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**Aspectos Genéticos e Ecológicos da Biologia Reprodutiva  
de *Vriesea gigantea* (Bromeliaceae)**

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## Resumo

Fatores como a biologia reprodutiva, sistema de cruzamento, fluxo gênico, distribuição espacial das plantas e dispersão de sementes tem um profundo efeito na composição e estrutura genética de populações naturais. Os sistemas de cruzamento de plantas e a fertilidade estão diretamente relacionados com questões evolutivas e de biologia da conservação. *Vriesea gigantea* é uma espécie endêmica da Mata Atlântica, autocompatível e que apresenta limitação de pólen em populações do sul do Brasil. Estas mesmas populações também apresentaram alta fertilidade, considerando parâmetros de produção de flores, frutos e sementes e características do pólen. As populações naturais de *V. gigantea* vêm sendo reduzidas em função da ação antropogênica, tanto pela destruição do habitat quanto pela coleta predatória. Recentes estudos moleculares revelaram que as populações de *V. gigantea* são geneticamente estruturadas, apresentam baixo fluxo gênico e alto coeficiente de endocruzamento. Estudos visando um melhor entendimento da evolução do sistema de cruzamento e conservação de espécies de bromélias não foram realizados até o momento. Neste contexto, a presente tese, está organizada em forma de três artigos. No **Capítulo II** a biologia reprodutiva e o sistema de cruzamento de *V. gigantea* foram caracterizados, através de experimentos de polinização manual e da análise de marcadores moleculares do tipo microssatélites, com o objetivo de compreender a manutenção da variação natural nos sistemas de cruzamento em espécies de plantas. Os resultados obtidos nos experimentos de polinização manual mostraram que *V. gigantea* é uma espécie não-autogâmica e dependente de polinizadores para frutificação. Nas populações analisadas, os polinizadores observados foram beija-flores e abelhas. A taxa de fecundação cruzada ( $t_m = 0,388$ ) e endocruzamento biparental ( $t_m - t_s = 0,144$ ) estão de acordo com o alto coeficiente de endocruzamento estimado ( $F = 0,312$ ), o qual provavelmente é consequência das altas taxas autofecundação devido ao comportamento dos polinizadores. A estrutura do “pool” genético de pólen foi alta ( $\Phi_{st} = 0,671$ ), correspondendo a um número efetivo de doadores de pólen de 0,75 indivíduos. A taxa de fecundação cruzada mostrou uma tendência geográfica, decrescendo do Norte para o Sul, contrastando com a estrutura do pólen, que aumenta do Norte para o Sul. *Vriesea gigantea* possui um sistema misto de cruzamento e

é um modelo interessante para compreensão da evolução de sistemas de cruzamento em escala latitudinal. Os padrões analisados foram formados pelo comportamento do polinizador e provavelmente podem sofrer influência da fragmentação do habitat, causando a diminuição do fluxo gênico e aumento do endocruzamento. No estudo descrito no **Capítulo III** o principal objetivo foi investigar se os padrões de sistema de cruzamento e fertilidade observados em *V. gigantea* ocorrem devido às condições ambientais ou à adaptação da espécie a autofecundação. As análises genéticas do sistema de cruzamento, utilizando marcadores moleculares do tipo microssatélites, mostraram que as taxas de fecundação cruzada ( $t_m$ ) foram moderadas e a estrutura do “pool” genético de pólen ( $\Phi_{FT}$ ) foi alta, variando entre populações e anos. A fertilidade de três populações naturais foi avaliada considerando a produção de flores, frutos e sementes, e a viabilidade das sementes. Nas três populações foi observada grande produção de flores, frutos e sementes, e uma alta viabilidade de sementes, com diferenças na taxa de frutificação entre as populações. Esses resultados sugerem que os padrões de sistemas de cruzamento e fertilidade observados nas populações de *V. gigantea* se devem a adaptação da espécie para a autofecundação e efeitos de borda, embora outras explicações adaptativas não possam ser descartadas. Considerando a variação espacial-temporal em sistemas de cruzamento e fertilidade, programas de conservação de *V. gigantea* devem incluir populações das bordas da distribuição da espécie. No **Capítulo IV**, para determinar o papel da dispersão e o recrutamento de sementes no fluxo gênico, colonização, e padrão de distribuição, a demografia e a dispersão de sementes de populações de *V. gigantea* foram analisados. Os resultados de demografia demonstraram que o recrutamento de plântulas é grande, 72,4% dessas desenvolvem-se em adultos. Entretanto a classe “juvenil” passa por um considerável decréscimo em sua porcentagem. Sementes de *V. gigantea* são dispersas em pequenas distâncias, logo, a maioria das sementes permanece na vizinhança da planta mãe. Esse padrão coincide com a distribuição agregada das plântulas de diferentes espécies de bromélias, as quais também permanecem em torno da planta mãe. Em suma, os resultados descritos nos capítulos que compreendem esta tese correspondem a mais um degrau de conhecimento para que haja uma compreensão efetiva dos processos biológicos, ecológicos e evolutivos envolvidos principalmente no sistema de cruzamento em plantas, utilizando *V. gigantea* como modelo.



## Abstract

Reproductive biology, mating systems, gene flow, population arrangement and seed dispersal have a profound effect on the genetic composition and structure of natural populations. Plant mating systems and fertility are directly related to many questions on plant evolution and conservation biology. *Vriesea gigantea* is a bromeliad species endemic to the Brazilian Atlantic Rainforest. It is self-compatible and showed pollen limitation in populations from Southern Brazil. These populations also showed high fertility level, considering flowers, pollen, fruits and seeds parameters. Its wild populations have been reduced by anthropogenic disturbance such as habitat destruction and predatory collection. Previous molecular studies revealed that *V. gigantea* populations are genetically structured, with low gene flow and high inbreeding coefficients. Patterns of among-population mating system and fertility in bromeliad species have never been thoroughly evaluated in order to understand the consequences for mating system evolution and species maintenance. In this context, the present thesis was divided in three manuscripts. In the **Chapter II**, the reproductive biology and mating system of *V. gigantea* were characterized (Bromeliaceae), using hand-pollination experiments and microsatellites nuclear markers analysis, in order to better understand the maintenance of natural variation in mating systems of plant species. Results from hand pollination experiments revealed that *V. gigantea* has mixed mating system. Observation of pollinators and nectar features indicated hummingbirds and bees as likely pollinators in the studied populations. Microsatellites marker-based estimates of outcrossing rate ( $t_m = 0.388$ ) and biparental inbreeding ( $t_m - t_s = 0.144$ ) agreed with the high inbreeding coefficient observed ( $F = 0.312$ ), caused most likely by high levels of selfing due to pollinator behavior. Strong pollen gene pool structure was observed across maternal plants ( $\Phi_{st} = 0.671$ ), corresponding to 0.75 effective pollen donors. Outcrossing rates showed a conspicuous decrease from North to South of the sampled area towards the range edge, in contrast to pollen pool structure which increased at this geographic scale. *Vriesea gigantea* is characterized by a mixed mating system and represents a promising model for studying the intraspecific evolution of plant mating systems in habitat affected by fragmentation. In the study presented in **Chapter III** we

investigate if mating system and fertility patterns are due to habitat conditions or due to species' mating system adaptation. Microsatellites nuclear markers showed that outcrossing rates ( $t_m$ ) were moderate and pollen gene pool structures ( $\Phi_{FT}$ ) were high, ranging across years and populations. The plant fertility of three wild populations was assigned considering flower production, fruit and seed set, and seed viability, during three consecutive years. In the three populations we observed high flower, fruit and seed production, and high seed viability, with differences in fruit set between populations. These results strongly suggest that the observed among-population patterns of flower, fruit and seed set, and mating system parameters in *V. gigantea* are attributable to the species adaptation for selfing and edge effects, though other adaptive explanations cannot be ruled out. Considering the spatial-temporal variation in mating system, fertility, and contemporary gene flow results, we considered that *V. gigantea* populations from edge of its distribution should be taking into account in conservation programs of the species. In the **Chapter IV** in order to determine the importance of seed supply for gene flow, colonization, and distribution, the demography and seed dispersal of *V. gigantea* populations were studied. Demography results demonstrated that seedling recruitment is high, since 72.4% of them developed into adults, although juveniles' class experienced considerable decrease on this percentage. *Vriesea gigantea* seeds are dispersed over short distance range, since it is likely that most seeds land in the mother plants' vicinity. This pattern coincides with the reportedly aggregated distribution of bromeliad seedlings around the mother plants. Finally, the results obtained in this study, helped us to increase de understanding of biological, ecological and evolutionary process involved in the mating system of plants, using *V. gigantea* as a model.

**CAPÍTULO I**  
Introdução Geral

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## Introdução Geral

### 1) A família Bromeliaceae

A família Bromeliaceae compreende aproximadamente 3.086 espécies distribuídas em 58 gêneros e três subfamílias: Pitcarnioideae, Bromelioideae e Tillandsioideae (Smith e Downs, 1974, 1977 e 1979; Luther, 2006). As bromélias possuem distribuição geográfica limitada à faixa intertropical das Américas, com uma única exceção no Oeste Africano – *Pitcairnia feliciana* (Smith e Downs, 1974). As espécies da família Bromeliaceae sofreram uma extensa radiação adaptativa, podendo ocupar ambientes extremos e apresentar hábitos que variam de terrestre a epífita (Ranker *et al.*, 1990). Podem ser encontradas desde o nível do mar até altitudes que chegam a 4000 metros, em regiões úmidas e desérticas, em locais com muita ou pouca luminosidade, apresentando adaptabilidade a vários tipos de ambientes (Leme e Marigo, 1993; Medina, 1990).

Espécies da família Bromeliaceae são utilizadas como plantas ornamentais em ambientes externos ou internos, sendo extremamente apreciadas no mundo todo, principalmente nos Estados Unidos, Europa e Japão, onde seu cultivo movimenta uma economia considerável (Paula e Silva, 2000). Além disso, podem ser utilizadas na fruticultura, como no caso do abacaxi, e na produção de xaropes, como *Bromelia antiacantha* (“bananinha-do-mato”; observação pessoal). Mais de 90 espécies de bromélias são utilizadas para os mais diversos fins: fibras, forragem, alimentação humana, ornamentação, em rituais místicos, entre outros (Bennett, 2000). Ainda, 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).

Está estimado que, de um total de 270.000 espécies de plantas, atualmente 34.000 estejam ameaçadas de extinção (IUCN, 1998), entre as quais estão diversas espécies de bromélias.

O Brasil é o maior centro de diversidade de bromélias, onde ocorre cerca de 50% das espécies da família. Bromeliaceae possui seu centro de diversidade genética no leste do Brasil, principalmente na Mata Atlântica (Reitz, 1983). Dentre as inúmeras espécies de bromélias descritas, aproximadamente 76 delas ocorrem no Rio Grande do Sul, havendo representantes das três subfamílias (Palma-Silva, 2003).

O interesse pelo cultivo de bromélias para a comercialização como planta ornamental é muito recente datado aproximadamente do início dos anos 1990 (Bered *et al.*, 2008). Atualmente, a crescente demanda de mercado vem sendo responsável pelo aumento na produção e comercialização destas plantas. No entanto, a intensificação do extrativismo ilegal, especialmente de espécies com ciclos de vida longos, vem reduzindo drasticamente muitas populações, principalmente aquelas oriundas da Mata Atlântica (Coffani-Nunes, 2002).

## 2) *Vriesea gigantea*

A subfamília Tillandsioideae, à qual pertence o gênero *Vriesea*, apresenta nove gêneros e aproximadamente 1100 espécies, sendo que os gêneros *Tillandsia*, *Vriesea* e *Guzmania*, representam o maior número de espécies, 518, 230 e 175, respectivamente (Costa, 2002). A espécie *V. gigantea*, assim como tantas outras espécies do gênero e também do restante da família, tem alto potencial ornamental e faz parte da lista de espécies ameaçadas de extinção no Estado do Rio Grande do Sul (SEMA: [www.sema.rs.gov.br](http://www.sema.rs.gov.br)). É uma planta exuberante, sendo que o indivíduo adulto com inflorescência chega a atingir 2,5m de altura (Reitz, 1983; Paggi *et al.*, 2007). Após a

frutificação ocorre reprodução vegetativa por brotamento; entretanto, a espécie não forma touceiras, encontrando-se no máximo três indivíduos clonais (Reitz, 1983). Pode apresentar tanto hábito terrestre como epífito (Smith e Downs, 1977). Está distribuída desde o Espírito Santo até o Rio Grande do Sul e é encontrada em uma faixa entre 5 a 500m de altitude (Smith e Downs, 1977; Reitz, 1983).

*Vriesea gigantea* possui cisternas que são utilizadas como uma importante fonte de recursos para muitas populações naturais de espécies associadas, principalmente de invertebrados. Alguns trabalhos sugerem uma modulação microclimática funcional para o estabelecimento de várias espécies animais nos tanques de *Tillandsia fasciculata* e *V. sanguinolenta* (Stuntz, et al., 2002). Desta forma, as bromélias participam de maneira ativa em processos ecológicos das matas, conforme Benzing (2000) relatou para diferentes espécies, sendo utilizadas como fonte de água e abrigo para muitas espécies de invertebrados e vertebrados. Schmidt (2003) observou que a composição da fauna de aranhas residentes em *V. gigantea* difere substancialmente daquela encontrada nos substratos arbóreos adjacentes da mesma região no Parque Estadual de Itapuã, Rio Grande do Sul, Brasil.

Com relação aos possíveis polinizadores, Vogel (1969) sugeriu com base em atributos florais relacionados à síndrome de polinização para quiropterofilia, que diversas espécies de *Vriesea*, seção *Xiphon*, podem ser polinizadas por morcegos, como por exemplo, *V. atra*, *V. bituminosa*, *V. gigantea* e *V. longicaulis*. Os espécimes de *V. gigantea* possuem flores branco-amareladas, corola tubular mais larga que as espécies de *Vriesea* polinizadas por beija-flores, e estigma do tipo copulato; as flores abrem ao entardecer e murcham na manhã seguinte, coincidindo com o período de atividade dos morcegos (Benzing, 1980); os morcegos alimentam-se do néctar que fica disponível no momento da

abertura das flores (Sazima *et al.*, 1995, 1999); a dispersão das sementes é anemocórica (Smith e Downs, 1977). Foram observadas também, visitas de morcegos da família Glossofagineae nas flores de *V. atra*, *V. longiscapa* e *V. gigantea*, na Serra do Mar e Serra da Mantiqueira, em São Paulo (Sazima *et al.*, 1995, 1999). Aparentemente, a espécie *Anoura caudifer* é o principal morcego glossofágneo, senão o único, que visita flores em locais de Mata Atlântica com grandes altitudes (Buzato *et al.*, 1994; Sazima *et al.*, 1994, 1999). A distribuição geográfica de espécies de morcegos glossofágneos no Rio Grande do Sul foi descrita por Fábian *et al.* (1999), no qual constatou-se que os morcegos da espécie *A. caudifer* ocorrem em áreas de Floresta Estacional Decidual do Rio Grande do Sul. Em geral, os morcegos visitam as flores seguindo um circuito denominado de “rota-de-captura”, que se caracteriza por visitas a flores distribuídas de forma dispersa, em plantas de porte pequeno a médio, com flores tubulares com bastante quantidade de néctar. Essas visitas são repetidas e regulares a plantas de uma determinada área, favorecendo a polinização cruzada e o fluxo gênico entre populações adjacentes (Webb e Bawa, 1983). Segundo Sazima *et al.* (1999), nas populações de *V. gigantea* da Serra da Mantiqueira – SP, de seis a sete flores abrem por noite, com comprimento médio da corola de 34,1mm. As flores produzem, em média, 152,8 µl de néctar por flor, com 17,85% de concentração de açúcar. O pólen tem sido encontrado, preferencialmente, na face e no tórax de morcegos (Sazima *et al.*, 1999).

Paggi *et al.* (2007), estudaram a fertilidade de populações do sul da distribuição da espécie, os indivíduos produziram um grande número de flores, frutos e sementes ( $\approx$  18.753 sementes por ciclo reprodutivo). A espécie é autocompatível e obsevou-se limitação de polinizadores no Parque Estadual de Itapuã (população da Praia do Araçá), o que foi considerado uma consequência da interrupção do mutualismo entre planta e

polinizador (morcegos; Paggi *et al.*, 2007), ou da baixa viabilidade do pólen observada nos indivíduos da Praia do Araçá (Palma-Silva *et al.*, 2008). Além disso, Palma-Silva (2008) estudou os padrões de diversidade genética de *V. gigantea* ao longo de sua distribuição geográfica, usando marcadores moleculares nucleares e plastidiais. Os principais resultados indicaram uma tendência latitudinal de diminuição da diversidade, do Norte para o Sul, partindo do equador, a qual é consistente com a expansão histórica da Mata Atlântica. Os resultados indicaram também, que a expansão da espécie parece ser impedida pela falta de fluxo gênico nas bordas da sua distribuição. Segundo Palma-Silva, (2008), considerando aspectos da conservação das populações de *V. gigantea*, prioridades deveriam ser dadas às populações com maior diversidade genética, do centro da distribuição (Paraná e São Paulo), e também às populações mais divergentes, das bordas da distribuição (Rio Grande do Sul e Espírito Santo).

### 3) Biologia reprodutiva: aspectos genéticos e ecológicos

Diferentes espécies de plantas podem apresentar exclusivamente um dos modos de reprodução, o assexual ou o sexual, e um sistema de cruzamento, autogamia ou alogamia, mas o mais comum é haver um modo de reprodução e sistema de cruzamento predominante, com eventos esporádicos do outro tipo (Bodanese-Zanettini e Cavalli, 2003). Estas características estão diretamente relacionadas com a composição genética das populações naturais, e seu entendimento é essencial para os programas de conservação de espécies que estão em risco de extinção (Wong e Sun, 1999; Bodanese-Zanettini e Cavalli, 2003). Outros trabalhos apontam que a reprodução vegetativa (Murawski e Hamirick, 1990) e os mecanismos de polinização também têm um efeito marcante na composição e



estrutura genética espacial e temporal de populações (Ritland e Jain, 1981; Ge e Sun, 1999).

#### a) Aspectos genéticos

A estimativa do sistema de cruzamento em plantas pode ser obtida através do uso de marcadores genéticos. Tradicionalmente, as isoenzimas foram os marcadores mais utilizados com este propósito. Atualmente, os marcadores microssatélites (ou SSR - “simple sequence repeats”) também estão sendo utilizados para esta finalidade. Os microssatélites consistem de pequenos motivos (um a seis nucleotídeos) repetidos lado a lado em regiões preferencialmente não codificadoras do DNA. São marcadores de natureza codominante, possibilitando, para um determinado *locus*, a distinção entre indivíduos homozigotos e heterozigotos. A grande vantagem na utilização de microssatélites está associada ao fato de, na maioria dos casos, representarem um único *locus*, o qual freqüentemente é multialélico (Parker *et al.*, 1998; Pinto, 2001). Deste modo, estes marcadores podem ser utilizados para ajudar a resolver problemas que variam desde a taxonomia, questões relacionadas à paternidade, à estrutura genética de populações e determinação do modo de reprodução (McDonald e Potts, 1997; Parker *et al.*, 1998).

Os métodos de estimativa direta da taxa de cruzamento estão baseados no modelo misto de cruzamento, o qual assume que todo o indivíduo de uma população produz uma porção  $s$  de suas sementes por autofecundação e uma porção  $t = 1-s$  por fecundação cruzada, através de pólen de diferentes plantas aleatoriamente disperso (Ritland e Jain, 1981; Ritland, 1990; 2002). Este modelo de análise permite estimar também a porção de indivíduos de uma determinada progênie proveniente de cruzamento entre indivíduos aparentados, além da correlação de autofecundação, variação nas taxas de autofecundação

entre famílias, e a correlação de paternidade, que é a probabilidade de dois indivíduos, da mesma progênie, serem irmãos completos (Ritland, 1989; 2002).

O fluxo gênico é outro fator chave na estrutura genética espacial das populações naturais de plantas. Biólogos evolutivos, interessados em processos microevolutivos, e biólogos conservacionistas, interessados no impacto das mudanças ambientais, necessitam de um método que estime em tempo real os processos de movimento gênico. O método do programa TwoGener analisa a estrutura genética de “pools” de pólen amostrados de plantas individuais relativos ao “pool” de pólen global (Smouse *et al.*, 2001), baseado na análise da AMOVA de Excoffier *et al.* (1992). Além disso, este método permite estimar a distância média de polinização entre dois indivíduos e o tamanho efetivo de vizinhança (Smouse *et al.* 2001). A maioria dos estudos que utilizam o método TwoGener abrange espécies de arbustos e árvores (Dick *et al.*, 2003; Hardy *et al.*, 2004; Veron *et al.*, 2005; Fernández-Manjarrés *et al.*, 2006; Gonzales *et al.*, 2006; O’Connell *et al.*, 2006; Cloutier *et al.*, 2007; Fénart *et al.*, 2007; Silva *et al.*, 2008), sendo que nenhum trabalho envolvendo bromélias ou epífitas foi relatado até o momento.

