



Sucesso reprodutivo, diversidade genética e fluxo de pólen de Dyckia distachya Hassler (Bromeliaceae), uma espécie altamente ameaçada de extinção

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*“Uma vez que nós aceitamos nossos limites,  
vamos além deles.”*

*Albert Einstein*

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**RESUMO**

*Dyckia distachya* Hassler é uma espécie rara, endêmica e exclusiva de ambientes reofíticos. Originalmente, essa bromélia ocorria em ilhas e margens rochosas da bacia do rio Uruguai, possuindo distribuição disjunta ao longo de 617 km, nos rios Pelotas e Uruguai, na divisa dos estados do Rio Grande do Sul e de Santa Catarina. Entretanto, devido ao aproveitamento do potencial hidrelétrico dessa bacia hidrográfica, sete das oito populações de *D. distachya* foram extintas, sendo conhecida atualmente apenas uma população na natureza. Visando a preservação dessa espécie, indivíduos foram coletados durante a construção das hidrelétricas e mantidos em coleções *ex situ*. Posteriormente, parte desses indivíduos resgatados foi introduzida em locais semelhantes aos de sua ocorrência natural. Neste contexto, este estudo foi realizado com o objetivo de avaliar o sucesso reprodutivo e aspectos genéticos da única população natural e de populações introduzidas, para obter informações que auxiliem na elaboração de estratégias de conservação para essa espécie. No **Capítulo II** é apresentado o estudo do sucesso reprodutivo da única população natural conhecida e de quatro populações introduzidas de *D. distachya*. Também foram avaliados a produção média de flores, frutos e sementes; o número médio de indivíduos reprodutivos e vegetativos; a germinação das sementes e a viabilidade do pólen; o tamanho médio dos *genets* e o número médio de *ramets* por *genet*. Os resultados mostraram diferenças significativas entre as populações introduzidas e a natural para todas as variáveis analisadas, sendo observado maior investimento em reprodução sexual nas populações introduzidas e maior investimento em propagação clonal na população natural. A produção média de flores, frutos e sementes foi superior nas populações introduzidas (34,1; 37,5; 87; respectivamente) quando comparadas com a população natural (2,7; 5,9; 31; respectivamente). A viabilidade do pólen também foi superior nas populações introduzidas (92 %) em relação a natural (47,9 %). Entretanto, o número médio de *ramets* por *genet* e o tamanho médio dos *genets* foi superior na população natural (297,6 e 2,172 m<sup>2</sup>, respectivamente) quando comparado com a média das populações introduzidas (5,9 e 0,122 m<sup>2</sup>, respectivamente). A introdução dessa

bromélia deve ser levada em consideração em programas de conservação, visto que as populações introduzidas estudadas parecem estar conseguindo se estabelecer em seu novo habitat, tendo sido observado inclusive o recrutamento de novos indivíduos através de sementes. No **Capítulo III** foram investigados a diversidade genética, o sistema de cruzamento e o fluxo de pólen da espécie em estudo, nas quatro populações introduzidas e na natural, utilizando marcadores moleculares microssatélites. Para as cinco populações, foram coletadas amostras de 130 indivíduos reprodutivos, das quais 50 foram utilizadas como plantas-mãe, provendo 700 plântulas. As populações introduzidas apresentaram altos níveis de diversidade genética ( $\widehat{H}_O = 0,466$ ) comparadas com a natural ( $\widehat{H}_O = 0,167$ ). A estimativa da taxa de fecundação cruzada nas populações introduzidas não diferiu de um ( $\widehat{t}_m = 0,965$ ), indicando que *D. distachya* é uma bromélia alógama. Porém, na população natural, esse valor foi diferente ( $\widehat{t}_m = 0,520$ ), sugerindo a ocorrência de algum tipo de perturbação nesse ambiente. O fluxo de pólen é mais restrito na população natural ( $\widehat{\phi}_{FT} = 0,296$ ) do que nas introduzidas ( $\widehat{\phi}_{FT} = 0,089$ ). A dispersão de pólen ocorre a curtas distâncias, parecendo ser influenciada pela combinação da densidade e da distribuição espacial dos indivíduos reprodutivos. Baseado nestes resultados, sugerimos que os índices de diversidade genética e fluxo de pólen sejam monitorados em longo prazo, na tentativa de evitar a redução da diversidade genética, a seleção local e a deriva genética. Além disso, recomendamos a criação de novas áreas de introdução, intermediárias as existentes, promovendo a formação de metapopulações. Um estudo mais aprofundado na população natural também deve ser realizado para saber o que realmente está ocorrendo naquele ambiente para serem tomadas medidas efetivas de conservação.

