativa para assegurar que a espécie não seja extinta, com a coleta de *ramets* das duas subpopulações, incluindo amostras das quatro áreas de maior densidade populacional (Capítulo III).

Porém, como a resposta das plantas em relação aos efeitos de fragmentação é espécie-específica (Dauber et al. 2010), é importante conservar as interações entre planta e polinizador, o funcionamento do ecossistema e o *fitness* das plantas em habitats

fragmentados. Por este motivo, neste trabalho, nós enfatizamos a importância da preservação do hábitat para garantir a persistência da única população conhecida de *A. winkleri*.

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