Alguns estudos baseados na estrutura e diversidade genética de populações, utilizando marcadores genéticos, discutiram o sistema de cruzamento em diferentes espécies de bromélias da subfamília Tillandsioideae (Soltis *et al.*, 1987; González-Astorga *et al.*, 2004; Cascante-Marín *et al.*, 2006; Barbará *et al.*, 2007; 2008; 2009). Soltis *et al.* (1987) e González-Astorga *et al.* (2004) estudaram espécies do gênero *Tillandsia*, através de marcadores isoenzimáticos. *Tillandsia ionantha* e *T. recurvata* apresentaram níveis diferentes de estruturação genética em função do sistema de cruzamento, sendo que *T. recurvata* exibiu características de uma espécie de autofecundação, com altos níveis de endocruzamento, e *T. ionantha* exibiu características de uma espécie de fecundação

cruzada, com pequena variação genética entre as populações (Soltis *et al.*, 1987). Populações de *T. achyrostachys*, espécie de fecundação cruzada, polinizada por animais, apresentaram baixos níveis de fluxo gênico, indicando um padrão de isolamento por distância, provavelmente devido à fragmentação do habitat (González-Astorga *et al.*, 2004). Nas espécies do gênero *Alcantarea*, polinizadas por morcegos, também os níveis de fluxo gênico foram considerados baixos, levando a uma forte estruturação das populações, principalmente na espécie *A. imperialis* ( $N_e m < 1$ ;  $F_{ST} = 0,434$ ; Barbará *et al.*, 2007). Nas demais espécies este padrão foi observado em menor intensidade (*A. geniculata* -  $N_e m \approx 1$ ;  $F_{ST} = 0,111$ , *A. glaziouana* -  $N_e m = 0,54$  a  $1,91$ ;  $F_{ST} = 0,217$ , and *A. regina* -  $N_e m = 1,03$ ;  $F_{ST} = 0,195$ ; Barbará *et al.*, 2007; 2008; 2009), sendo que *A. glaziouana* e *A. regina* também apresentaram propagação clonal (assexuada). *Tillandsia fasciculata* e *Guzmania monostachia* apresentaram uma alta ocorrência de autogamia espontânea, o que está de acordo com as altas taxas de autofecundação obtidas com análises de microssatélites ( $s = 0,972$  e  $0,993$ , respectivamente; Cascante-Marín *et al.*, 2006).

#### b) Aspectos ecológicos

Estudos considerando aspectos ecológicos do sistema reprodutivo, tais como características florais, experimentos de polinização e identificação de polinizadores, revelaram que existem diferentes sistemas de cruzamento entre as espécies de bromélia. Algumas populações frutificam após a fecundação por autogamia ou alogamia, exibindo uma variedade de mecanismos que vão desde a dioecia até a auto-incompatibilidade (Martinelli, 1994; Benzing, 1994, 2000). Em geral, a estrutura floral de bromélias é especializada de forma a prevenir autofertilização espontânea e facilitar a fecundação cruzada mediada por animais (Martinelli, 1994; Benzing, 2000; Canela e Sazima 2003). A

subfamília Tillandsioideae (~1100 espécies) apresenta uma grande variação na morfologia e hábito de vida (Smith e Downs, 1977), estudos recentes têm documentado uma tendência para a autogamia para esta subfamília (Martinelli, 1994; Bush e Beach, 1995; Cascante-Marín *et al.*, 2005). Entretanto, a protoginia ocorre em cerca de 150 membros do gênero *Tillandsia* (Gardner, 1982). Além disso, diversas espécies do gênero *Vriesea* são autocompatíveis, porém exibem características florais, como dicogamia e hercogamia, que facilitam a fecundação cruzada (Martinelli, 1994).

A família Bromeliaceae apresenta uma ampla radiação adaptativa no que se refere a vetores de polinização, como invertebrados e principalmente vertebrados, beija-flores e morcegos (Benzing, 2000), está entre as poucas famílias de plantas em que a polinização por vertebrados predomina sobre a entomofilia (Sazima *et al.*, 1989; Vogel, 1990; Martinelli, 1994). Interessantemente, em *Bromelia antiacantha*, foram observadas características para ornitofilia e melitofilia (Canela e Sazima, 2005). Em sua grande maioria, as espécies do gênero *Vriesea* são polinizadas por beija-flores e morcegos (Martinelli, 1994). Em geral, as espécies com antese noturna e polinizadas por morcegos apresentam maior média de volume de néctar ( $\approx 200\mu\text{l}$ ) com menor concentração de açúcar ( $\approx 14\%$ ).

A fertilidade das populações pode ser afetada pela destruição de habitats e conseqüentemente, pela fragmentação destas populações. Em espécies autocompatíveis, a redução do tamanho efetivo da população pode levar ao endocruzamento e redução da variabilidade genética intrapopulacional (Bodanese-Zanettini e Cavalli, 2003). O efeito negativo da fragmentação das populações pode também reduzir os recursos atrativos para polinizadores, como a quantidade de néctar, além da redução da qualidade do habitat para as espécies (Kéry e Matthies, 2004). Diversas características relacionadas à fertilidade de

plantas vêm sendo utilizadas para determinar a viabilidade de populações, tais como o tamanho das plantas, a produção de flores, frutos e sementes, o padrão de frutificação, a limitação de pólen e a viabilidade de sementes (Colas *et al.*, 2001; Ishii e Kadono, 2002; McIntosh, 2002; Burne *et al.*, 2003; Ortiz *et al.*, 2003; Buide, 2004; Clark-Tapia e Molina-Freaner, 2004; Johnson *et al.*, 2004; Kéry e Matthies, 2004; Caruso *et al.*, 2005; Hampe, 2005; Paggi *et al.*, 2007).

#### 4) Evolução do sistema de cruzamento em plantas

A evolução do sistema misto de cruzamento em plantas tem sido discutida nos últimos 20 anos (Lande e Schemske, 1985; Schemske e Lande, 1985; Goodwillie *et al.*, 2005; Porcher e Lande, 2005). Estudiosos da biologia evolutiva de plantas vêm tentando explicar a manutenção da estabilidade de sistemas mistos de cruzamento, com taxas de fecundação cruzada e de autofecundação moderadas, fenômeno este que não é mencionado pela maioria dos estudos genéticos teóricos baseados em duas maiores forças evolutivas: a vantagem automática da autofecundação (reprodução assegurada) e a depressão por endocruzamento (Lande e Schemske, 1985; Schemske e Lande, 1985; Goodwillie *et al.*, 2005; Porcher e Lande, 2005; Charlesworth, 2006). Embora a distribuição de sistemas de cruzamento seja considerada bimodal, taxas de autofecundação intermediárias ( $0,2 < s \leq 0,8$ ) ocorrem em uma considerável fração de populações naturais (Schemske e Lande, 1985; Vogler e Kalisz, 2001; Barrett, 2003; Goodwillie *et al.*, 2005). Evidências empíricas sugerem que taxas de autofecundação intermediárias podem ser estáveis em muitas espécies; além disso, plantas polinizadas por animais são caracterizadas por apresentarem uma ampla variação nas taxas de fecundação cruzada (Barrett, 2003; Goodwillie *et al.*, 2005; Porcher e Lande, 2005). Segundo Goodwillie *et al.* (2005), o sistema misto de

cruzamento de diversas espécies deve ter um significado adaptativo que ainda não foi completamente entendido. Recentes estudos práticos e teóricos sugerem que o futuro entendimento destes padrões virão de uma melhor integração entre estudos que envolvam mecanismos florais, genéticos, ecológicos e da identificação de como as pressões seletivas variam no espaço e no tempo (Barrett, 2003; Goodwillie *et al.*, 2005; Medrano *et al.*, 2005; Porcher e Lande, 2005; Karasawa *et al.*, 2007; Koslow e Clay, 2007; Mazer *et al.*, 2007).

### 5) Objetivo Geral

A presente tese está inserida em um projeto amplo que visa contribuir para os estudos genéticos e biológicos de plantas neotropicais, com ênfase na família Bromeliaceae. Esta tese teve como objetivo geral investigar aspectos genéticos e ecológicos da biologia reprodutiva, biologia da polinização, modo de reprodução e fertilidade de *V. gigantea*, visando contribuir para o conhecimento da evolução de diferentes mecanismos de reprodução na família Bromeliaceae e também para o desenvolvimento de estratégias para a conservação e manejo das populações de *V. gigantea* e de espécies relacionadas.

## **CAPÍTULO II**

### **Reproductive biology and mixed mating system in the Neotropical rainforest species *Vriesea gigantea* (Bromeliaceae)**

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1 **REPRODUCTIVE BIOLOGY AND MIXED MATING SYSTEM IN THE**  
2 **NEOTROPICAL RAINFOREST SPECIES *VRIESEA GIGANTEA***  
3 **(BROMELIACEAE)**

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5  
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15  
16  
17 **KEY WORDS:** Brazilian Atlantic rainforest, inbreeding depression, outcrossing rate,  
18 pollen structure, pollination biology, TwoGener.

19  
20  
21 **Running title:** MIXED MATING SYSTEM IN *VRIESEA GIGANTEA*

22  
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26 *Abstract* – Mating systems and gene flow have a profound effect on the genetic structure of  
27 natural populations. Our goal was to characterize the reproductive biology and mating  
28 system of *Vriesea gigantea* (Bromeliaceae) in order to better understand the maintenance  
29 of natural variation in plant mating, using the neotropical Bromeliaceae family as an  
30 example. Results from hand pollination experiments revealed that *V. gigantea* has mixed  
31 mating system. Observation of pollinators and nectar features indicated hummingbirds and  
32 bees as likely pollinators in the studied populations. Microsatellites marker-based estimates  
33 of outcrossing rate ( $t_m = 0.388$ ) and biparental inbreeding ( $t_m - t_s = 0.144$ ) agreed with the  
34 high inbreeding coefficient observed ( $F = 0.312$ ), caused most likely by high levels of  
35 selfing due to pollinator behavior. Strong pollen gene pool structure was observed across  
36 maternal plants ( $\Phi_{st} = 0.671$ ), corresponding to 0.75 effective pollen donors. Outcrossing  
37 rates showed a conspicuous decrease from North to South of the sampled area towards the  
38 range edge, in contrast to pollen pool structure which increased at this geographic scale.  
39 *Vriesea gigantea* is characterized by a mixed mating system and represents a promising  
40 model for studying the intraspecific evolution of plant mating systems in habitat affected  
41 by fragmentation.

42

43

44 During the past 20 years, plant evolutionary biologists have tried to explain the  
45 maintenance of stable mixed mating systems, which is not accounted for by most  
46 theoretical genetic studies based on two major evolutionary forces: automatic advantage of  
47 selfing (reproductive assurance) on one hand and inbreeding depression on the other  
48 (Porcher and Lande 2005). Although, the distribution of mating systems is generally  
49 considered bimodal, intermediate selfing rates ( $0.2 < s \leq 0.8$ ) occur in an appreciable  
50 fraction of natural populations (Schemske and Lande 1985; Vogler and Kalisz 2001;

51 Barrett 2003; Goodwillie et al. 2005). Empirical evidence suggests that many mating  
52 systems with intermediate selfing rates might be stable. Moreover, animal-pollinated plants  
53 are characterized by a wide variation in outcrossing rates (Barrett 2003; Goodwillie et al.  
54 2005; Porcher and Lande 2005). The adaptive significance of mixed mating has not been  
55 fully explained for species studied in-depth so far (Goodwillie et al. 2005). Recent  
56 theoretical and empirical work suggests that future progress will come from a better  
57 integration of studies of floral mechanisms, genetics, ecology and the recognition of how  
58 selective pressures vary in space and time (Barrett 2003; Goodwillie et al. 2005; Porcher  
59 and Lande 2005; Karasawa et al. 2007; Koslow and Clay 2007; Mazer et al. 2007).

60 Bromeliaceae is considered one of those families with the highest richness in  
61 different pollination modes (ornithophily, chiropterophily, entomophily, mixed/unspecific,  
62 autogamy) in South America (Sazima et al. 1999; Kessler and Krömer 2000). The floral  
63 structure of bromeliads is generally specialized in a manner that prevents spontaneous self-  
64 fertilization and facilitates animal-mediated outcrossing (Martinelli 1994; Benzing 2000;  
65 Canela and Sazima 2003). In several species studied *in situ* and in cultivation, floral  
66 morphology and pollination experiments indicated the existence of diverse mating systems  
67 among bromeliads (Benzing 2000). The Tillandsioideae subfamily comprises ~1100  
68 species with great variation in morphology and life habit (Smith and Downs 1977), and  
69 recent studies have documented a tendency towards autogamy in this subfamily (Martinelli  
70 1994; Bush and Beach 1995; Cascante-Marín et al., 2005); however, protogyny occurs  
71 almost without exception among ≈150 members of the genus *Tillandsia* (Gardner 1982).  
72 Several species of the genus *Vriesea* are also self-compatible despite of exhibiting floral  
73 features to facilitate outcrossing (Martinelli 1994). Some reports on mating systems of  
74 bromeliads are based on inferences from population genetic data in a few species (Soltis et  
75 al. 1987; González-Astorga et al. 2004; Cascante-Marín et al. 2006; Barbará et al. 2007,

76 2008, 2009; Palma-Silva 2008). Available studies based on the TwoGener approach  
77 concerned only scrubs and trees species (Fernández-Manjarrés et al. 2006; O’Connell et al.  
78 2006; Cloutier et al. 2007; Fénart et al. 2007; Silva et al. 2008).

79         In this study we attempt to understand the mechanisms that contributed to the  
80 maintenance of natural variation in mating systems in *V. gigantea*, filling important gaps in  
81 the knowledge of the reproductive biology of Bromeliaceae. Specifically we address the  
82 following questions: (1) What do hand pollinations experiments of flowers tell us about the  
83 nature of the species’ mating system? (2) Is the species dependent on animals for  
84 pollination, if yes, what is the influences of pollinator behavior on the genetic structure of  
85 the populations? (3) How do outcrossing rates and genetic structure of pollen pool vary  
86 towards the range edge, and what are the relative roles of true selfing vs. matings between  
87 close relatives in generating the observed variation? (4) In which direction does inbreeding  
88 depression evolve towards the range edge? We use our combined experimental pollination  
89 and molecular marker data to discuss the maintenance of mixed mating in this ecologically  
90 important member of the Bromeliaceae family of flowering plants.

91

92

### 93 *Material and Methods*

#### 94 **STUDY SPECIES**

95 *Vriesea gigantea* Gaud. (Tillandsioideae) is an epiphytic, saxicolous and terrestrial  
96 bromeliad that occurs in the Brazilian Atlantic Rainforest (Smith and Downs 1977), and is  
97 very appreciated as an ornamental plant. Wild populations of *V. gigantea* have been  
98 destroyed or restricted to small size due to habitat destruction, fragmentation and predatory  
99 collection practices. Its distribution ranges from Espírito Santo to Rio Grande do Sul States,  
100 Brazil (Smith and Downs 1977; Figure 1). It was described as a bat-pollinated species

101 (Sazima et al. 1999) and its vegetative structure forms tanks that are able to hold many  
102 liters of water, supporting a rich diversity of associated animal life (Benzing 2000).  
103 Typical inflorescences have a central axis with several branches on each side and one  
104 flower on each side of the lateral axis (Reitz 1983; Figure 2A). The flowers are tube-  
105 shaped with three petals and their color agrees with the chiropterophilous syndrome,  
106 suggesting outcrossing as the predominant mating system of this species (Vogel 1969;  
107 Figure 2F and 2G). Flowers at the base and center of the inflorescence open first, while  
108 flowers in the apical position open later (Reitz 1983; Benzing 2000).

109 Paggi et al. (2007) studied the fertility of *V. gigantea* in a population from the  
110 Southern portion of its distribution. The population produced large number of flowers,  
111 fruits, and highly viable seeds. The species is self-compatible and exhibited pollinator  
112 limitation, which was considered a consequence of habitat fragmentation, and specifically,  
113 a disruption of the bat pollinator-plant mutualism (Paggi et al. 2007). Palma-Silva et al.  
114 (2008) studied the pollen viability and meiotic behavior in seven populations of *V.*  
115 *gigantea* from southern Brazil. The pollen grains were highly viable, but one population  
116 (Praia do Araçá in the Itapuã State Park), showed a low pollen viability, which may also  
117 contribute to pollen limitation in that population (Palma-Silva et al. 2008).

118

## 119 **STUDY AREAS**

120 This study was conducted in three populations of *V. gigantea* (Maquiné Itapuã and Taim),  
121 located in the Brazilian Atlantic Rainforest, in the East of the Brazilian state of Rio Grande  
122 do Sul (Table 1 and Figure 1), where the climate is classified as humid subtropical (Cfa) by  
123 the Köppen Climate Classification System. Fieldwork was conducted during the flowering  
124 and fruiting seasons, from January to September of 2005, 2006, 2007 and 2008. The  
125 individuals of *V. gigantea* studied in the Maquiné population are located outside of the

126 Ecological Reserve of Serra Geral. Besides, the location has undergone several instances of  
127 habitat disturbance due to clearing for farming. The Itapuã population, on the other hand, is  
128 included in a protected area: Itapuã State Park, protected since the 1970's. Nevertheless,  
129 prior to this time this area has suffered from severe disturbance due to deforestation and  
130 stone extraction. The population from Taim occurs next to a conservation unit (Taim  
131 Ecological Station) but has continuously been affected by anthropogenic disturbance due  
132 to cattle farming and rice cropping.

133

## 134 **REPRODUCTIVE BIOLOGY**

### 135 *Hand-pollination experiments*

136 We investigated the reproductive biology of *V. gigantea* using controlled hand-pollination  
137 experiments with three pollination treatments and control during the flowering season of  
138 January and February 2005. This study was carried out in the Itapuã State Park in four  
139 subpopulations of *V. gigantea* (Table 1). Controlled hand-pollination experiments tested  
140 for agamospermy (AG), spontaneous selfing (autonomous, SS) and open outcrossing (O),  
141 Table 2. Flowers under natural conditions of pollination were used as controls (C). Three  
142 to five flowers were used per treatment in sixteen plants. Plastic mesh bags (maximum  
143 mesh size 0.9 mm) were used to exclude visitors. When necessary, flowers were  
144 emasculated before anther dehiscence (Table 2). Fruit set and number of seeds per fruit  
145 were recorded as described by Paggi et al. (2007). Data on the fruit set and the mean  
146 number of seeds obtained from pollination treatments were analyzed using the Kruskal-  
147 Wallis non-parametric test (SAS, Version 8, SAS Institute, Cary, USA).

148

### 149 *Nectar features*

150 The nectar volume and sugar concentration were analyzed during the flowering season of

151 January and February 2006/2007 in the three studied populations. Nectar sugar  
152 concentration was measured with a pocket refractometer (Bellingham and Stanley, Ltd,  
153 England) at a room temperature 24°C. The data collected in BRIX were transformed for  
154 sugar % with a conversion table. Nectar volume was measured using calibrated  
155 micropipettes in a random sample of flowers protected from visitors with a mesh bag.  
156 These measurements were done throughout anthesis, at four 2-h intervals of unvisited  
157 flowers ( $n = 98$  flowers, from 34 plants). Stigmatic receptivity ( $n = 20$ ) at different times  
158 after anthesis was tested using the  $H_2O_2$  10V catalase activity method (Zeisler 1938).

159

#### 160 *Floral visitors*

161 Detailed observations on the floral biology of the three studied populations and visitor  
162 behavior were made during the flowering season of January and February 2008. Visitors  
163 were observed directly or through binoculars for records about behavior along the  
164 inflorescence length and pollen deposition place, on 20 individuals from ~1400 to 0400 h  
165 over 3 days in each population (~120 h). Some visitors were photographed and identified  
166 by a specialist. The following sequence of floral parameters was also noted: anthesis,  
167 number of open flowers per day, and pollen release. Data from nectar features and floral  
168 visitors were analyzed by the ANOVA followed by the Tukey test to determine whether  
169 the differences among hours were significant (SAS, Version 8, SAS Institute, Cary, USA).

170

#### 171 *Pollen germination*

172 During the flowering season of January and February 2008, hand self- and cross-  
173 pollination treatments were performed in order to analyze pollen tube growth and number  
174 of penetrated and non-penetrated ovules. This procedure was carried out in the Itapuã State  
175 Park population. Hand self- and cross-pollinated flowers were fixed in 3:1 (ethanol: glacial

176 acetic acid) at 48, 72 and 96 h following pollination to allow further observation using a  
177 fluorescence microscopy and aniline blue staining (Martin 1959). Five to seven pistils per  
178 sample per treatment were prepared for examination, totaling 33 pistils. The proportion of  
179 penetrated ovules in self- and cross-pollinated pistils was assessed by scraping out ovules  
180 and scoring for the presence of pollen tube ‘tails’ at the micropyle. Comparisons of the  
181 proportion of penetrated ovules in self- and cross-pollinated pistils were tested with a t-test  
182 of means (SAS, Version 8, SAS Institute, Cary, USA).