**Reproductive success, genetic diversity and pollen flow of *Dyckia distachya* Hassler (Bromeliaceae), a species highly endangered**

**ABSTRACT**

*Dyckia distachya* Hassler is a rare, endemic and exclusive species of rheophytic environments. Originally, this bromeliad occurred on islands and rocky shores of the Uruguay River basin, having disjunct distribution over 617 km in Pelotas and Uruguay rivers, bordering the states of Rio Grande do Sul and Santa Catarina. However, due to the exploitation of the hydroelectric potential of this basin, seven of the eight populations of *D. distachya* were extinguished, being currently known only one population in nature. Aiming the preservation of this species, individuals were collected during the construction of hydroelectric and maintained in ex situ collections. Later, some of these rescued individuals were introduced in similar places to their natural occurrence. In this context, this study was carried out to evaluate the reproductive success and genetic aspects of the only natural population and introduced populations, to obtain information to assist in the development of conservation strategies for this species. In **Chapter II** is presented the study of reproductive success of the only known natural population and four introduced populations of *D. distachya*. Were also evaluated the average production of flowers, fruits and seeds; the average number of reproductive and vegetative individuals; seed germination and pollen viability; the average size of genets and the average number of ramets per genet. The results showed significant differences between natural and introduced populations for all variables analyzed, being observed higher investment in sexual reproduction in the introduced populations and greater investment in clonal propagation in the natural. The average production of flowers, fruits and seeds were higher in introduced populations (34.1; 37.5; 87; respectively) when compared with the natural population (2.7; 5.9; 31; respectively). Pollen viability was also higher in introduced populations (92 %) in relation to natural (47.9 %). However, the average number of ramets per genet and the average size of the genets were higher in the wild population (297.6 and 2,172 m<sup>2</sup>, respectively) when compared to the average of the introduced populations (5.9 and 0.122 m<sup>2</sup>, respectively). The introduction of this bromeliad should be considered in conservation programs, since the introduced populations studied seem to be getting to

settle in their new habitat, having been observed including the recruitment of new individuals from seeds. In **Chapter III** were investigated the genetic diversity, mating system and pollen flow of this species in the four introduced populations and natural one, utilizing microsatellite markers. For the five populations, samples from 130 reproductive individuals were collected, of which 50 were used as mother plants, providing 700 seedlings. Introduced populations showed high levels of genetic diversity ( $\widehat{H}_O = 0.466$ ) compared with the natural ( $\widehat{H}_O = 0.167$ ). The estimated outcrossing rate in introduced populations did not differ from one ( $\widehat{t}_m = 0.965$ ), indicating that *D. distachya* is an allogamous bromeliad. However, in natural population, this value was different ( $\widehat{t}_m = 0.520$ ), suggesting the occurrence of some kind of disturbance in this environment. Pollen flow is more restricted in natural population ( $\widehat{\phi}_{FT} = 0.296$ ) than in introduced ( $\widehat{\phi}_{FT} = 0.089$ ). The pollen dispersal occurs over short distances, appearing to be influenced by the combination of density and spatial distribution of reproductive individuals. Based on these results, we suggest that the rates of genetic diversity and pollen flow should be monitored in the long term, in an attempt to avoid a reduction in genetic diversity, site selection and genetic drift. Additionally, we recommend the creation of new areas of introduction, intermediate of those already existing, promoting the formation of metapopulations. A further study in natural population must also be done to find out what is really occurring in that environment for effective conservation measures are taken.



## Capítulo I

### *Introdução Geral*

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## INTRODUÇÃO GERAL

### 1. *Conservação de espécies*

Estima-se a existência de 250 a 420 mil espécies de plantas no mundo (Prance *et al.*, 2000; Govaerts, 2001; Bramwell, 2002; Thorne, 2002; Scotland e Wortley, 2003; Wortley e Scotland, 2004; Joppa *et al.*, 2011; Mora *et al.*, 2011), sendo que aproximadamente um quarto delas está correndo um sério risco de extinção (Pitman e Jorgensen, 2002; Millennium Ecosystem Assessment, 2005; Smith *et al.*, 2011). Além disso, muitas espécies podem se tornar extintas antes mesmo de serem descritas, principalmente as que apresentam distribuição geográfica restrita e ocorrem em locais menos explorados (Mora *et al.*, 2011). Essas espécies são fundamentais do ponto de vista da conservação, pois podem possuir ecótipos geneticamente distintos, extremamente importantes para a proteção do potencial evolutivo das espécies (Frankham *et al.*, 2009).