183

#### 184 **MATING SYSTEM ANALYSIS**

##### 185 *Plant material*

186 Natural-pollinated maternal seeds were collected randomly from five mother-plants in  
187 Taim, 10 in Maquiné and 11 in Itapuã population, totaling 26 mother-plants (MP), during  
188 the fruiting set season of August 2006. The MP were apart from each other by 5m. Four to  
189 thirty seedlings of each MP were used for genetic analysis, totaling 527 individuals (26 MP  
190 and 501 progenies, 19.3 seedlings per MP, on average). In order to obtain the seedlings,  
191 seeds were sterilized and placed in petri dishes containing ½ MS medium (Murashige and  
192 Skoog 1962) with B5 vitamins (Gamborg et al. 1968). The petri dishes were incubated in a  
193 climate-control chamber (Digmec – YTWC-1) with relative humidity near 100 % and  
194 photoperiod of 16 h light at 25 °C and 8 h dark at 22 °C for six months. Fresh leaves (~5  
195 cm<sup>2</sup>) from the MP and seedlings were collected and stored in liquid nitrogen until DNA  
196 extraction. Total genomic DNA was extracted using the protocol described by Doyle and  
197 Doyle (1990). The localization of each mother-plant was plotted using a GPS (Global  
198 Positioning System).

199

##### 200 *Microsatellite markers*

201 Eight microsatellite loci previously characterized for the bromeliad species *Tillandsia*  
202 *fasciculata*, *Guzmania monostachia* (Boneh et al. 2003 – loci: e6b and CT5) and *V.*  
203 *gigantea* (Palma-Silva et al. 2007 – loci: VgA04, VgA06, VgB06, VgF01, VgF02 and  
204 VgG02) were selected for this study. The forward primer of each locus was labeled at the  
205 5' end with a fluorescent dye (6-FAM or JOE). All PCR amplifications were performed in  
206 a PE Applied Biosystems 9700 thermocycler as described by the authors. Microsatellite  
207 alleles were resolved on a 3100 DNA Analyser (Applied Biosystems) and were precisely  
208 sized against ROX (Applied Biosystems) molecular size standard using GENOTYPER 3.7  
209 software (Applied Biosystems).

210

#### 211 *Progeny pair analysis and inbreeding depression*

212 The multilocus outcrossing rate ( $t_m$ ) was estimated under the mixed mating model  
213 described by Ritland (2002), with the software Mltr 3.0 (Ritland 1990). This procedure  
214 allowed us to estimate the multilocus outcrossing rate ( $t_m$ ), the mean single-locus  
215 outcrossing rate ( $t_s$ ), the difference between estimates ( $t_m - t_s$ ) which represents the  
216 outcrossing rate between related individuals (=biparental inbreeding), and the inbreeding  
217 coefficient of the maternal parents ( $F$ ). We tested the significance level of  $t_m$  and  $F$  by a  
218 one-tailed Student's  $t$ -test based on the null hypothesis that  $t_m = 1$  and  $F = 0$  (SAS, Version  
219 8, SAS Institute, Cary, USA). Mltr also estimates the correlated mating parameters: the  
220 correlation of selfing ( $r_s$ ), which reflects variation in the selfing rates among families, and  
221 the correlation of paternity ( $r_p$ ), which is the probability that the two siblings are  
222 outcrossed full-sibs (Ritland 1989, 2002). Standard errors were estimated based on 1000  
223 bootstraps between individuals within a progeny array.

224 The inbreeding depression was estimated from the inbreeding coefficient ( $F$ ) of  
225 adult plants relative to the expected  $F$  of progeny based on the selfing rate ( $s = 1 - t_m$ ;



226 Ritland 1990). According to Goodwillie et al. (2005) the inbreeding depression can be  
 227 estimated as:

228

229

$$230 \quad \delta = 1 - 2 \left[ \frac{(1-s)F}{s(1-F)} \right]$$

231

232

233 *Pollen pool genetic structure analysis*

234 The TwoGener procedure was used to analyze the genetic structure of pollen pools ( $\Phi_{FT}$ )  
 235 sampled by individual plants relative to the global pollen pool (Smouse et al. 2001) based  
 236 on the AMOVA approach of Excoffier et al. (1992). We computed the 99% confidence  
 237 interval of  $\Phi_{FT}$  by bootstrapping among loci with 1000 replicates. Computations were  
 238 performed using the POLDISP 1.0 software (Robledo-Arnuncio et al. 2007).

239 Because inbreeding is significant in the *V. gigantea* adult population (Palma-Silva  
 240 2008) and it could increase the observed values of pollen genetic structure, we corrected  
 241 the estimate of  $\Phi_{FT}$  using the formula described in Austerlitz and Smouse (2001):

242

243

$$244 \quad \Phi'_{FT} = \Phi_{FT} (1 + F)$$

245

246

247 where  $\Phi_{FT}$  is the pollen structure parameter due only to limited pollen dispersal and  $F$  is  
 248 the adult inbreeding coefficient as described by Fernández-Manjarrés et al. (2006).

249 The estimate of  $\Phi_{FT}$  can be used to further calculate the variance in pollen flow

250 ( $\sigma^2$ ), the average distance of pollination ( $\delta$ ), the effective neighborhood pollination area  
251 ( $A_{ep}$ ), and the effective number of pollen donors  $N_{ep}$  (Austerlitz and Smouse 2001;  
252 Fernández-Manjarrés et al. 2006). ANOVA followed by Tukey test were performed in  
253 order to verify whether there are differences among populations and the pattern of mating  
254 system parameters along the geographic distribution of the studied samples, using families  
255 as repetitions. All analyses were performed using the SAS (Version 8, SAS Institute, Cary,  
256 USA) software package.

257

258

## 259 *Results*

### 260 **REPRODUCTIVE BIOLOGY**

#### 261 *Hand-pollination experiments*

262 The average rate of natural fruit set was 47.9% under natural conditions of pollination  
263 (control). Fruit set was significantly lower in the open treatment (O) than in the control,  
264 only 17.7% ( $\pm 8.8$ ) ( $F = 3.43$ ,  $P = 0.0409$ ). The result obtained in the spontaneous selfing  
265 treatment (SS) was not different from the control (Table 3). Considering the number of  
266 seeds per fruit, the control flowers produced the largest number of seeds, 237.5 ( $\pm 44.3$ ) ( $F$   
267  $= 8.13$ ,  $P = 0.0005$ ). In the agamospermy treatment, only one plant from the Praia do Araçá  
268 population produced seeds, probably due to pollen contamination during emasculation.  
269 Variation in seed set was high among populations, under natural pollination (control  
270 treatment), the mean number of seeds in the Morro da Grota population was 3.8 times  
271 higher than in Praia do Araçá (Table 3).

272

#### 273 *Floral biology: anthesis, nectar and floral visitors*

274 Prior to bud opening in *V. gigantea* the style lengthens so that the stigma slightly exceeds

275 the anthers (Figure 2E). The stigma was wet, i. e. receptive, only immediately following  
276 anthesis. Anther dehiscence usually occurred later, and pollen release was sometimes  
277 postponed by up to 1 h, in agreement with protogyny for *V. gigantea*. Anthesis started at  
278 different times for the three studied populations and it was characterized by discrete  
279 separation and outward curvature of petal tips. In Maquiné, the northward population,  
280 anthesis started at dusk, usually between 1830 and 1900 h ( $n = 20$  flowers). In Itapuã State  
281 Park the beginning of the anthesis varied greatly from 1200 to 1700 h ( $n = 20$ ); and in  
282 Taim, the anthesis for all observed individuals ( $n = 20$ ), started around 1600 h. By  
283 approximately one hour after anthesis the flowers were open and the anthers were exposed.  
284 The flowers exhibited herkogamy, the spatial separation of anthers and stigmas within  
285 flowers, which generally reduces intraflower self-pollination (Figure 2F). The flowers  
286 lasted just one night, and petals, stamens, and the style abscised during the following days.  
287 On average, six flowers per inflorescence opened per day, considering all studied  
288 populations (data not shown).

289 Both nectar volume ( $F = 3.87$ ,  $P = 0.0062$ ) and sugar concentration ( $F = 6.04$ ,  $P =$   
290  $0.0003$ ) varied significantly throughout the day (Figure 3A and 3B). At the onset of  
291 anthesis, flowers had 43  $\mu\text{l}$  of nectar, on average. Nectar production increased until it  
292 reached its peak around six and eight hours after anthesis (mean = 79.8  $\mu\text{l}$ ), with no  
293 difference between the beginning and end of anthesis (Figure 3A). Sugar concentration  
294 was greatest between four and eight hours after anthesis (mean = 14.8 %); it decreased to  
295 half this value at ten hours after anthesis (Figure 3B).

296 During the flowering seasons of 2007 and 2008, hummingbirds and bees were  
297 observed as diurnal flower visitors of *V. gigantea*. Two bee species were observed, namely  
298 *Apis mellifera* and *Trigona spinipes*; they visited flowers of *V. gigantea* in all individuals  
299 and populations. *Apis mellifera*, was observed as an effective pollinator (Figure 2B).

300 Because of their behavior, the number of visits per flower and inflorescence was difficult  
301 to estimate - they visited all open flowers of the same inflorescence, rarely moving  
302 between inflorescences, thus promoting self-pollination by autogamy and geitonogamy  
303 (Figure 2G). Occasionally, *A. mellifera* individuals avoided flowers with *T. spinipes*.  
304 Individuals of *T. spinipes* spent substantial amounts of time in the same flower or  
305 inflorescence. They rarely moved among plants and frequently destroyed the flowers,  
306 “stealing” pollen and later nectar through holes at the corolla base, affecting  
307 hummingbird/bee visits through aggressive interactions (Figure 2H).

308 Hummingbirds visited all open flowers in each day, and often visited each flower  
309 five to 10 times during anthesis; visits to a flower varied from one to 15s. Pollen deposition  
310 was observed on the hummingbirds’ bill and head, which seems to be an efficient way of  
311 pollen transfer (Figure 2C). Most hummingbird visits (69%) occurred from four to six  
312 hours after anthesis with a peak of  $\approx 3.5$  visits per hour in each flower (Figure 3C). The  
313 variation in the number of hummingbird visits throughout the day ( $F = 15.86$ ;  $P = 0.0001$ )  
314 was correlated with nectar production ( $r^2 = 0.792$   $P = 0.043$ ). *Amazilia fimbriata* was the  
315 only hummingbird species observed in Itapuã State Park and Taim Ecological Station, it  
316 always visited *V. gigantea* inflorescences at regular intervals (around five minutes). In  
317 Maquiné four hummingbird species were observed: *Amazilia fimbriata*, *Thalurania*  
318 *glaucoptis*, and two unidentified species. They visited every flower on each inflorescence  
319 before moving on to another inflorescence and sometimes returned to one they had visited  
320 previously. Between visits, the hummingbirds commonly remained perched nearby. *T.*  
321 *glaucoptis* and *A. fimbriata* frequently showed aggressive behavior and interacted  
322 agonistically with intruding hummingbirds of another or the same species; they also  
323 excluded and chased bees. None bats were observed visiting the plants in ~120 h of  
324 observation. In the Taim Ecological Station bats were observed and one individual was

325 captured, it was identified as a species probably belonging to the genus *Myotis*  
 326 (*Vespertilionidae*), which is an insectivore genus.

327

328 *Pollen germination*

329 Fluorescence microscopy showed that both self- and cross-pollen grains germinated on the  
 330 stigma and produced tubes growing toward the ovary (Figure 2D). The proportion of  
 331 ovules penetrated by self- and cross-pollen tubes was significantly different in all time  
 332 period tested. After 48 h of self-pollination and cross-pollination, 9.5% and 22.8% of the  
 333 ovules were penetrated respectively ( $F = 5.63$ ,  $P = 0.0451$ ). After 72 h, around 90% of  
 334 ovules were penetrated in the cross-pollination treatment, while in the self-pollination 76%  
 335 of ovules were penetrated ( $F = 33.47$ ,  $P = 0.0001$ ). This difference increased after 96 h in  
 336 which 92% of cross-pollinated ovules were penetrated, and 68% of self-pollinated ovules  
 337 were penetrated ( $F = 10.69$ ,  $P = 0.0114$ ). We conclude that *V. gigantea* presents cryptic  
 338 self-incompatibility.

339

340 **MATING SYSTEM**

341 *Progeny pair analysis and inbreeding depression*

342 The outcrossing rate ( $t_m$ ) was highly variable among populations, ranging from 0.176 to  
 343 0.477, with an average of 0.388, which means that 38.8% of the progenies resulted from  
 344 outcrossing events considering all populations (Table 4). A significant self-fertilization rate  
 345 ( $s = 1 - t_m$ ) was detected ( $s = 0.612$ ) since the mean of  $t_m$  was significantly lower than 1 for  
 346 all populations ( $P < 0.0001$ ). The outcrossing rate between relatives (biparental inbreeding;  
 347  $t_m - t_s$ ) ranged from 0.069 to 0.141, with a mean of 0.144. The mean correlation the  
 348 paternity was  $r_p = 0.631$ , which allowed us to conclude that approximately 24.5% ( $t_m \cdot r_p$ ) of  
 349 the offspring were derived from correlated outcrossing (full sibs) and 14.3% [ $t_m (1 - r_p)$ ]

350 derived from random mating, related as half sibs for all studied populations (Table 4).

351 The estimated number of effective pollinators  $N_{ep}$  ( $1/r_p$ ) was only 1.58 (Table 4).

352 Thus, considering outcrossing events, the *V. gigantea* families in these populations are

353 mainly full sibs, i.e., randomly chosen pairs of outcrossed progeny from a maternal family

354 tend to have the same pollen parent. Individual plant estimates revealed significant

355 correlation of selfing (among-plant variation of outcrossing rate) only within the Maquiné

356 population. The distribution of individual outcrossing rates for this population indicates

357 that this correlation probably arose from a few families exhibiting significant selfing ( $s >$

358 0.20; data not shown). The inbreeding coefficient ( $F$ ) based on genotypic frequencies of

359 maternal plants was positive and significantly greater than zero ( $F = 0.312$ ;  $P < 0.0001$ ;

360 Table 4). The estimated inbreeding depression ( $\delta$ ) for all *V. gigantea* populations was

361 0.274, and for Maquiné and Itapuã populations this parameter was pronounced, (0.509 and

362 0.443 respectively) whereas outcrossing rates there were high (0.477 and 0.336);

363 conversely, in Taim population, inbreeding depression was low,  $\delta = -0.0877$  (Table 4). All

364 mating system parameters showed a characteristic trend towards the southern margin of the

365 species' range of increasing ( $F$ ,  $N_{ep}$ ) and decreasing ( $r_p$ ,  $r_s$ ,  $t_m$ ,  $t_s$ ,  $t_m - t_s$  and  $\delta$ ) (Figure 4).

366 The multi- and single-locus outcrossing rate and the correlation of paternity showed

367 differences among populations, being lowest in the Taim population (Figure 4).

368

369 *Pollen poll genetic structure*

370 The genetic structure of pollen pools in the *V. gigantea* populations was high,  $\Phi'_{FT} = 0.532$ ,

371 indicating significant differentiation of the pollen pools of different mother plant (Table 5).

372 Considering the significant inbreeding found in the adult population ( $F_{IS} = 0.261$ ; Palma-

373 Silva 2008), we corrected the pollen structure parameter and found a slightly greater value

374 of  $\Phi_{st} = 0.671$  (Table 5). The effective neighbourhood pollination area ( $A_{ep}$ ) was 173m<sup>2</sup> for

375 the species, and the average distance of pollination ( $\delta$ ) was only 147m. Also for the  
376 TwoGener approach,  $\Phi_{st}$  showed a characteristic increase towards the Southern margin of  
377 the species' range (Figure 4).

378

379

## 380 *Discussion*

### 381 **REPRODUCTIVE BIOLOGY**

382 Fruit set under natural conditions (control) in wild populations of *V. gigantea* is very  
383 similar with that reported for other bromeliads. In the closely related species, *V.*  
384 *friburgensis* (Smith and Downs 1977), in the same study area, 44% of flowers produced  
385 fruits (Silveira 2006), and in *Bromelia antiacantha* (Bert.), 50% of flowers developed into  
386 fruits (Canela and Sazima 2005). *Vriesea gigantea* is self-compatible and exhibits  
387 herkogamy and protogyny as strategies to prevent self-pollination. Self-compatibility is  
388 widespread in several species in Bromeliaceae (Martinelli 1994; Benzing 2000); but most  
389 self-compatible species need pollinator services and have several floral mechanisms such  
390 as dichogamy and herkogamy to hinder autonomous self-pollination (Martinelli 1994;  
391 Benzing 2000; Canela and Sazima 2003). Related species of *V. gigantea* from different  
392 genera in the Tillandsioideae subfamily (*Werauhia gladioliflora*, *Guzmania monostachia*  
393 and *Tillandsia fasciculata*) are fully self-compatible like *V. gigantea*, but showed  
394 autogamy as a reproductive strategy (Cascante-Marín et al. 2005, 2006).

395         Considering fruit and seed set in natural conditions, the results indicated a relatively  
396 low fruit and seed production in open treatment comparing to control (Table 3). Some  
397 factors may be responsible for this observation in *V. gigantea* population, such as pollen  
398 discounting and pollen limitation (Canela and Sazima 2005; Paggi et al. 2007). In the  
399 spontaneous selfing treatment, fruit and seed set occur in moderate ratios in *V. gigantea*.

400 However, considering the high fruit and seed production observed in the control treatment  
401 we suggest that selfing and outcrossing may occur in this species. Paggi et al (2007),  
402 comparing the control treatment with hand- pollination selfing treatment in *V. gigantea*,  
403 concluded that this species suffer pollen limitation in Itapuã State Park population. In  
404 addition, individuals of *Trigona spinipes* visiting *V. gigantea* flowers were recorded; they  
405 can contribute to pollen limitation due to their behavior, which affects pollinator visits  
406 through aggressive interactions as reported for *B. antiacantha* (Renner 1983; Canela and  
407 Sazima 2005). In summary, *V. gigantea* is a self-compatible species with mixed mating  
408 system in the Itapuã State Park populations. As the control treatment is higher than the  
409 open treatment, we can suggest that much of the seeds produced in the control treatment  
410 can occur from geitonogamy or selfing mediated by pollinators.

411         The results from pollen germination confirmed that *V. gigantea* is self-compatible,  
412 since pollen from self- and cross-pollination germinated in the pistil and penetrated the  
413 ovules. However, pollen from self-fertilization germinated slower than pollen from cross-  
414 fertilization, hence *V. gigantea* showed a preference for cross-pollination. Martinelli  
415 (1994) reported similar results for sixteen other species of the genus *Vriesea*. Self-  
416 incompatibility was also observed for species of the genera *Aechmea*, *Bilbergia* and  
417 *Quesnelia* (Martinelli 1994). Canela and Sazima (2003) also observed signs of  
418 incompatibility in *Aechmea pectinata*, such as curvature and thickening of the callose  
419 deposit on the extremity of the pollen tubes.

420         *Vriesea gigantea* also presents some floral features related to quiropterophily, such  
421 as tubular-shaped, big flowers, abundant nectar, absence of odor, long distance between  
422 nectar and reproductive organs (Vogel 1969), long anthesis and flowering period ( $\cong 12$  h  
423 and  $\cong 3$  months, respectively). All of these features favor outcrossing, which is also true for  
424 the foraging behavior of bats (Vogel 1969; Webb and Bawa 1983). It is noteworthy in this



425 context that Sazima et al. (1999) reported bat visitation in flowers of *V. gigantea* in a  
426 population from Serra do Mar, São Paulo State, southeastern Brazil. Taken together, their  
427 studies and ours indicate that bats, bees and hummingbirds serve as pollinators for this  
428 species across the species' range. Similar results as ours were observed in species of the  
429 genus *Guzmania* from Bolivia (Krömer et al. 2008), in which flowers were also open  
430 during the day and were scentless, which suggested that they were also pollinated by  
431 hummingbirds, as was indeed observed for *G. killipiana* in Ecuador (Dziedziuch et al.  
432 2003). The behavior of the hummingbirds (*T. glaucopsis* and *A. fimbriata*) in *V. gigantea*  
433 populations was classified as territorial (Feinsinger and Colwell 1978). They can behave  
434 aggressively and exclude hummingbird intruders, which contributes to increased selfing  
435 rates through autonomous or geitonamous selfing (Canela and Sazima 2003).

436

#### 437 **MIXED MATING SYSTEM**

438 The outcrossing rate observed in the *V. gigantea* populations agrees with a mixed mating  
439 system (Table 4; Lande and Schemske 1985). Despite the fact that *V. gigantea* presents  
440 characteristics of an outcrossing species, as described above, different factors may explain  
441 the intermediate outcrossing rate in these populations. Pollinator-mediated selfing and  
442 geitonogamy could contribute to high selfing rates in *V. gigantea*, since each plant  
443 produces many flowers concurrently at anthesis and the observed diurnal pollinators (bees  
444 and hummingbirds) often visit several flowers consecutively on the same plant, which also  
445 reduce the number of effective pollinators. Secondly, although *V. gigantea* has floral  
446 mechanisms to prevent self-pollination, such as protogyny, herkogamy, and cryptic SI, it is  
447 self-compatible (Paggi et al. 2007). Finally, pollen limitation was observed in the Itapuã  
448 State Park population (Paggi et al. 2007), which has been put forward as an important  
449 factor responsible for high selfing rates in many species with mixed mating systems

450 (Porcher and Lande 2005).

451         Likewise, pollen discounting, the reduction of male reproductive success by  
452 outcrossing, may accompany an increase in selfing rate, due to a decrease in amounts of  
453 exported pollen (Porcher and Lande 2005). Pollen discounting is a major factor favoring  
454 the maintenance of stable mixed mating systems in plants under a wide range of  
455 conditions, but strongly depends on inbreeding depression (Holsinger 1986; Porcher and  
456 Lande 2005). The estimated inbreeding depression ( $\delta$ ) for *V. gigantea* and pollen limitation  
457 and discounting observed in the studied population (Table 4; Paggi et al. 2007) are in  
458 agreement with the mixed mating maintenance model (Porcher and Lande 2005). Mixed  
459 mating systems cannot be explained by the opposing effects of inbreeding depression and  
460 the automatic advantage of selfing without additional features of pollination biology. In  
461 view of this premise, our results on the pollination biology of *V. gigantea* (discussed  
462 above), are complementary to genetic results and also in accordance with Porcher and  
463 Lande's mixed maintenance model (2005).

464         The TwoGener analysis revealed that the genetic composition of pollen pools was  
465 significantly differentiated among populations and that pollen-mediated gene movement  
466 was severely limited in all populations ( $\Phi_{st} = 0.671$ ; Table 5). The effective pollination  
467 neighbourhood ( $A_{ep}$ ) around a maternal plant was only  $173m^2$ , suggesting that maternal  
468 plants are preferentially pollinated by pollen drawn from near neighbors, about 147m away  
469 ( $\delta$ , average distance of pollination; Table 5). Furthermore, the low  $N_{ep}$  values observed  
470 (Table 4) suggest that a majority of seeds within each fruit are sired by only one or two  
471 pollen donors, with a few progeny having different fathers, due either to pollen carryover  
472 or multiple pollinator visits. All these parameters help us to understand the ecological  
473 conditions under which *V. gigantea* has experienced a reduction of gene flow and high  
474 levels of inbreeding in the margins of its distribution range. Knowledge of several aspects

475 of population biology is necessary to predict risks to population viability in the face of  
476 habitat fragmentation (Ohara et al. 2006).

477         The outcrossing rates and pollen pool parameters showed a characteristic trend  
478 towards the Southern margin of the species' range, with increased selfing and stronger  
479 pollen pool structure towards the range edge (Figure 4). Maquiné population (northward)  
480 has more different species of pollinators (different hummingbird species) than all other  
481 studied populations and subpopulations. This difference could easily produce different  
482 patterns of mating among families, resulting in high correlation of selfing ( $r_s$ ), and high  
483 outcrossing rates ( $t_m$ ); at the same time, these features may result in weaker pollen pool  
484 structure in Maquiné population ( $\Phi_{st} = 0.491$ ; Table 5). The inbreeding depression  
485 estimated for Maquiné ( $\delta > 0.5$ ) may also contribute to high  $t_m$ , since it is a force in  
486 opposition to selfing. In contrast, at the range edge (south), Taim population presents low  
487 levels of outcrossing, correlation of selfing, and inbreeding depression, but strong pollen  
488 pool structure. As discussed above, different factors may account for this pattern in this  
489 marginal population, where only territorial hummingbirds and bees were observed as  
490 pollinators. Consequently, low levels of inbreeding depression in this population make  
491 possible the evolution of high levels of selfing ( $s = 0.824$ ). In addition, as discussed by  
492 Charlesworth and Charlesworth (1979), inbreeding depression is the only general factor  
493 strong enough to prevent the evolution of self-fertilization in most species. Alternatively,  
494 the studied populations may differ in the degree of dominance of deleterious alleles,  
495 resulting in differences in the relative ease with which they are purged, (Table 4, Taim  
496 population). Variation in mating systems has been documented in many plant species  
497 including bromeliads (Barbará et al. 2008, 2009), and the most common factors put  
498 forward as being responsible are edge effects (Sun and Ritland 1998), habitat  
499 fragmentation, and population history (Ohara et al. 2006). In our present study on *V.*

500 *gigantea*, threefold evidences (hand-pollination experiments, pollinator observations, and  
501 molecular marker-based inference of mating system parameters) indicate that ecological  
502 patterns, as pollination biology, cannot be ignored in studies addressing the maintenance of  
503 mixed mating in plants.

504

505

## 506 *Conclusions*

507 In summary, we propose a scenario for the maintenance of mixed mating in *V. gigantea*,  
508 with opposing trends of selfing and inbreeding depression across the sampled portion of its  
509 range. Furthermore, as mentioned earlier, in contrast to several floral features adapted to  
510 cross-pollination, other aspects evaluated, such as pollinators, inbreeding depression,  
511 outcrossing rates and pollen pool structure suggest that *V. gigantea* could adapt to high  
512 levels of selfing, or could even be in transition from outcrossing to inbreeding. Similar  
513 transitions among mating systems within and among related species of bromeliads have  
514 been documented previously (Barbará et al. 2008; 2009) but the underlying mechanisms  
515 were not addressed. Here we show that variation in the mating system of *V. gigantea* likely  
516 has been shaped by pollinator behavior on one hand and habitat fragmentation on the other,  
517 effectively decreasing gene flow and increasing inbreeding at the range edge. A good  
518 reference data set on genetic variation and reproductive biology in bromeliad species with  
519 different mating systems will be necessary to assess the impact of habitat fragmentation on  
520 biological diversity in this large, ecologically important family of flowering plants.

521

522

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536

537

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692

### 693 LEGENDS OF FIGURES

694 **Figure 1.** Map showing the current geografic distribution of *V. gigantea* (Reitz 1983).

695 Locality of the three study sites from Rio Grande do Sul State, Brazil: (A) Maquiné city;

696 (B) Itapuã State Park; and (C) Taim Ecological Station.

697

698 **Figure 2.** Adult individual, pollinators and ovules, and Anthesis floral features and floral

699 visitors of *V. gigantea* in the rainforest, southern Brazil. (A) Individual showing the large

700 inflorescence characteristics for this species in the fruiting season. Photo: Courtesy of CM  
 701 Zanella. (B) An individual of *Apis mellifera* visiting a flower, touching the stigma, Itapuã  
 702 State Park, RS, Brazil. Photo: GM Paggi. (C) A male of *Amazilia fimbriata* visiting a  
 703 flower, touching the stigma with its head, Taim Ecological Station, RS, Brazil. Photo:  
 704 Courtesy of M Bruxel. (D) Ovules showing pollen germination on a flower fixed 48h after  
 705 manual self-pollination, arrow showing a penetrated ovule. Photo: Courtesy of NSJr  
 706 Bittencourt. (E) Prior to bud opening, the stigma appears. (F) Flower exhibiting  
 707 herkogamy, the spatial separation of anthers and stigmas within flowers. (G) *Apis mellifera*  
 708 visiting the nectary of a *V. gigantea* flower. (H) *Trigona spinipes* “stealing” pollen of a *V.*  
 709 *gigantea* flower.

710

711 **Figure 3.** Nectar features and number of visits in *V.gigantea*. Variation in (A) nectar  
 712 volume ( $F = 3.87$ ;  $P = 0.0062$ ); (B) sugar concentration in nectar ( $F = 6.04$ ;  $P = 0.0003$ );  
 713 and (C) number of hummingbird visits per flower ( $F = 15.86$ ;  $P = 0.0001$ ). a, b, c – Means  
 714 with the same letter are not significantly different in the  $t$ -tests (5%).

715

716 **Figure 4.** Mixed mating and pollen pool structure parameters. The populations are:  
 717 Maquiné (black bars), Itapuã State Park (grey bars) and Taim Ecological Station (white  
 718 bars). (A) Parameters that increasing from north to south:  $F$ , (estimated) parental  
 719 inbreeding ( $F = 1.50$ ;  $P = 0.2453$ );  $\Phi_{st}$ , corrected pollen pool genetic structure parameter;  
 720 and  $N_{ep}$ , effective number of pollen donor from the sibling pair model ( $F = 1.12$ ;  $P =$   
 721  $0.3450$ ). (B) Parameters that decreasing from north to south:  $r_p$ , correlation of paternity ( $F$   
 722  $= 4.08$ ;  $P = 0.0288$ );  $t_m$ , multilocus outcrossing rate ( $F = 5.69$ ;  $P = 0.0106$ );  $t_s$ , single-  
 723 locus average outcrossing rate ( $F = 7.06$ ;  $P = 0.0036$ );  $(t_m - t_s)$ , biparental inbreeding ( $F =$   
 724  $0.99$ ;  $P = 0.3856$ ); and  $\delta$ , inbreeding depression ( $F = 1.01$ ;  $P = 0.3886$ ).

725 **TABLES**

726 **Table 1**

727 Natural populations and subpopulations of *V. gigantea* in the Atlantic Rainforest, Rio Grande do Sul, Brazil. Location, coordinates, mean of  
728 temperature, annual rainfall, and plant habit.

729

<i>Population</i>	<i>Subpopulaïton</i>	<i>Conservation Unit/City</i>	<i>Geographical coordinates</i>	<i>Mean of temperature</i>	<i>Annual rainfall</i>	<i>V. gigantea habit</i>
Maquiné		Serra Geral Ecological Reserve/Maquiné	29° 48' S, 50° 16' W	21.5 °C	1650 mm/year	epiphytic
	Trilha do Fenômeno		30° 21' 40,3" S, 51° 01' 51,1" W			epiphytic/ saxicolous
Itapuã	Praia do Araçá	Itapuã State Park/Viamão	30° 21' 31,5" S, 51° 02' 41,1" W	17.5 °C	1200 mm/year	epiphytic/ saxicolous
	Pedra da Visão		30° 22' 40,2" S, 51° 01' 44,7" W			epiphytic/ saxicolous
	Morro da Grota		30° 22' 13,3" S, 51° 01' 42,2" W			epiphytic/ saxicolous
Taim		Taim Ecological Station/Rio Grande	31° 56' S, 52° 25' W	18 °C	1100 mm/year	epiphytic

730

731 **Table 2**

732 Manual pollination experiments for assessing the mating system of *V. gigantea*.

733

<i>Manipulation</i>	<i>Pollination treatment</i>			
	Agamospermy	Spontaneous selfing	Open	Control
Bagging *	✓	✓		
Emasculation **	✓		✓	
Sample ***	36	48	35	47

734 \* Bagging excludes insect or vertebrate visitors.

735 \*\* Emasculation removes all anthers.

736 \*\*\* Number of flowers used in each treatment

737 **Table 3**

738 Rate of fruit setting (%) and mean number of seeds in four *V. gigantea* subpopulations from Itapuã State Park, RS, Brazil. Treatments: AG,  
739 agamospermy; SS, spontaneous selfing; O, open; C, control; standard errors in parentheses.

740

Subpopulations	Treatments							
	AG	SS	O	C	AG	SS	O	C
	fruit set (%)				n° seed			
Morro da Grota	0	27.7 (12.5)	0	66.7 (14.9)	0	105.1 (51.7)	0	395.3 (81.9)
Praia do Araçá	33.3 (33.3)	44.4 (11.1)	33.3 (33.3)	44.4 (29.4)	41.11 a (33.56)	154.1 (68.5)	16.2 (13.5)	102.9 (69.2)
Trilha do Fenômeno	0	27.6 (12.5)	16.7 (10.5)	33.3 (18.3)	0	102.9 (52.5)	64.9 (49.5)	187.7 (82.9)
Pedra da Visão	0	11.1 (11.1)	33.3 (33.3)	44.4 (22.2)	0	56.7 (56.7)	34.3 (34.3)	186.6 (82.6)
All	5.6 (0.6) c	27.1 (6.3) ab	17.7 (8.8) b	47.9 (9.6) a	10.28 (6.44) c	104.5 (27.9) b	40.8 (23.0) b	237.5 (44.3) a

741 a and b – Means with the same letter in the lines are not significantly different by the Kruskal-Wallis Test (5%).

742 **Table 4**

743 Estimates of mating system parameters (SE in parentheses) for three *V. gigantea* populations from Rio Grande do Sul State, Brazil.

744

	<i>Parameter</i>	<i>Maquiné</i>	<i>Itapuã</i>	<i>Taim</i>	<i>All</i>
Mixed mating system model*	<i>F</i>	0.212 (0.011)	0.355 (0.008)	0.718 (0.012)	0.372 (0.004)
	$t_m$	0.477 (0.028)	0.336 (0.029)	0.176 (0.033)	0.388 (0.017)
	$t_s$	0.337 (0.022)	0.225 (0.021)	0.106 (0.020)	0.244 (0.011)
	$(t_m - t_s)$	0.141 (0.018)	0.111 (0.020)	0.069 (0.015)	0.144 (0.012)
	$r_p$	0.714 (0.070)	0.476 (0.093)	0.386 (0.309)	0.631 (0.044)
	$r_s$	0.603 (0.083)	0.121 (0.096)	-0.021 (0.157)	-0.200 (0.014)
	$N_{ep} (1/ r_p)$	1.4	2.1	2.59	1.58
	Full sibs ( $t_m \cdot r_p$ )	0.341	0.159	0.068	0.245
	Half sibs [ $(t_m (1 - r_p))$ ]	0.136	0.176	0.108	0.143
Inbreeding depression**	$\delta$	0.509	0.443	-0.087	0.274

745 \*Mixed mating system parameters: *F*, (estimated) parental inbreeding;  $t_m$ , multilocus outcrossing rate;  $t_s$ , single-locus average outcrossing rate;  $(t_m - t_s)$ , biparental inbreeding;  $r_p$ ,

746 correlation of paternity; and  $N_{ep}$ , effective number of pollen donor. \*\* Estimated based on Goodwillie et al. (2005).



**Table 5**

Estimates of pollen pool structure parameters for three *V. gigantea* populations from Rio Grande do Sul State, Brazil.

	<i>Parameter</i>	<i>Maquiné</i>	<i>Itapuã</i>	<i>Taim</i>	<i>All</i>
TwoGener Model*	$\Phi_{st}$	0.491	0.768	0.928	0.671
	$\Phi'_{FT}$	0.401	0.516	0.659	0.532
	$N_{ep}$	1.02	0.65	0.54	0.75
	$\delta$ (m)	137	403	245	147
	$A_{ep}$ (m <sup>2</sup> )	150	1302	480	173
(Palma-Silva 2008)	$F_{IS}$	0.225	0.489	0.408	0.261

\*TwoGener model parameters:  $\Phi_{st}$ , corrected pollen structure parameter;  $\Phi'_{FT}$ , observed (uncorrected for inbreeding) pollen structure parameter;  $N_{ep}$ , effective number of pollen donors;  $\delta$ , average distance of pollination; and  $A_{ep}$ , effective neighborhood pollination area.