Considerando os critérios da União Internacional para a Conservação da Natureza (IUCN, 2008) uma espécie é considerada *extinta* quando não há dúvidas de que o último indivíduo desapareceu e *extinta na natureza* quando é conhecida apenas por sobreviver em cativeiro ou como uma população naturalizada fora de sua área de ocorrência natural. Ainda, uma espécie *ameaçada* é aquela que corre um alto risco de extinção na natureza.

A perda ou a degradação dos habitats, a introdução de espécies exóticas, a poluição e as rápidas mudanças climáticas são os principais fatores que ameaçam uma espécie (Secretariat of the Convention on Biological Diversity, 2010; IUCN, 2013; Gauthier *et al.*, 2013). A fragmentação dos habitats representa uma ameaça para muitos ecossistemas (Young *et al.*, 1996), principalmente devido à possibilidade de restrição do fluxo gênico entre indivíduos de uma população, favorecendo a ocorrência de cruzamentos endogâmicos e levando a redução da diversidade genética (Frankham *et al.*, 2009). A fragmentação muitas vezes vem acompanhada da redução do tamanho populacional, ocasionando um gargalo genético, onde os indivíduos remanescentes apresentarão apenas uma amostra do *pool* gênico original (Young *et al.*, 1996). Aliado a isso, quanto menor for o número de indivíduos no local de ocorrência de uma determinada espécie, maior é a chance dela se tornar extinta devido a fatores estocásticos (Frankham *et al.*, 2008; Gauthier *et al.*, 2010).



As populações com um pequeno número de espécimes apresentam maior vulnerabilidade à perda da diversidade genética, devido ao aumento da endogamia estar relacionada fundamentalmente ao tamanho efetivo populacional (Frankham, 2005). Essas populações que permanecem pequenas e isoladas por diversas gerações também poderão perder alelos em decorrência da deriva genética. A diminuição da heterozigosidade e o aumento da endogamia podem resultar no acúmulo de alelos deletérios, provocando uma diminuição nas taxas de fecundidade, aumento da mortalidade e redução da taxa de crescimento das plantas (Young *et al.*, 1996).

A redução da heterozigosidade leva a uma diminuição do valor adaptativo e da viabilidade das populações fragmentadas, com isso, espera-se uma menor proporção de locos polimórficos e uma redução do número de alelos por loco nessas populações (Young *et al.*, 1996). Por outro lado, a redução da diversidade genética observada em longo prazo, geralmente está associada com as respostas da espécie em relação às mudanças impostas pelas pressões de seleção, podendo aumentar a probabilidade de extinção de uma espécie (Young *et al.*, 1996; O'Grady *et al.*, 2006).

Devido aos fatores mencionados acima, a manutenção da diversidade genética deve ser o principal objetivo das ações de conservação, sendo esta necessária para a evolução das espécies (Frankham, 2010). A conservação dos recursos genéticos pode ocorrer através da preservação de amostras fora do seu habitat natural, conhecido como conservação *ex situ*, e/ou através da proteção das populações em seus locais de ocorrência, chamado de conservação *in situ* (Cohen *et al.*, 1991; Frankham *et al.*, 2009). As espécies também podem ser preservadas em condições ambientais semelhantes ao seu habitat, quando seu ambiente natural estiver ameaçado ou for destruído, denominada de conservação *inter situ* (Guerrant *et al.*, 2004). As diferentes formas de conservação devem manter populações geneticamente distintas na tentativa de evitar a perda da diversidade genética de uma espécie (Thornton *et al.*, 2008).

Muitas vezes as espécies ameaçadas precisam de mais de um método de conservação para reduzir os riscos de extinção (Yokogawa *et al.*, 2013), sendo eles complementares uns aos outros (Cohen *et al.*, 1991). A conservação *ex situ* é importante para a manutenção da diversidade genética (Guerrant *et al.*, 2004), sendo capaz de gerar conhecimento para as medidas *in situ* e/ou *inter situ* (Cochrane, 2004) e fornecer material para ser utilizado em programas de translocação de espécies (Guerrant *et al.*, 2004; Frankham *et al.*, 2008;

Godefroid *et al.*, 2011). A qualidade da amostragem dos indivíduos para a conservação *ex situ* é de grande relevância, pois deve garantir a representatividade do material em relação à diversidade da espécie (Frankham *et al.*, 2008).