**FIGURES**

**Figure 1**

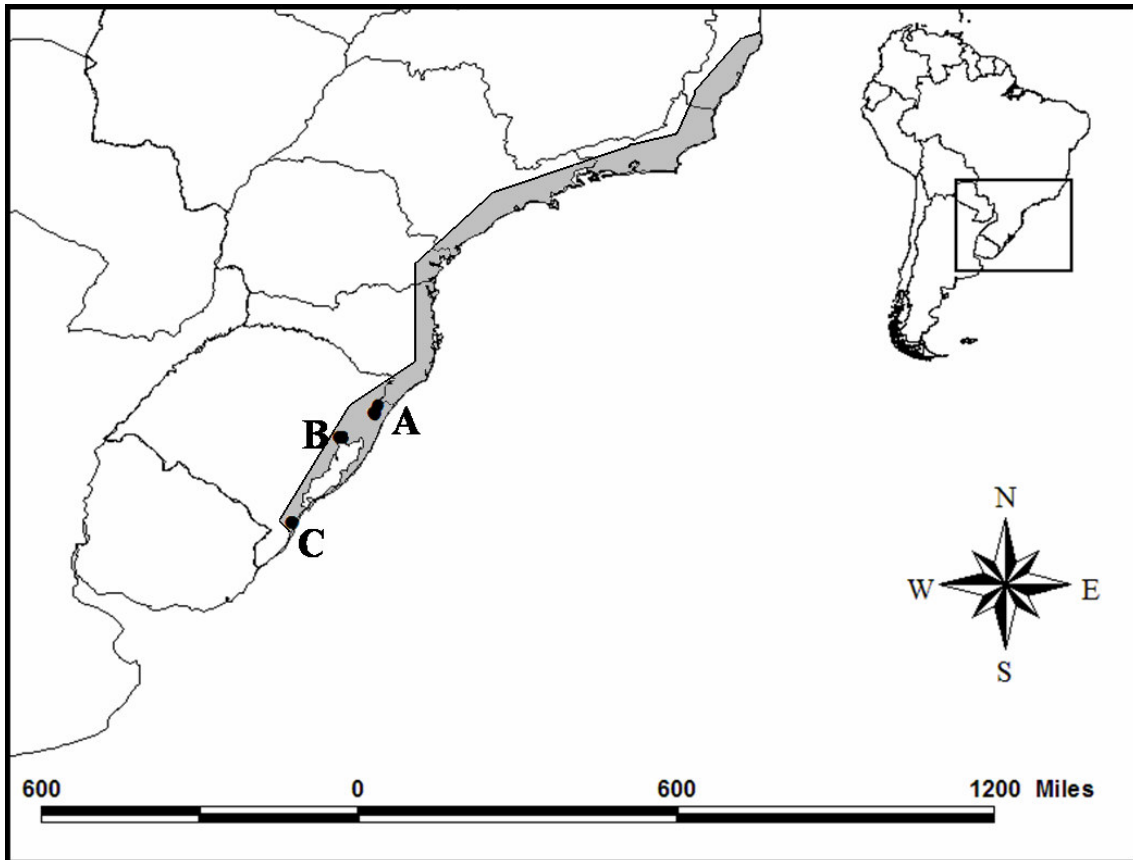


Figure 2

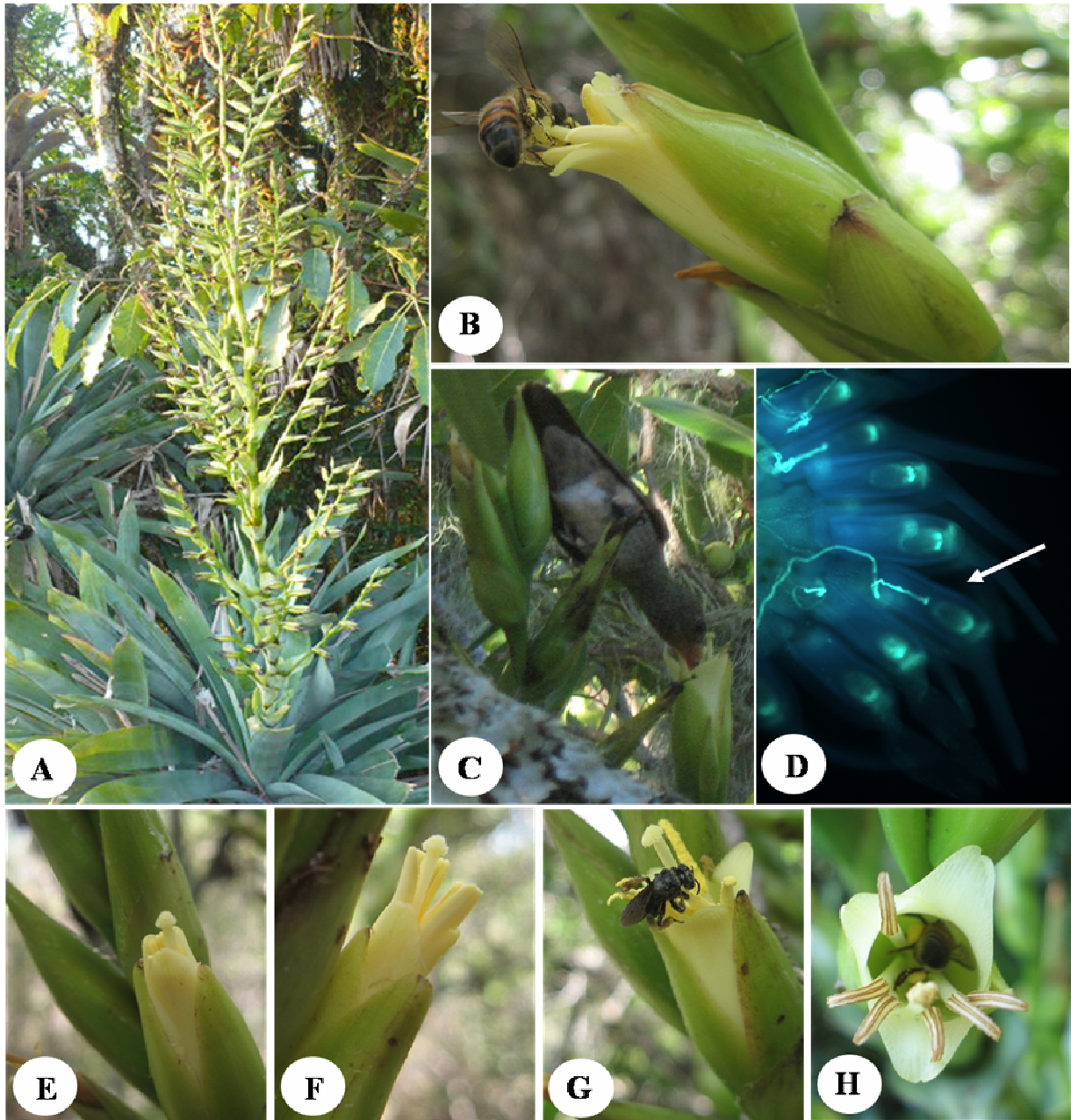


Figure 3

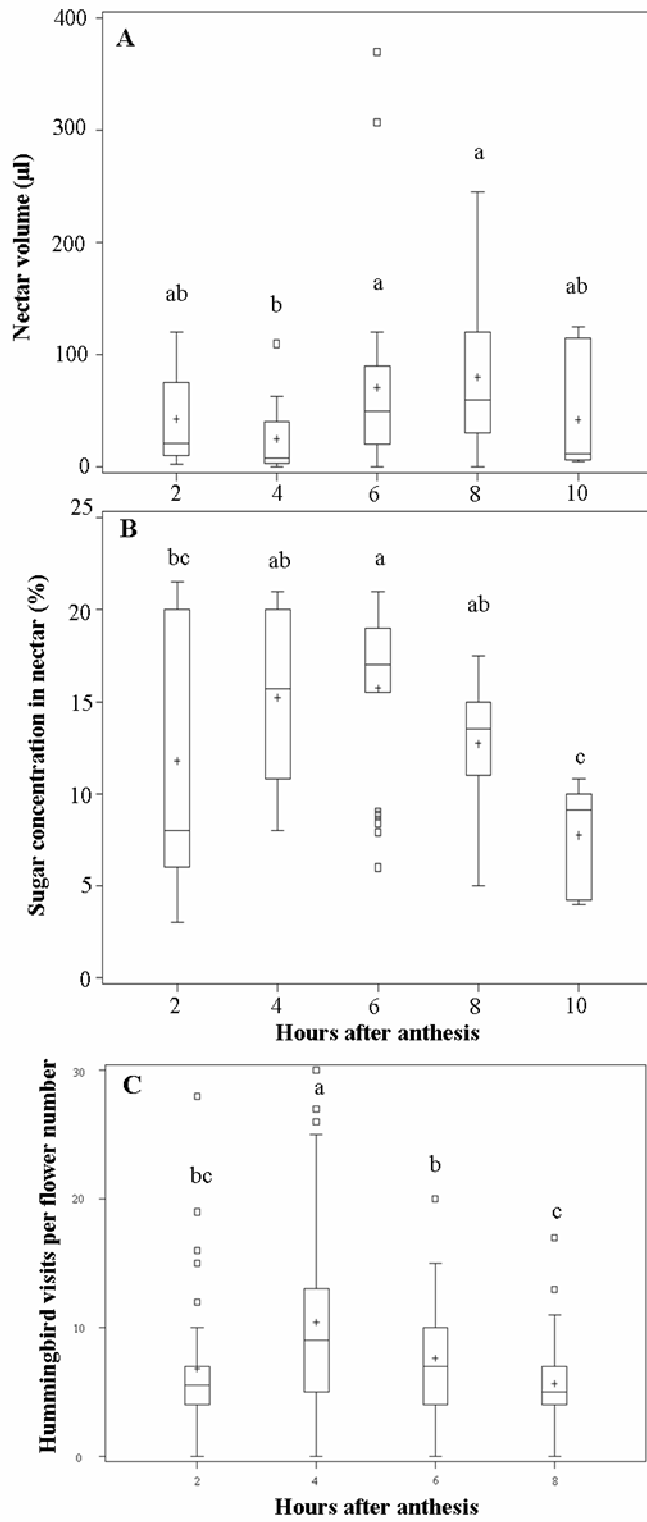
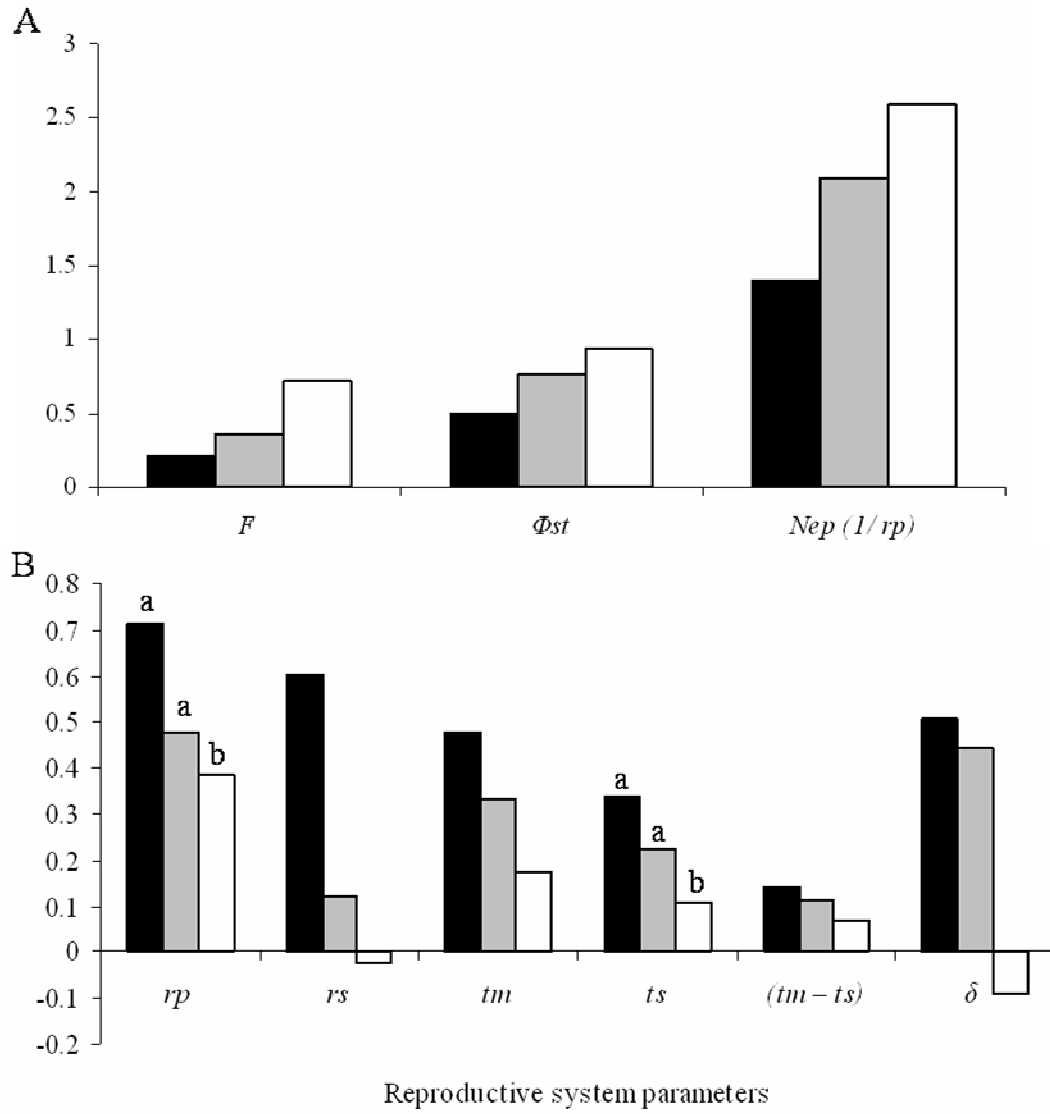


Figure 4



ANNEX

**Annex 1**

Genetic variability parameters within populations of *V. gigantea* mother-plants based on eight microsatellites loci. Number of individuals sampled (N), average number of alleles per locus (A), expected heterozygosity ( $H_e$ ) observed heterozygosity ( $H_o$ ) and P-values (P).

Populations	N	A	$H_o$	$H_e$
Maquiné	10	4.06	0.567	0.708
Itapuã	11	3.32	0.364	0.627
Taim	5	1.38	0.075	0.108
All	26	3.73	0.335	0.500

### **CAPÍTULO III**

Spatial-temporal variation of mating system and fertility of  
*Vriesea gigantea* (Bromeliaceae) wild populations at the species'  
southern range margin

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A ser submetido para *Heredity*

Spatial-temporal variation of mating system and fertility of *Vriesea gigantea* (Bromeliaceae) wild populations at the species' southern range margin

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**Keywords:** Atlantic Rainforest; bromeliad; gene flow; outcrossing rate; reproductive success; TwoGener.

**Running title:** Mating system and fertility variation of *Vriesea gigantea* GM Paggi et al.



Plant mating systems and fertility are directly related to many questions on plant evolution and conservation. Patterns of among-population mating system and fertility in bromeliads have been thoroughly evaluated in few studies in order to understand the consequences for mating system evolution and species conservation. *Vriesea gigantea* is endemic to the Brazilian Atlantic Rainforest, is self-compatible and pollinator dependent to fruit set. Previous molecular studies revealed that *V. gigantea* populations are structured, with low gene flow and high selfing rates. Our aim was to investigate if mating system and fertility patterns are due to habitat conditions or due to species' mating system adaptation. In the present study, outcrossing rates ( $t_m$ ) were moderate and pollen pool structures ( $\Phi_{FT}$ ) were high, ranging across years and populations. The plant fertility of three wild populations was assigned considering flower production, fruit and seed set, and seed viability, during three consecutive years. In the three populations we observed high flower, fruit and seed production, and high seed viability, with differences in fruit set between populations. These results strongly suggest that the observed among-population patterns of flower, fruit and seed set, and mating system parameters in *V. gigantea* are attributable to the species adaptation for selfing and edge effects, though other adaptive explanations cannot be ruled out. Considering the spatial-temporal variation in mating system and fertility, and contemporary gene flow results, we considered that *V. gigantea* populations from edge of its distribution should be taking into account in conservation programs of the species.

## Introduction

Mating systems have important effects on the amount and structuring of genetic variation within and among plant populations. Likewise, variations in the gene flow via pollen transfer and seed dispersal also contribute to the shape of genetic diversity within populations (Hamrick, 1982). In spite of this, mating systems studies of plants are commonly characterized only by field studies of pollination and occasionally by genetic parameters, which should provide detailed information about mating system and gene flow. One of these parameters is the outcrossing rate estimate, which can be generated by examining progeny arrays from plants pollinated naturally, using a maximum-likelihood model to exclude progeny resulting from self-fertilization (Ritland and Jain, 1981). Besides, evolutionary biologists interested in microevolutionary processes and conservation biologists interested in the impact of landscape change require a method that measures the real time process of gene movement. Smouse *et al.* (2001) presented a two-generation approach to study pollen poll genetic structure (TwoGener) that allows us to quantify heterogeneity among the male gamete pools sampled by maternal plants scattered across the landscape and to estimate mean pollination distance and effective neighborhood size.

Population viability depends on survival and fecundity parameters, which will be a consequence from habitat conditions (biotic and abiotic). However, since it would be difficult to increase individual survivorship without modifying the whole ecosystem, quantifying those factors that affect reproduction success is an important step in developing possible management strategies for plant species (Colas *et al.*, 2001). Several features associated with plant fertility have been used to determine the population viability,

such as plant size, flower production, fruit and seed set, pattern of fruit production, pollen limitation and seed viability (Colas *et al.*, 2001; Ishii and Kadono, 2002; McIntosh, 2002; Burne *et al.*, 2003; Ortiz *et al.*, 2003; Buide, 2004; Clark-Tapia and Molina-Freaner, 2004; Johnson *et al.*, 2004; Kéry and Matthies, 2004; Caruso *et al.*, 2005; Hampe, 2005). Fruit and seed production in plants can be limited by the availability of pollen and abiotic resources. Pollen limitation of seed production can be the result of fewer pollinator visits or a decrease in the quantity or quality of pollen deposited per visit (Huang and Guo, 2002; Ashman *et al.*, 2004; Buide, 2004). Fertility patterns associated with mating system analyses can provide a better understanding of those processes in reproductive success of natural plant populations.

Some species, such as bromeliads, show a remarkable stability of spatial distribution, probably because they live in a stable, naturally fragmented habitat condition. These species are generally poor competitors, well adapted to drought and wind, and their colonizing ability is probably hindered by landscape structure, i.e. a limited number of suitable sites surrounded by large patches of unsuitable habitat (Benzing, 1990, 2000; Winkler *et al.*, 2007). Barbará *et al.* (2007) studying the population genetic structure and species cohesion of ‘inselbergs’ endemic bromeliad species, *Alcantarea imperialis* and *A. geniculata* (Bromeliaceae), showed that population differentiation is pronounced in both taxa, especially in *A. imperialis*. Also gene flow in this species is considerably lower than expected from the literature on plants in general and in Bromeliaceae in particular (Palma-Silva, 2008).

In the present study we investigated patterns of spatial-temporal variation in mating system and fertility in three populations of *V. gigantea* in three years. Our aim was to investigate if parameters obtained from populations are due to habitat conditions or if all

the studied parameters are traits of species' mating system adaptation. Specifically, it aims: (1) to estimate mating system parameters, including outcrossing rate and pollen genetic structure; (2) to quantify fertility of *V. gigantea* populations, including flower production, fruit and seed set, and seed germination; (3) to verify what extent of mating system parameters effects influence the reproductive success of populations at the species' southern range margin. The results will help us to suggest population management strategies that could increase the species probability of stability.

## Material and Methods

### Study species

*Vriesea gigantea* is a self-compatible bromeliad which presents high fertility, a high selfing rate and the occurrence of inbreeding depression in populations from its southern range margin (Paggi *et al.*, 2007; Palma-Silva *et al.*, 2008; Paggi *et al.*, *in prep*). In the Itapuã State Park population, Paggi *et al.* (2007) observed pollinator limitation, which was considered a consequence of habitat fragmentation, or low pollen viability observed in Praia do Araçá subpopulation (Itapuã State Park; Palma-Silva *et al.*, 2008). Additionally, nuclear and plastidial molecular markers revealed latitudinal trend of decreasing diversity from North to South away from the equator, consistent with historical forest expansion from the Northern half of the present distribution range (Palma-Silva 2008). Further, species expansion appears to be impeded by lack of gene flow at the current range edges.

## Study sites

This study was conducted in three wild populations at the species' southern range margin of *V. gigantea*, Brazilian Atlantic Rainforest, east of Rio Grande do Sul state, Brazil (Table 1 and Figure 1). Fieldwork was conducted from January to September of 2005 (only in Itapuã State Park population), and 2006, 2007, 2008 in all populations (Maquiné, Itapuã and Taim). Sample sizes used for fertility and mating systems analysis in each reproductive cycle are indicated in Table 2.

## Mating system analysis

*Plant material and microsatellite markers* - Open-pollinated maternal seeds were collected from each mother-plant (MP) per year, and were used for genetic analysis. Fresh leaves (~5 cm<sup>2</sup>) from the MP and seedlings were collected and stored in liquid nitrogen until DNA extraction. Total genomic DNA was extracted using the protocol described by Doyle and Doyle (1990). The localization of each mother-plant was plotted using a GPS (Global Positioning System). Different microsatellite loci were used for each sampled year. In the analyses of Itapuã population (2005), four nuclear microsatellite loci previously characterized for *V. gigantea* (Palma-Silva *et al.*, 2007 – loci: VgA04, VgA06, VgB06, and VgF02), were used. In the analyses of 2006 for all populations, also four microsatellite loci were used, one described for *Guzmania monostachia* (Boneh *et al.*, 2003 – loci: CT5) and three for *V. gigantea* (Palma-Silva *et al.*, 2007 – loci: VgB06, VgF01 and VgG02). In the analyses in 2008, two loci (VgF01 and VgG02; Palma-Silva *et al.*, 2007) were used in all populations. The forward primer of each locus was labeled at the 5' end with the M13 fluorescent tag (6-FAM). All PCR amplifications were performed in a PE Applied Biosystems 9700 thermocycler as described by the authors. Microsatellite alleles were

resolved on a 3100 DNA Analyser (Applied Biosystems) and were precisely sized against ROX molecular size standard using GENOTYPER 3.7 software (Applied Biosystems).

*Progeny pair analysis* - The progeny pair analysis was performed under the mixed mating model described by Ritland (2002), using the MLTR 3.0 software (Ritland, 1990). Estimates of outcrossing rate ( $t_m$ ), inbreeding coefficient of maternal parents ( $F$ ), and correlation of selfing ( $r_s$ ) (Ritland, 1989; 2002) were obtained. Standard errors were estimated based on 1000 bootstraps between individuals within a progeny array.

*Pollen structure analysis* - TwoGener method was performed to analyze the genetic pollen pool structure among individual plants sampled relative to the global pollen pool of a population (Smouse *et al.*, 2001); this procedure is based on the AMOVA approach of Excoffier *et al.* (1992). Estimates of pollen pool structure ( $\Phi_{FT}$ ), average distance of pollination ( $\delta$ ), effective neighborhood pollination area ( $A_{ep}$ ) and effective number of pollen donors ( $N_{ep}$ ) were performed using the POLDISP 1.0 software (Robledo-Arnuncio *et al.*, 2007). We computed the 99% confidence interval of  $\Phi_{FT}$  by bootstrapping among loci with 1000 replicates.

### Fertility analysis

The following parameters of fertility were used in each population and plant: (a) total number of flowers per plant; (b) fruit set, i.e. the fraction of flowers developing into a mature fruit; (c) number of seeds per fruit (from one fruit randomly collected from 25 plants): estimated by comparing the weight of 20 seeds with the weight of all seeds from each fruit. To analyze seed germination rate, twelve plants and two fruits per plant (30

seeds per fruit) were used. Seeds were germinated as described by Paggi *et al.* (2007). Germination was monitored daily for 30 days.

### Statistical analyses

Fertility parameters, flower production, fruit set, number of seeds and seed germination were analyzed by the ANOVA followed by the Tukey test to verify differences among populations and years. Possible correlation between outcrossing rate ( $t_m$ ) and reproductive success (fruit set) considering all populations and years were test with regression linear analyses. All the statistical analyses were performed using SAS software (Version 8, SAS Institute, Cary, USA).

## Results

### Mating system

Inbreeding coefficient ( $F$ ) based on genotypic frequencies of maternal plants was positive and significantly greater than zero in all populations (except in Taim-2008), suggesting an excess of homozygotes ( $P < 0.0001$ ; Table 3). The outcrossing rate ( $t_m$ ) was variable among populations and years within populations, ranging from 0.107 to 0.44 (Table 3). A significant self-fertilization rate ( $s = 1 - t_m$ ) was detected since the  $t_m$  was significantly lower than one for all populations and years ( $P < 0.0001$ ). The correlation of selfing ( $r_s$ ) in Maquiné population was high which means that some families were high selfers and others high outcrossers. In the other populations, the values of  $r_s$  were lower, indicating less variation of selfing among families. The estimates obtained in Taim – 2008, should be

viewed with caution, because only one of the two *loci* analyzed in this year (VgF01 and VgG02) was polimorphic.

The differentiation of allelic frequencies among the pollen pools ( $\Phi_{FT}$ ) of *V. gigantea* ranged from 0.319 to 0.598, indicating great variation in the distribution of the pollen pool from plant to plant overall populations and years (Table 4). The average distance of pollination ( $\delta$ ) ranged from 77 to 299m, and the effective neighbourhood pollination area ( $A_{ep}$ ) ranged from 47 to 232m<sup>2</sup>. The lower average distance of pollination and effective neighbourhood pollination area were observed in Itapuã – 2008, when reproductive adult density was bigger (17.42 plants/km<sup>2</sup>; data not showed). The effective number of pollen donors ( $N_{ep}$ ) overall populations and years were low, ranging from 0.77 to 1.56. Estimates for Taim – 2008 could not be computed because one *locus* was monomorphic.

### Fertility

The number of seeds per fruit ranged from 293 to 1079, with a mean of 598.43 (SE  $\pm$  35.18), and no differences among populations were detected (Table 5). The seed viability and germination rate of *V. gigantea* were very high. After 15 days in culture, an average of 86.81% (SE  $\pm$  2.46) of seeds from open-pollinated flowers germinated. Taim population showed the lower germination rate, 72.96% (SE  $\pm$  6.29). The mean number of flowers per plant was 169.19 (SE  $\pm$  5.62), considering all populations and years, without differences among them (Table 5). The fruit set per plant ranged from 23.27 (SE  $\pm$  5.98) to 75.92% (SE  $\pm$  5.62) with an overall mean of 47.63% (SE  $\pm$  8.24), considering populations and years. Differences of fruit set were detected among populations in each year. Taim population yielded more fruits in 2006 (75.92  $\pm$  5.62). Itapuã population showed a decrease



on fruit set in 2006 ( $23.27\% \pm 5.98$ ; Table 5). Patterns of fruit set observed in populations of *V. gigantea* were not significantly correlated with outcrossing rates ( $R^2 = 0.155$ ;  $P = 0.381$ ; Figure 2).

## Discussion

### Spatial-temporal variation of mating system and fertility of *Vriesea gigantea*

Variation in time and space of outcrossing rates can be due to historical and biological context of each plant species (Loveless and Hamrick, 1984). Interpopulation variation in outcrossing rates for three species of *Lupinus*, was considered a result from fluctuations in bee activity (Schemske and Lande, 1985). Similarly, the wide variation observed in outcrossing rates for self-fertile *Collinsia sparsiflora* var. *arvenis*, was considered due to environmental variation, as outcrossing depends strongly on flower density in this species (Schemske and Lande, 1985). However, the interpopulation variation in outcrossing rates observed in *V. gigantea* associated with some reproductive biology traits of the species, e.g. self-compatibility and moderate inbreeding depression (Paggi *et al.*, *in prep*), suggest a historical effect, where genetic and environmental factors are determinants of mating system in this species, as a consequence from habit (epiphytic), habitat fragmentation and edge effects adaptation.

Patterns of fruit set observed in populations of *V. gigantea* were not significantly correlated with outcrossing rates (Figure 2), indicating that outcrossing rates have no significant influence in the reproductive success (fruit set). Besides, results from outcrossing rates confirm that selfing occurs with high frequency ( $0.555 < s < 0.893$ ; not

including Taim-2008), which must ensure reproduction in *V. gigantea* populations at the species' southern range margin. Fertility studies, including populations from Southern edge of the distribution, revealed that those populations are fertile, though Itapuã population shows strong evidences of pollen limitation, considering qualitative (pollen viability) and quantitative (pollinator limitation) components (Paggi *et al.*, 2007; Palma-Silva *et al.* 2008). These findings highlight the assumptions of Porcher and Lande (2005) model of self-fertilization evolution, in which mixed mating systems with high selfing rates could be maintained by strong pollen limitation and pollen discount, and low inbreeding depression, as discussed for *V. gigantea* populations by Paggi *et al.* (*in prep*). In overall populations and years we observed high flower, fruit and seed production, and high seed viability, Taim population showing the highest fruit set production. These results may be due low inbreeding depression observed in Taim population ( $\delta = -0.0877$ ; Paggi *et al.* *in prep*) compared to Maquiné and Itapuã populations, where inbreeding depression was high ( $\delta = 0.509$  and  $0.443$ , respectively; Paggi *et al.*, *in prep*). Variation in time and space of outcrossing rates and fertility observed in *V. gigantea* populations can be due to pollinator behavior or flower plant density, but also an adaptation to selfing due to edge effects (Sun and Ritland, 1998) and habitat fragmentation (Ohara *et al.*, 2006).

#### Patterns of gene flow and fertility in the species' range margin

Fragmented habitats have a profound effect in gene flow of natural plant populations. Previous molecular studies, using nuclear microsatellites markers, indicated that *V. gigantea* populations at the range margin have increased drift due to reduced gene flow ( $N_e m = 0.688$ ) among marginal populations and smaller effective population size ( $N_e$ ) compared to the central populations (from Paraná, São Paulo and Rio de Janeiro States;

Palma-Silva, 2008), which are in continuous forest. Additionally, the observed outcrossing rate interpopulation variation of *V. gigantea*, ( $0.107 < t_m < 0.445$ ; Table 3) and the high levels of pollen pool genetic structure ( $0.319 < \Phi_{FT} < 0.598$ ; Table 4), are in agreement with the previous estimation of gene flow, which was low among populations from the southern range margin related by Palma-Silva (2008). On the other hand, even with low gene flow and effective populations size (Palma-Silva, 2008), moderate inbreeding depression (Paggi *et al.*, *in prep*), high genetic structure of pollen pool and low effective number of pollen donors, fertility parameters revealed that populations are fertile with a significant fruit and seed production (this study). These results suggest that the observed among-population patterns mating system and fertility parameters in *V. gigantea* are attributable to the species adaptation for selfing and edge effects, though other adaptive explanations cannot be ruled out.

### Consequences for evolution and conservation

In this study we observed spatial-temporal variation of mating system and fertility of *V. gigantea* wild populations at the species' southern range margin. Two-generation analyses revealed almost zero gene flow among populations, since distance among them are bigger than average distance of pollination ( $\delta$ ), and, consequently, the pollen pool structure were very high (Table 4). Regarding the rapid ongoing fragmentation in the Atlantic Rainforest 'hotspot' of South America, it is expected that isolation by distance and low gene flow will contribute to increase genetic differentiation among these populations (Palma-Silva, 2008). Considering the spatial-temporal variation in mating system and fertility results, we considered that *V. gigantea* populations from edge of its distribution should be taking into account in conservation programs of the species. Large-scale conservation strategy in order

to protect all populations at the species' southern range margin will ensure continuous survival and maintenance of evolutionary potential of these *V. gigantea* populations.

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## Legends of figures

**Figure 1** Locality of the three study sites from Rio Grande do Sul State, Brazil: (A) Maquiné city; (B) Itapuã State Park; and (C) Taim Ecological Station.

**Figure 2** Relation between fruit set and outcrossing rate of *V. gigantea*, regarding all populations and years.



Tables

**Table 1** Natural populations of *V. gigantea* in the Atlantic Rainforest, Rio Grande do Sul State, Brazil. Locations, coordinates, mean temperature, annual rainfall, and plant habit.

<i>Population</i>	<i>Conservation Unit/City</i>	<i>Geographical coordinates</i>	<i>Mean temperature</i>	<i>Annual rainfall</i>	<i>V. gigantea habit</i>
Maquiné	Serra Geral Ecological Reserve/ Maquiné	29° 48' S, 50° 16' W	21.5 °C	1650 mm/year	epiphytic
Itapuã	Itapuã State Park/ Viamão	30° 21' S, 51° 01' W	17.5 °C	1200 mm/year	epiphytic/ saxicolous
Taim	Taim Ecological Station/ Rio Grande	31° 56' S, 52° 25' W	18 °C	1100 mm/year	epiphytic

**Table 2** Sample sizes per population, per year in fertility (adult plants) and mating systems analysis (adult plants/progenies) of *V. gigantea* populations.

<i>Analysis</i>	<i>Fertility</i>				<i>Mating system</i>			
	<i>Population</i>	2005	2006	2007	<i>Total</i>	2005	2006	2008
Maquiné	-	13	12	25	-	10/191	14/276	24/467
Itapuã	26	16	15	57	12/182	11/162	14/273	37/435
Taim	-	12	15	27	-	5/91	12/212	20/303

**Table 3** Estimates of mating system parameters (standard error in parentheses) of *V. gigantea* populations and years.

Populations	Maquiné			Itapuã			Taim		
	2006/ 4 loci	2008/ 2 loci	2005/ 4 loci	2006/ 4 loci	2008/ 2 loci	2008/ 4 loci	2006/ 4 loci	2008/ 2 loci	
$F$	0.240 (0.091)	0.267 (0.233)	0.411 (0.015)	0.291 (0.107)	0.293 (1.89)	0.611 (0.455)	0.611 (0.455)	-0.200 (0.270)	
$t_m$	0.445 (0.087)	0.300 (0.082)	0.299 (0.030)	0.296 (0.079)	0.107 (0.079)	0.152 (0.215)	0.152 (0.215)	1.200 (0.076)	
$r_s$	0.452 (0.208)	0.710 (0.185)	0.326 (0.109)	-0.175 (0.066)	-0.200 (0.427)	-0.200 (0.481)	-0.200 (0.481)	0.406 (0.235)	

\*Mixed mating system parameters: inbreeding coefficient of maternal parents ( $F$ ); multilocus outcrossing rate ( $t_m$ ); correlation of selfing ( $r_s$ ).

**Table 4** Estimates of pollen pool genetic structure parameters of *V. gigantea* populations and years.

<i>Populations</i>	<i>Maquiné</i>		<i>Itapuã</i>		<i>Taim</i>		
	2006	2008	2005	2006	2008	2006	2008
Parameters*							
$\Phi_{FT}$	0.401	0.319	0.572	0.465	0.598	0.569	-
$\delta$ (m)	152	235	170	150	77	299	-
$A_{ep}$ (m <sup>2</sup> )	184	441	232	181	47	718	-
$N_{ep}$	1.25	1.56	1.36	0.77	0.82	0.81	-

\*TwoGener model parameters: pollen structure parameter ( $\Phi_{FT}$ ); average distance of pollination ( $\delta$ ); effective neighborhood pollination area ( $A_{ep}$ ); and effective number of pollen donors ( $N_{ep}$ ).

**Table 5** Fertility parameters: Number of seeds per fruit, germination rate, flower production and fruit set of *V. gigantea*, in the three studied populations and years. Values shown are means  $\pm$  1 SE.

Parameters	N° of seeds			Germination (%)			Flower production			Fruit set (%)		
	2007 <sup>1</sup>	2008 <sup>2</sup>	2008 <sup>2</sup>	2005 *	2006 <sup>3</sup>	2007 <sup>4</sup>	All <sup>5</sup>	2005 *	2006 <sup>6</sup>	2007 <sup>7</sup>	All <sup>8</sup>	
Population	700.27 $\pm$ 117.93	89.75 $\pm$ 3.17 <b>a</b>	-	201.23 $\pm$ 20.66	174.08 $\pm$ 20.97	188.20 $\pm$ 14.67	-	48.21 $\pm$ 4.96 <b>b</b>	46.70 $\pm$ 5.48 <b>ab</b>	47.49 $\pm$ 3.61 <b>b</b>		
Maquiné	506.73 $\pm$ 65.25	93.6 $\pm$ 2.97 <b>a</b>	151 $\pm$ 12.49	154.13 $\pm$ 11.34	181.73 $\pm$ 13.57	160.46 $\pm$ 7.43	43.38 $\pm$ 5.41	23.27 $\pm$ 5.98 <b>c</b>	42.15 $\pm$ 4.41 <b>b</b>	37.08 $\pm$ 3.31 <b>b</b>		
Itapuã	601.35 $\pm$ 35.87	72.96 $\pm$ 6.29 <b>b</b>	-	178.00 $\pm$ 14.51	161.93 $\pm$ 10.09	169.07 $\pm$ 8.52	-	75.92 $\pm$ 5.62 <b>a</b>	63.24 $\pm$ 5.73 <b>a</b>	68.88 $\pm$ 4.16 <b>a</b>		
Taim	598.43 $\pm$ 35.18	86.81 $\pm$ 2.46	151 $\pm$ 12.49	176.05 $\pm$ 9.28	172.48 $\pm$ 8.39	169.19 $\pm$ 5.62	43.38 $\pm$ 5.41	46.59 $\pm$ 4.67	50.98 $\pm$ 3.24	47.63 $\pm$ 2.49		
All <sup>8</sup>												

<sup>1</sup>  $F = 1.76$ ,  $P = 0.1954$ ; <sup>2</sup>  $F = 6.63$ ,  $P = 0.0024$ ; <sup>3</sup>  $F = 2.42$ ,  $P = 0.1021$ ; <sup>4</sup>  $F = 0.49$ ,  $P = 0.6148$ ; <sup>5</sup>  $F = 2.00$ ,  $P = 0.1403$ ; <sup>6</sup>  $F = 21.82$ ,  $P < 0.0001$ ; <sup>7</sup>  $F = 4.93$ ,

$P = 0.0124$ ; <sup>8</sup>  $F = 18.29$ ,  $P < 0.0001$ ; \* Difference among populations not tested; † Differences among years: flower production,  $F = 1.51$ ,  $P = 0.2263$ ; and fruit set,  $F = 0.70$ ,  $P = 0.4988$ .

Figures

**Fig. 1**

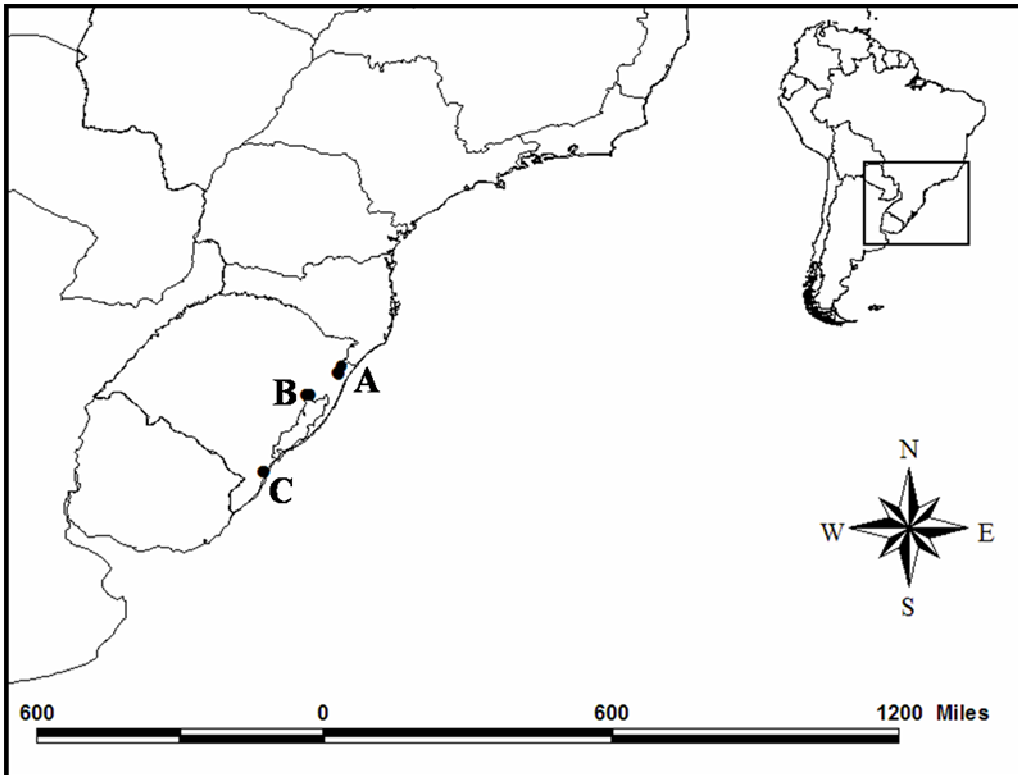
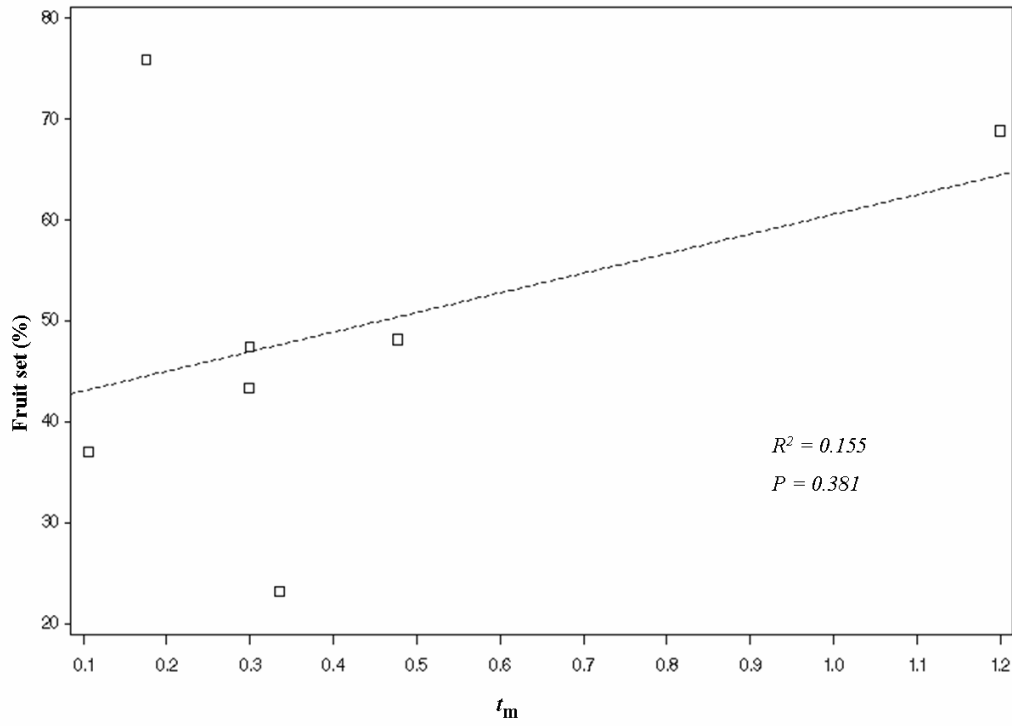


Fig. 2



## Annex

### Annex 1

Genetic variability parameters within populations of *V. gigantea* mother-plants based microsatellites loci. Number of individuals sampled (N), average number of alleles per locus (A), expected heterozygosity ( $H_e$ ) observed heterozygosity ( $H_o$ ) and P-values (P).

Populations	<i>Maquiné</i>		<i>Itapuã</i>		<i>Taim</i>		
Parameters/Years	2006	2008	2005	2006	2008	2006	2008
<i>n loci</i>	4	2	4	4	2	4	2
N	10	14	12	11	14	5	12
A	4.06	2.5	4	3.32	1.93	1.38	1.8
$H_o$	0.567	0.463	0.228	0.364	0.282	0.075	0.287
$H_e$	0.708	0.393	0.524	0.627	0.301	0.108	0.225



## **CAPÍTULO IV**

### Demography structure and seed dispersal in *Vriesea gigantea* populations (Bromeliaceae) from Southern Brazil

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A ser submetido para *Plant Ecology*

**Demographic structure and seed dispersal in *Vriesea gigantea* populations**

**(Bromeliaceae) from Southern Brazil**

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**Abstract** Population arrangement and seed dispersal of plants have great consequences to genetic structure of populations. *Vriesea gigantea* is a bromeliad from Southeastern Brazil, self-compatible and pollinator dependent for fruit set. Populations from Southern Brazil showed high fertility level, considering flowers, pollen, fruits and seeds. Previous molecular studies revealed that *V. gigantea* populations are genetically structured, with low gene flow and high selfing rates. In order to determine the importance of seed supply for gene flow, colonization, and distribution, we studied the demography and seed dispersal of *V. gigantea* populations. Demography results demonstrated that seedling recruitment is high, since 72.4% of them developed into adults, although juveniles' class experienced considerable decrease on this percentage. *Vriesea gigantea* seeds are dispersed over short distance range, since it is likely that most seeds land in the mother plants' vicinity. This pattern coincides with the reportedly aggregated distribution of bromeliad seedlings around the mother plants.

**Keywords** Atlantic Rainforest, Conservation, Gene flow, Seed recruitment

## Introduction

Population arrangement, seed dispersal patterns and mating systems of plants are key determinants influencing the genetic structure and evolutionary dynamics of natural populations (Loveless and Hamrick 1984; Levin et al. 2003). Studies to explain patterns of demography have mainly focused on environmental factors, such as light, temperature, humidity and altitude (Castro-Hernández et al. 1999; Zotz and Vollrath 2002; van Dunné

2001; Winkler et al. 2007). However, such habitat characteristics can only partly explain the observed distribution of epiphytes, which has often been described as clumped and patchy (Benzing 1981; Bennett 1986).

The pattern of seed dispersal is the most important factor influencing spatial and genetic structure within a population (Geng et al. 2008). In seed dispersal studies, the most common practice utilizes a ground network of seed traps (Greene and Calogeropoulos 2002); direct observations (Watkinson 1978) and genetic analyses (e.g., Godoy and Jordano 2001) are used much less frequently. In any approach, data interpretation should address uncertainties involved not only with dispersal, but also with predispersal (e.g., pollination, seed production, and predispersal seed loss), and postdispersal (e.g., seed predation, germination, and seedling competition) processes (Nathan and Muller-Landau 2000; Schupp and Fuentes 1995). However, knowledge of dispersal-related life-history traits and other non-physiological attributes that possibly can account for the spatial distribution of epiphytes, such as reproductive biology, breeding system, population genetics and demography, is still required for Bromeliaceae family (Soltis et al. 1987; Izquierdo and Piñero 2000; Canela and Sazima 2005; Cascante-Marín et al. 2005, 2006; Zotz et al. 2005; Cavallari et al. 2006; Barbará et al. 2007, 2008, 2009; Paggi et al. 2007; Palma-Silva 2008; Paggi et al. *in prep*).