A translocação também é uma importante ferramenta de conservação *in situ* e/ou *inter situ* (Godefroid *et al.*, 2011), referindo-se a qualquer movimento de organismos vivos de uma área para outra, sendo reconhecidos três tipos: (a) *introdução* é a dispersão intencional ou acidental de um organismo fora do seu alcance natural; (b) *reintrodução* é o movimento intencional de um organismo para um local da sua área de distribuição natural que tenha desaparecido ou sido extinto; e (c) *repopoamento* é o movimento intencional de indivíduos com o objetivo de reconstituir uma população existente (IUCN, 1987).

A introdução pode ser vista como uma possibilidade de conservação quando não há mais locais adequados para que seja realizada a reintrodução de uma população ou espécie em sua área de distribuição natural (IUCN, 2013). Portanto, o termo introdução expressa o estabelecimento de uma população ou espécie em um novo ecossistema, semelhante ao seu ambiente de ocorrência natural (IUCN, 2013), determinando o método de conservação *inter situ* (Guerrant *et al.*, 2004).

Os diferentes tipos de translocação têm como objetivo principal o estabelecimento de populações autossustentáveis, com ampla diversidade genética e elevado *fitness* reprodutivo (Frankham *et al.*, 2009). O sucesso de uma translocação pode ser avaliado através da capacidade de realização dos processos básicos do ciclo de vida de uma planta, tais como, o estabelecimento, a reprodução e a dispersão, devendo ser levado em consideração o recrutamento de novos indivíduos através de sementes (Pavlik, 1996). As questões genéticas também são fundamentais para a obtenção de êxito, devendo ser avaliada a depressão endogâmica, a viabilidade reprodutiva, a adaptação local, o efeito fundador e o potencial evolutivo das populações translocadas (Menges, 2008).

As translocações devem ser realizadas com muito cuidado, visando evitar a ocorrência de gargalos genéticos. Isso é particularmente importante se a população doadora encontra-se nessa condição, se uma pequena parte das populações contribuiu com a maioria dos propágulos ou se o tamanho da população introduzida é muito pequeno (Hufford e Mazer, 2003). A situação ideal de diversidade genética é obtida quando uma população translocada contém níveis de diversidade semelhantes ao da população selvagem (Menges, 2008).

A escolha das áreas onde serão realizadas as translocações merece uma atenção especial, pois as populações translocadas terão de se adaptar as condições do novo ambiente, estando suscetíveis a herbivoria e a competição com outras espécies (Menges, 2008; Godefroid *et al.*, 2011). Indivíduos mal adaptados ao novo ambiente poderão ter sua taxa de sobrevivência reduzida, podendo comprometer o sucesso da translocação (Thornton *et al.*, 2008). O monitoramento contínuo e o manejo dessas novas populações são necessários para garantir o estabelecimento e a persistência das plantas, principalmente das espécies que demoram a alcançar a maturidade reprodutiva (Cochrane, 2004).

Estudos envolvendo fertilidade, viabilidade, estruturação genética e sucesso reprodutivo das populações translocadas em comparação com as naturais, quando possível, servem como uma ferramenta importante no processo de conservação de espécies ameaçadas de extinção.

## **2. A bela família Bromeliaceae**

A família Bromeliaceae Juss. é composta por plantas perenes que possuem uma beleza singular, apresentando uma ampla variedade de formas, cores e tamanhos (Benzing, 2000). As espécies são típicas do continente Americano, sendo encontradas desde os estados da Virgínia, Texas e Califórnia, nos Estados Unidos (latitude 37°) até o norte da Patagônia, na Argentina (latitude 44°), tendo como exceção a espécie *Pitcairnia feliciana* (A. Chev.) Harms e Mildbr., descoberta no Oeste da África, na região da Guiné (Porembsky e Arthlott, 1999), parecendo ser o resultado de um evento recente de dispersão a longa distância (Givnish *et al.*, 2004; 2011).