The mating system plays also an important role in genetic variation within and among populations (Hamrick et al. 1979; Ritland 1988; Brown 1990; Lemes et al. 2007). The predominance of selfing results in low genetic diversity and high genetic structure among populations. High levels of gene flow are expected to maintain genetic cohesion among populations, whereas low levels are related to genetic differentiation among populations due to genetic drift and local selection (Bittencourt and Sebbenn 2007). Although genetic diversity of

bromeliads populations is frequently high, species show low levels of gene flow and high genetic structure among populations (e.g. *Tillandsia ionantha*, Soltis et al. 1987; *T. achyrostachys*, González-Astorga et al. 2004; *T. fasciculata*, Cascante-Marín 2005; *Alcantarea imperialis*, *A. geniculata*, *A. glaziouana*, and *A. regina*, Barbará et al. 2007, 2009; *V. gigantea*, Palma-Silva 2008).

*Vriesea gigantea* (Gaud., Tillandsioideae) is an epiphytic bromeliad that occurs in the Atlantic Rainforest, Brazil. Sazima et al. (1999) studying populations from southeastern Brazil, described *V. gigantea* as a bat-pollinated species, in addition Paggi et al. (*in prep*) reported hummingbirds and bees as *V. gigantea* pollinators in populations from southern Brazil. The anthesis time of *V. gigantea* varies greatly between populations from south, ranging from 1pm to 7pm, and flowers stay open for 12 hours (Paggi et al. *in prep*). Flowering period occur in the summer and seed dispersal during the winter and spring (Sazima et al. 1999; Paggi et al. *in prep*). Fruits are dehiscent and seeds are small with a plumose coma at one side, which enables the seed to stay in the air longer and promotes adhesion to bark-like structures (Smith and Downs 1977; Reitz 1983; Benzing 2000). As many other bromeliad species, *V. gigantea* is very appreciated as an ornamental plant and its wild populations have been destroyed or restricted to small size due to habitat destruction, fragmentation and predatory collection (Bered et al. 2008).

Paggi et al. (2007) studied the fertility and pollen limitation of *V. gigantea* in a population from Southern Brazil. The individuals produced large number of flowers, fruits, and seeds ( $\approx 18,753$  seeds in each reproductive cycle). *Vriesea gigantea* is self-compatible and showed pollinator limitation in subpopulations from Itapuã State Park, which was considered a consequence of a disruption of the bat pollination mutualism (Paggi et al. 2007), or low pollen

viability observed in Praia do Araçá (Itapuã State Park; Palma-Silva et al. 2008). Furthermore, Palma-Silva (2008) studied the patterns of genetic diversity of *V. gigantea* across its geographical distribution. The main results indicate latitudinal trend of decreasing diversity from North to South away from the equator. The mating system analyses with nuclear microsatellites revealed relatively low outcrossing rate in populations from southern Brazil pollen pool and highly genetic structured across maternal plants, indicating relatively low gene flow via pollen (Paggi et al. *in prep*).

The present study concerns an investigation of demography and seed dispersal distance of *V. gigantea*, in the Brazilian Atlantic rainforest, Itapuã State Park, Brazil. The main goal of this work was to determine the relative importance of seed supply for gene flow, colonization, and distribution of *V. gigantea*. These results allied with those obtained in the genetic structure (Palma-Silva 2008) reproductive biology (Paggi et al. *in prep*) and fertility studies (Paggi et al. 2007; Palma-Silva et al. 2008) will provide a better understanding of the species biology and will supply a diagnostic of species threat status which could help conservation programs.

## **Methods**

### Study sites

Field work was performed in the Itapuã State Park (30° 20' and 30° 27' S, and 50° 50' and 51° 05' W), Viamão, Rio Grande do Sul State (RS), southern Brazil (Fig. 1). The study area

comprises three subpopulations (1 km apart from each other) randomly selected: Trilha do Fenômeno (TF), Praia do Araçá (PA), and Morro da Grota (MG), Fig. 1. The vegetation consists of Atlantic Rainforest and the area was hardly altered by human intervention until 1970s. *Vriesea gigantea* is found in several subpopulations in the Itapuã State Park, and have been protect since 1970s, when the Park was created. Nevertheless, prior to this time, the populations of *V. gigantea* were damaged by deforestation and stone extraction.

### Demography

Population demography was analyzed sampling plots on a total area of 4 km<sup>2</sup> within Itapuã State Park. Two plots of 400 m<sup>2</sup> per populations were sampled. The populations were relatively dense and all plants within the plots were counted. Plants were divided into four classes according to their development: seedlings (until 5 cm); juveniles (6 to 30 cm); adults (>30 cm without inflorescence); and reproductive adults (with inflorescence). Data were analyzed by the ANOVA followed by the Tukey test to determine whether the differences among size classes were significant (SAS software, Version 8, SAS Institute, Cary, USA).

### Seed dispersal

Seeds were collected from August to November of 2008. Four areas in the Trilha do Fenômeno and two in the Morro da Grota subpopulation were sampled. Eight seed traps were placed per sample area, resulting in a total of 48 traps. A trap consisted of a 60X60 cm piece of 3mm plastic mesh supported at the edges by nylon lines attached with nylon cord at the

next trees. The distance between adjacent traps varied between 0.5 and 10 m. The distance of the nearest dispersing fruiting plant to each trap was recorded. Traps were monitored for seeds attachment in intervals of 10 days during four months. Trapped seeds were removed and counted in the laboratory. To test for the effect of the distance to the nearest dispersing plant on the number of seeds per trap, we divided the traps into four distance classes: (I) closer than 1 m; (II) 1 to 3 m; (III) 3 to 8 m; and (IV) more than 8 m. Two traps were placed radially in each distance class per sampled area. In order to identify differences among distance classes of seed dispersal, an ANOVA followed by the Tukey test was performed (SAS software, Version 8, SAS Institute, Cary, USA).

## **Results**

### Demography

The three studied populations exhibited all size classes: seedlings, juveniles, adults and reproductive adults. The proportion of individuals in each class was very similar among populations (Fig. 2). Adults and reproductive adults were less frequent in Araçá population, only 0.096 and 0.026 per m<sup>2</sup>, respectively. The number of seedlings in each plot ranged from 32 to 158 individuals with a mean of 85.67 individuals in 400 m<sup>2</sup> or 0.21 individuals per m<sup>2</sup> (Table 1). The juveniles size class was obtained taking into account individuals of different life-stage categories (see Methods), hence probably from different years of *V. gigantea* reproduction, consequently this class was more frequent in all populations, 0.45 per m<sup>2</sup> on



average (Table 1). Adults and reproductive adults' classes exhibited the smallest mean number of plants per m<sup>2</sup>, 0.11 and 0.05, respectively. Considering seedlings' class, 72.4% of them may develop into adults, and 29.0% may reproduce in a year. On the other hand, considering juveniles' class, only 9.4% will develop into reproductive adults.

### Patterns of seed dispersal

As expected, more seeds were trapped in the closer trap regarding a dispersing bromeliad (Fig. 3). Traps located within one meter of a dispersing plant received significantly more seeds than traps beyond a distance of one meter, on which the number of trapped seeds decreased sharply. In class (I) an average number of 51.6 ( $\pm$  19.9) seeds, was found per trap, in (II) 15.5 ( $\pm$  7.3), (III) 0.84 ( $\pm$  0.31) and in (IV) of 1.00 ( $\pm$  0.87), on average (Fig. 3). The Kruskal-Wallis test comparisons revealed a significant difference between class I and class III, and class I and IV ( $P < 0.0001$ ; Fig. 3).

## **Discussion**

### Demography and seed dispersal

The relative contribution of each life stage (size class) to the local population is an indicator of the recruitment rate. Populations with high recruitment rates will have a higher number of

individuals in the smallest size-classes (seedlings and juveniles; Cascante-Marín 2005). Conversely, populations with a low recruitment will mainly be composed of adult individuals (Oostermeijer et al. 1994). Different from many other bromeliads that reproduce asexually (Benzing 2000), *V. gigantea* produce clones, which are attached to their mother plants, so they can be easily identified. In this species the recruitment by sexual reproduction yielded a large number of seedlings, thus *V. gigantea* showed a population structure characterized by high seed recruitment in the Itapuã State Park (Table 1; Fig.2). Some species from Tillandsioideae sub-family showed equivalent feature of recruitment in Central America, *Tillandsia brachycaulos*, (Mondragón et al. 2004), *Guzmania monostachia*, *Catopsis nutans* and *T. fasciculata* (Cascante-Marín 2005), *Werauhia sanguinolenta* (Zotz et al. 2005), and *C. sessiliflora*, *T. deppeana* and *T. multicaulis* (Winkler et al. 2007).

During the seed trapping experiment a very large part of the seeds was trapped in the close neighborhood of dispersing plants. This is in agreement with earlier suggestions (Zotz et al. 1999; van Dunné 2001) and observations (García-Franco and Rico-Gray 1988; Cascante-Marín 2005) for other bromeliad species. We can not be sure that the nearest dispersing bromeliad was in fact the source of the trapped seeds but the clear negative distance-seed density correlation suggests that long distance dispersal is indeed relatively rare, as predict by models on wind dispersal (Greene and Johnson 1989; Bullock and Clarke 2000). In agreement, the branches and stems of the host tree as well as other plants directly around dispersing bromeliads were often covered with seeds (Fig. 4A). Moreover, it is likely that a considerable part of the seeds never becomes airborne. Frequent rains in the winter season, cause the seeds to cluster and form masses of seeds that are deposited either on the own inflorescence or on vegetative parts of the mother plants, where they sometimes even

germinate (Fig.4B). Similar observations were reported in a study of seed dispersal patterns of Tillandsioideae species, *Guzmania monostachia*, *Tillandsia fasciata*, *T. tricolor* and *Werauhia gladioliflora* (Cascante-Marín 2005). The tendency of bromeliad seedlings to establish near their mother plants suggest that seed dispersal mostly occurs in the same host trees (Benzing 1978; Zotz 1997; van Dunné 2001; Cascante-Marín 2005). *Vriesea gigantea* has small seeds that feature a hairy appendage at one end which enhances air buoyancy and dispersal by wind (Benzing 2000). Nevertheless, wind-dispersed seeds of epiphytic bromeliads apparently do not disperse very far from the mother plant (García-Franco and Rico-Gray 1988).

#### Gene flow

Gene flow within and among populations is usually affected by pollen and propagule dispersal (Geng et al. 2008). Indirect measure of gene flow by genetic studies of several bromeliad species revealed that pollen dispersal is greater than propagule dispersal, confirming the studies concerning seed dispersal and demography, discussed above. These studies reported low gene flow and high population genetic structure for different bromeliad species, which may be considered as reproductive system consequences (Sarhou et al. 2001; Sgorbati et al. 2004; Barbará et al. 2007, 2008; Cascante-Marín 2005), long-term effects of fragmentation (Soltis et al. 1987; Sarhou et al. 2001; González-Astorga et al. 2004; Barbará et al. 2007, 2008), or species dependence upon host availability (González-Astorga et al. 2004).

Results of seed dispersal observed in this study contribute to understand the genetic patterns observed in *V. gigantea* populations. The previous results on *V. gigantea* obtained by our research team have revealed that this species is self-compatible, with low gene flow

among populations (Palma-Silva 2008), and high levels of selfing rate due to pollinator behavior (bees and hummingbirds), high pollen structure (Paggi et al. *in prep*) and experiences pollen limitation in one of the southern populations (Paggi et al. 2007). Accordantly, Palma-Silva (2008) estimated a ratio of 3.3 of pollen flow to seed flow indicated an asymmetry between them, being gene flow via seed less efficient than via pollen, resulting in a stronger genetic structure for chloroplast genome than nuclear genome. This pattern of gene flow is also confirmed by comparing the maximum seed dispersal distance (about 8m) with the results from pollen genetic structure analyses (Paggi et al. *in prep*), which estimated an average distance of pollination of 147m. Moreover, Paggi et al. (*in prep*) observed a moderate level of biparental inbreeding, that could be explained by pollinators' behavior or by the patterns of seed dispersal distance and the agglomerate distribution of epiphytes (García-Franco and Rico-Gray 1988).

#### Conservation outcome

In this study we observed high seed recruitment in *V. gigantea* populations, thus population persistence strongly relies on the survival of adult plants. Consequently harvesting bromeliads for ornamental or other purposes should be avoided or restricted to immature individuals. Fertility studies, including populations from Southern edge of the distribution, revealed that these populations are fertile, though Itapuã subpopulation shows strong evidences of pollen limitation, considering qualitative (pollen viability) and quantitative (pollinator limitation) components (Paggi et al. 2007; Palma-Silva et al. 2008). Conservations programs including *V.*

*gigantea* and also other bromeliad species, should taking into account reproductive biology, fertility, and demographic parameters in order to maintain the poplato evolutionary potential.

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## Legends of figures

**Fig. 1** Studied populations of *V. gigantea* in Southern Brazil: (A) Itapuã State Park, Rio Grande do Sul State; (B) Itapuã State Park – Trilha do Fenômeno (TF), Praia do Araçá (PA), and Morro da Grota (MG).

**Fig. 2** Size classes distribution of *V. gigantea* in the three studied populations: Praia do Araçá (PA), Trilha do Fenômeno (TF), and Morro da Grota (MG), from Itapuã State Park, Rio Grande do Sul, Brazil.

**Fig. 3** Average number of *V. gigantea* seeds per trap in four distance classes from the nearest dispersing plant. Error bars represent  $\pm$  one standard error.

**Fig. 4** (A) Branches and stems of a host tree covered with *V. gigantea* seeds. (B) *V. gigantea* seeds clustered and deposited on the own inflorescence parts of the mother plants.

**Tables**

**Table 1** Mean number of plants per plot and per  $m^2$  in different size classes  $\pm$  SE = Standard error.

<i>Classes</i>	<i>400 m<sup>2</sup></i>	<i>1 m<sup>2</sup></i>
Seedlings	85.7 $\pm$ 17.7	0.21 $\pm$ 0.04 ab
Juveniles	178.7 $\pm$ 47.6	0.45 $\pm$ 0.12 a
Adults	44.0 $\pm$ 11.3	0.11 $\pm$ 0.03 b
Reproductive adults	17.8 $\pm$ 5.9	0.05 $\pm$ 0.02 b

a and b – Means with the same letter are not significantly different by the Tukey Test (5%).

Figures

Fig. 1

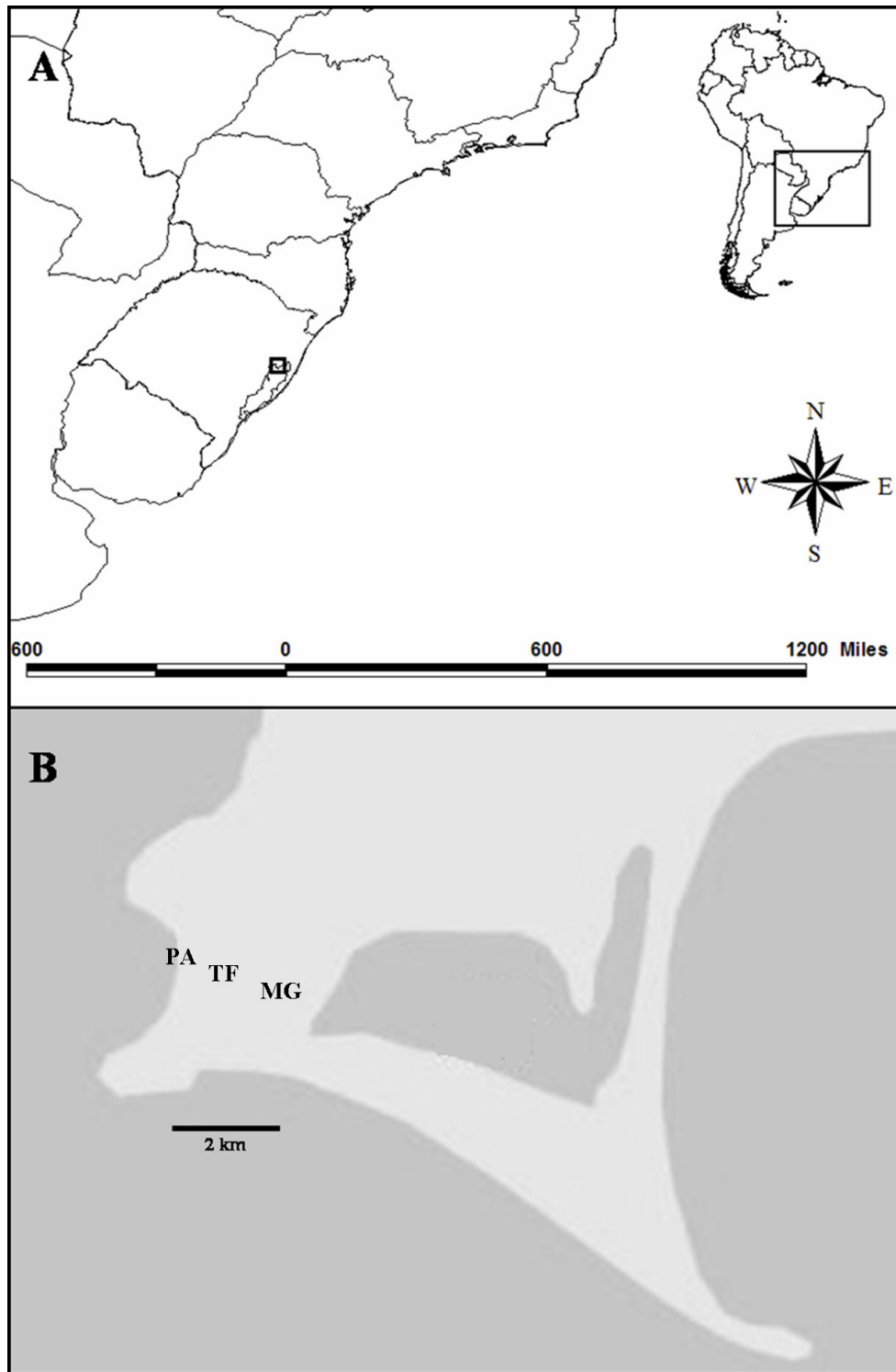


Fig. 2

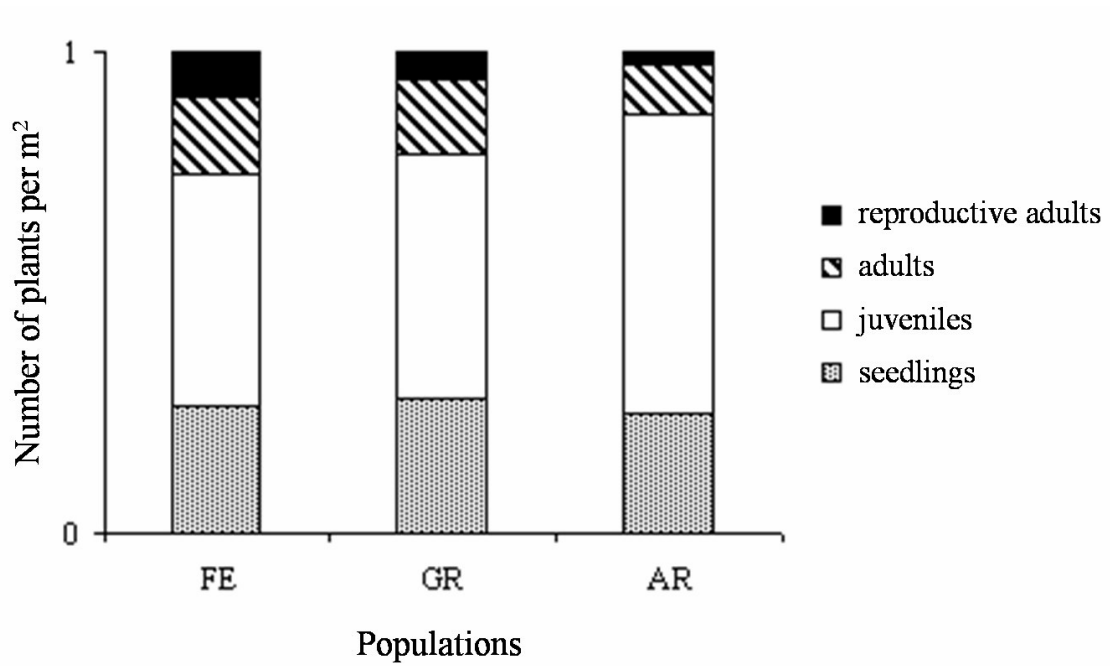
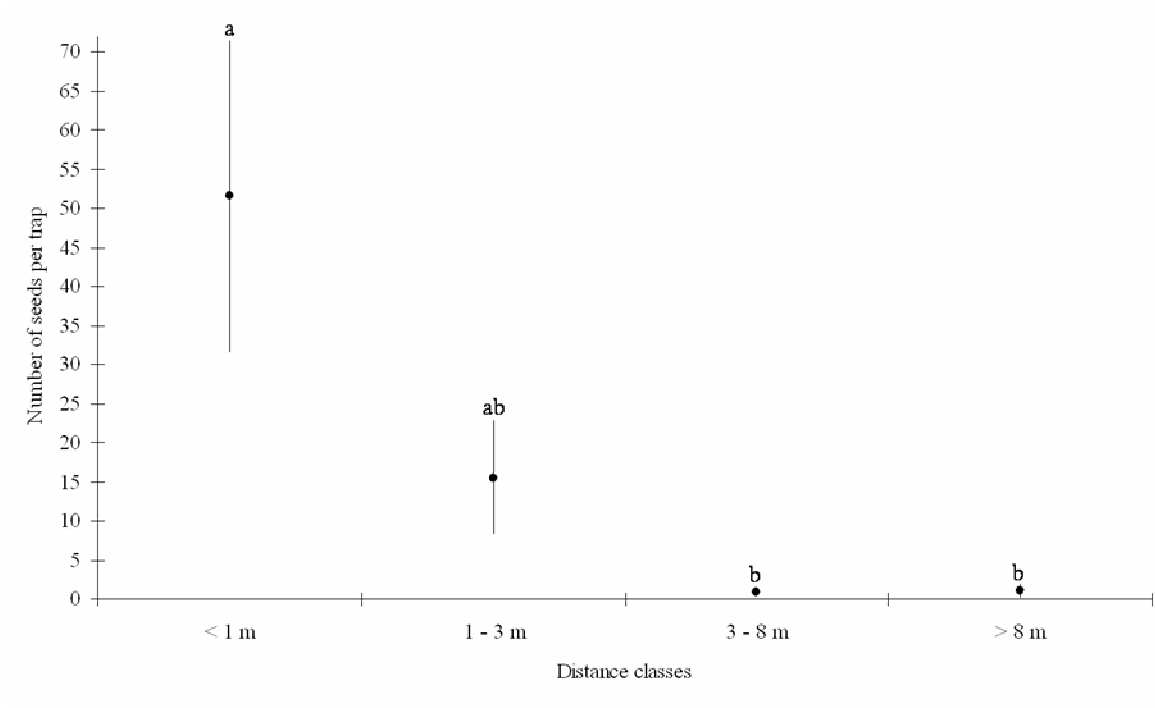
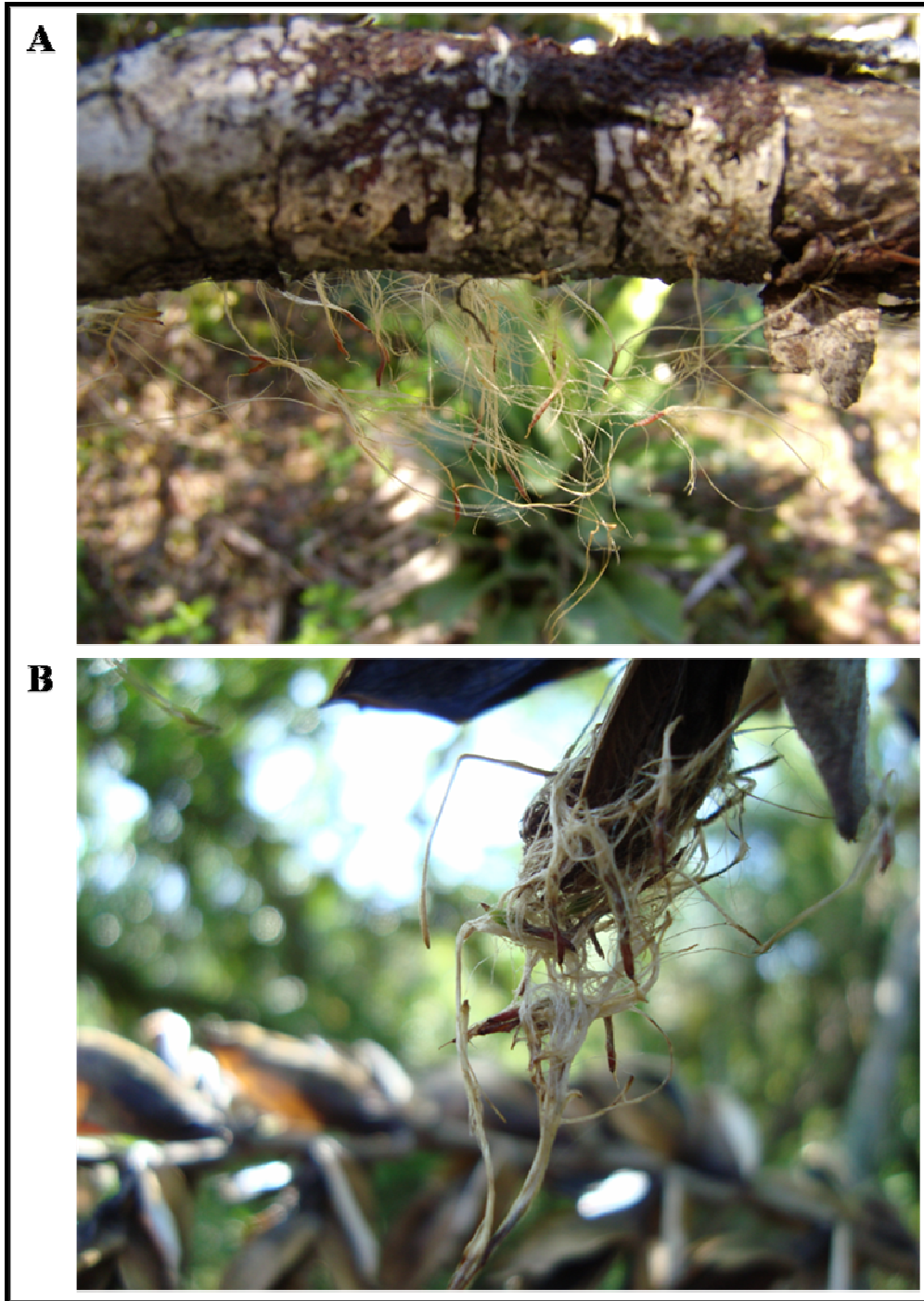


Fig. 3





**Fig. 4**



## **CAPÍTULO V**

### **Considerações Finais**

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## Considerações Finais

A presente tese está dividida em três artigos relacionados a um projeto amplo que tem como objetivo contribuir para o entendimento de questões relacionadas à evolução de famílias de plantas neotropicais, com ênfase na família Bromeliaceae. O conjunto de dados obtidos nestes trabalhos descreve um panorama inicial que pode contribuir para a compreensão da evolução do sistema de cruzamento em bromélias, principalmente daquelas espécies que podem sofrer efeitos de borda, seja devido ao seu padrão de distribuição, no caso de espécies epífitas, seja devido à fragmentação do habitat, no caso da Mata Atlântica. Ainda, este estudo contribuirá para o esclarecimento dos padrões de diversidade e estrutura genética das populações de *V. gigantea*, relatados por Palma-Silva (2008), e para o desenvolvimento de estratégias de conservação e manejo das populações de *V. gigantea* e de espécies relacionadas.

Sendo uma bromélia endêmica da Mata Atlântica, e também uma espécie alvo de coleta predatória, *V. gigantea* se tornou objeto deste estudo devido à ameaça que suas populações vêm sofrendo. Nos primeiros estudos, foi identificado que *V. gigantea* é uma espécie autocompatível, produzindo um grande número de flores, frutos e sementes viáveis (Paggi *et al.*, 2007). Além disso, os estudos moleculares revelaram que as populações de *V. gigantea* estão estruturadas, com baixo fluxo gênico e altas taxas de endocruzamento (Palma-Silva, 2008). Com o objetivo de entender os padrões genéticos (estrutura de populações) e ecológicos (fertilidade) das populações de *V. gigantea*, uma investigação mais aprofundada de sua biologia reprodutiva se fez necessária.

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. Embora a maioria das bromélias ocorra em áreas com altos índices de devastação, padrões de sistemas de cruzamento e fertilidade em populações de bromélias ainda foram avaliados em conjunto com a finalidade de entender as conseqüências para a evolução, manutenção e conservação das espécies.

O estudo da biologia reprodutiva de *V. gigantea* (Capítulo II) identificou que ela é uma espécie não-autogâmica e dependente de polinizadores para produção de frutos. Neste contexto, a observação dos polinizadores, ajudou a identificar beija-flores e abelhas como os principais polinizadores nas populações amostradas. Entretanto, morcegos foram descritos como polinizadores de *V. gigantea* em populações de São Paulo (Sazima *et al.*, 1999). O comportamento territorial de abelhas e beija-flores auxiliou a explicar os resultados obtidos com as análises de progênie, os quais mostraram que a taxa de fecundação cruzada em *V. gigantea* é de baixa a moderada, com altos níveis de autofecundação, compatível com um sistema misto de cruzamento (Lande e Schemske, 1985). Além disso, as taxas de fecundação cruzada apresentaram uma tendência geográfica, decrescendo do Norte para o Sul, contrastando com a estrutura genética do “pool” de pólen, que aumenta nesta direção, o que também está de acordo com a diminuição norte-sul do fluxo gênico nestas populações, observada por Palma-Silva (2008). Os padrões observados nesta tese foram formados possivelmente pelo comportamento dos polinizadores e provavelmente podem sofrer influência da fragmentação do habitat, o que leva a uma diminuição do fluxo gênico e aumento do endocruzamento (Anexos I e II).

Segundo o modelo para evolução em direção à autofertilização, descrito por Porcher e Lande (2005), sob limitação de pólen (devido à baixa frequência de polinizadores ou baixa densidade de plantas) e desconto de pólen (redução do sucesso reprodutivo masculino, devido à diminuição de pólen exportado), mesmo com bases genéticas mais realísticas para depressão por endocruzamento e limitação de pólen, sistemas mistos de cruzamento não podem ser explicados pelos efeitos opostos da depressão por endocruzamento e da vantagem automática da autofecundação sem adicionar características da biologia da polinização. Portanto, os dados supramencionados associados à limitação de pólen observada na população de Itapuã (Paggi *et al.*, 2007), contribuem para a compreensão de quais fatores biológicos estão influenciando na manutenção de um sistema misto de cruzamento em *V. gigantea*. Tais fatores correspondem à: limitação de pólen (Paggi *et al.*, 2007), comportamento de polinizadores (Capítulo II), desconto de pólen (altos valores para  $\Phi_{FT}$ , Capítulos II e III), moderados índices de depressão por endocruzamento (Capítulo II), e altas taxas de autofecundação (Capítulos II e III; Anexos I e II).

Nos estudos de fertilidade (Paggi *et al.*, 2007; Capítulo III) foi observada grande produção de flores, frutos e sementes, e uma alta viabilidade de sementes, com algumas diferenças entre populações. Esses resultados sugerem que os padrões de fertilidade e parâmetros de sistemas de cruzamento entre populações de *V. gigantea* podem ser atribuídos à adaptação da espécie para o endocruzamento e efeitos de borda, embora outras explicações adaptativas não possam ser ignoradas. Considerando a variação espacial-temporal em sistemas de cruzamento e fertilidade, e os resultados de fluxo gênico, as populações das bordas da distribuição de *V. gigantea* também devem ser consideradas em programas de conservação da espécie.

O papel da dispersão e recrutamento de sementes para o fluxo gênico, colonização, e distribuição de populações de *V. gigantea* também foi investigado com o objetivo de contribuir para a compreensão do panorama geral já abordado anteriormente (Capítulo IV). Os resultados mais relevantes referem-se à dispersão das sementes. As sementes de *V. gigantea* são dispersas a pequenas distâncias, logo, a maioria das sementes permanece na vizinhança da planta mãe (Anexos I e II). Esse padrão coincide com a distribuição agregada das plântulas de diferentes espécies de bromélias, as quais permanecem em torno da planta mãe. Além disso, o padrão de dispersão de sementes de *V. gigantea* observado no Capítulo IV, coincide com os resultados obtidos em análises da estrutura filogeográfica e fluxo gênico histórico, utilizando microsatélites de cloroplastos, as quais revelaram uma assimetria entre o fluxo de pólen e sementes, sendo o fluxo gênico via semente menos eficaz do que via pólen (Palma-Silva, 2008).

Em suma, os resultados descritos nos capítulos que compreendem esta tese correspondem a mais um degrau de conhecimento para que haja uma compreensão efetiva dos processos biológicos, ecológicos e evolutivos envolvidos principalmente no sistema de cruzamento em plantas, utilizando *V. gigantea* como modelo.

## **CAPÍTULO VI**

### **Referências Bibliográficas dos Capítulos I e V**

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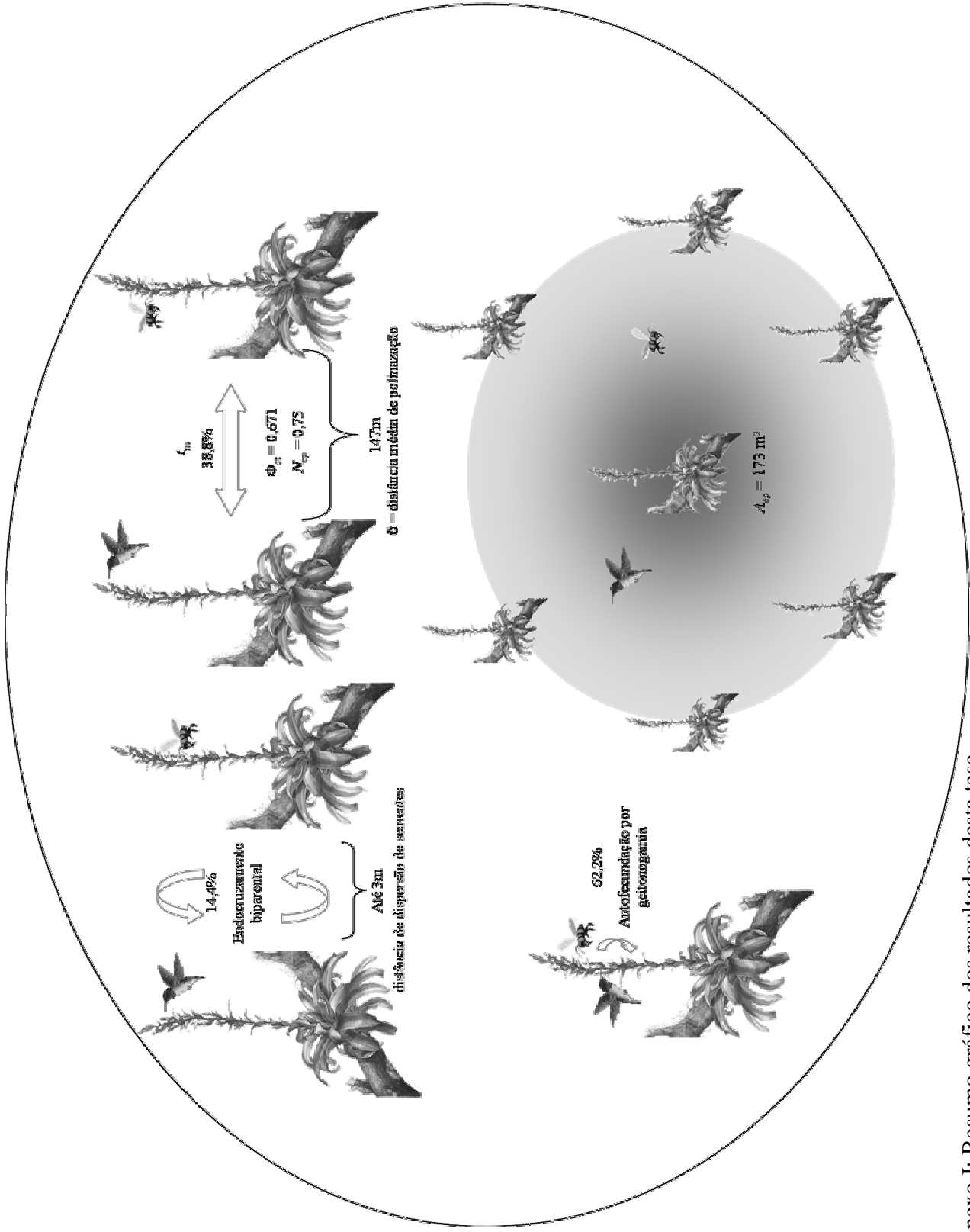
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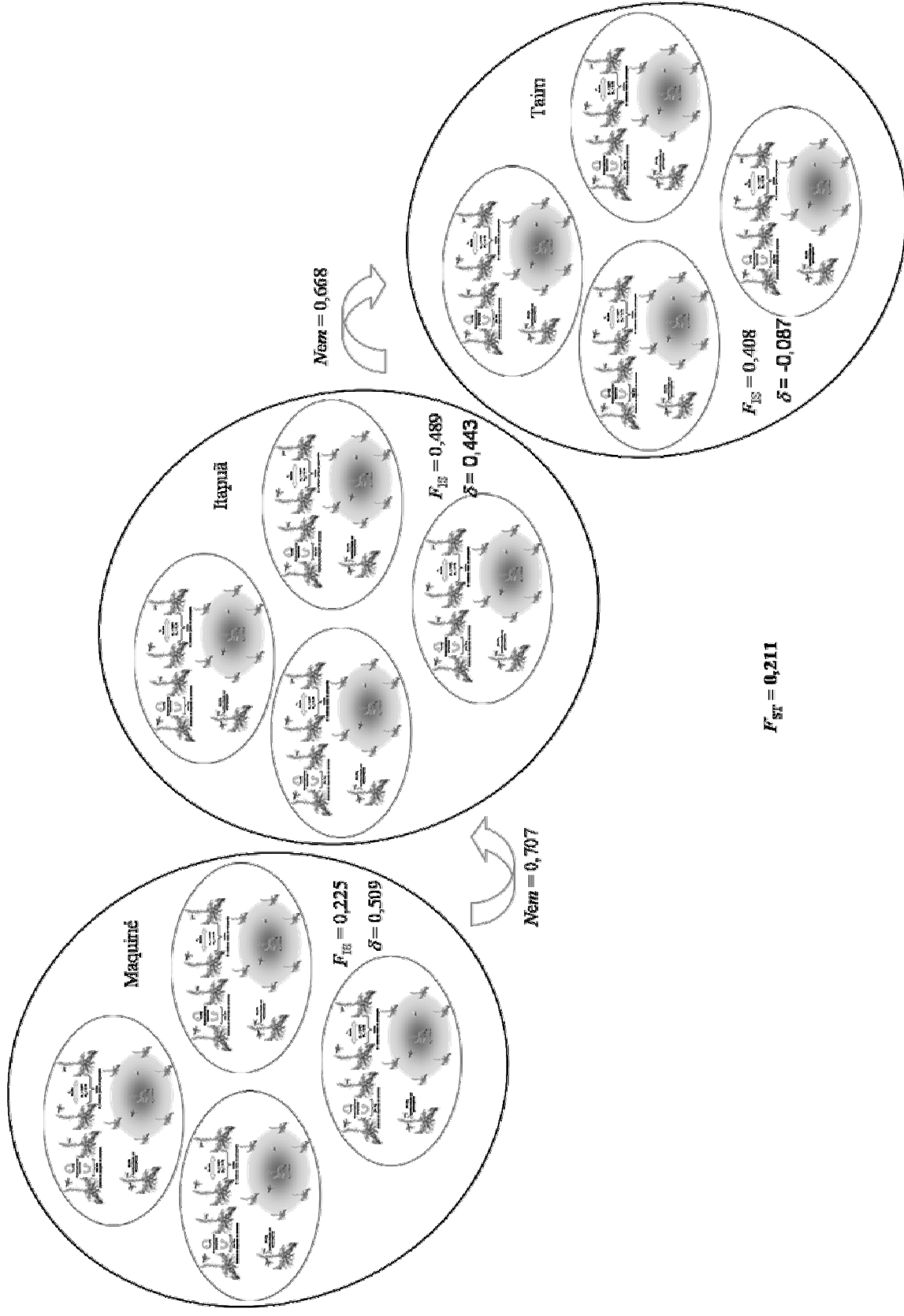
**ANEXOS**

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Anexo I: Resumo gráfico dos resultados desta tese.



Anexo II: Resumo gráfico dos resultados de análises da estrutura genética de populações (Palma-Silva, 2008) e de depressão por endocruzamento desta tese.