As espécies dessa família ocupam distintos habitats, podendo ser terrestres, rupícolas, epifíticas, saxícolas ou reófitas, variando de plantas delicadas e de pequeno porte, como *Tillandsia recurvata* (L.) L., com alguns centímetros de comprimento, até plantas de grande porte, como *Puya raimondii* Harms, encontrada nos Andes, chegando a alcançar mais de 10 metros de altura (Smith e Downs 1974; Reitz 1983). As bromélias não são plantas parasitas, pois suas raízes têm a função de fixação e absorção de água (Coffani-Nunes, 2002). As plantas podem ser encontradas desde o nível do mar até elevadas altitudes (4.000 m), em regiões desérticas e úmidas, em locais propensos a inundações

regulares e com baixa ou alta luminosidade. Podem ainda florescer em temperaturas elevadas ou em temperaturas próximas a 0 °C (Benzing, 2000).

Existem três centros de diversidade para a família Bromeliaceae, uma na região norte dos Andes até o México e as Antilhas, outra no Planalto das Guianas e a terceira no leste do Brasil (Smith e Downs, 1974). Essa família compreende aproximadamente 3.140 espécies, distribuídas em 58 gêneros e divididas em oito subfamílias: Brocchinioideae, Bromelioideae, Hechtioideae, Lindmanioideae, Navioideae, Pitcairnioideae, Puyoideae e Tillandsioideae (Givnish *et al.*, 2007, 2011). Aproximadamente metade das espécies de bromélias pode ser encontrada no Brasil (Leme, 1997), principalmente na Floresta Atlântica (Reitz, 1983; Rundel e Dillon, 1998), tornando o país o mais importante centro de diversidade desse grupo (Leme, 1997).

Entretanto, a Floresta Atlântica é considerada uma das mais ameaçadas de extinção no mundo (Heringer e Montenegro, 2000), possuindo atualmente menos de 4% de sua área original de matas primárias e outros 4% em florestas secundárias (Reserva da Biosfera da Mata Atlântica, 2013). A conservação desse ambiente é fundamental para a manutenção da diversidade da família Bromeliaceae, tendo sido observados 31 gêneros contendo 803 espécies de bromélias, sendo 653 delas endêmicas. Segundo listas oficiais de espécies ameaçadas, cerca de 40% das espécies de bromélias registradas nos domínios da Floresta Atlântica encontra-se em alguma categoria de ameaça: 54 espécies estão incluídas na categoria *criticamente em perigo*, 89 *em perigo*, 182 *vulneráveis* e 17 *raras*. Além disso, três espécies foram indicadas como extintas na natureza: *Cryptanthus fosterianus* L.B. Smith, *Neoregelia binotii* (Antoine) L.B. Smith e *Nidularium utriculosum* Ule (Martinelli *et al.*, 2008).

As bromélias possuem importância econômica relevante, sendo cultivadas especialmente como plantas ornamentais a partir do início da década de 1990 (Coffani-Nunes, 2002), também utilizadas como alimentação, cosméticos, fontes de fibras, plantas medicinais, entre outros (Reitz, 1983). Entretanto, devido a grande procura dessas espécies, o extrativismo ilegal vem se intensificado nos últimos anos, contribuindo para o aumento do número de plantas vulneráveis, ameaçadas de extinção ou até mesmo em extinção (Bered *et al.*, 2008). Entre as plantas da família Bromeliaceae o abacaxi (*Ananas comosus* (L.) Merr.) é a espécie de maior importância econômica, muito apreciada na alimentação humana, sendo a quarta fruta tropical mais produzida no mundo (Chwee e Ahmad, 2008).

Outra espécie de grande importância é *Neoglaziovia variegata* (Arr. Cam.) Mez, utilizada como fonte de fibras (Moreira *et al.*, 2006). Na medicina natural, se destaca *Bromelia antiacantha* Bertol., na produção de xaropes caseiros (Zanella *et al.* 2011).

Além disso, as bromélias também desempenham um importante papel ecológico, possibilitando o acúmulo de água nos tanques formados pela disposição das folhas, bem como, sendo fonte de néctar e de frutos carnosos (Benzing, 2000). Podem servir ainda de abrigo e local de reprodução para muitas espécies, tais como, anfíbios, insetos, mamíferos e pássaros (Benzing, 2000; Givnish *et al.*, 2011). Apesar da importância econômica e ecológica dessa família e o número de trabalhos referentes a mesma estar aumentando, a bibliografia científica ainda é consideravelmente reduzida (Zanella *et al.*, 2012a).

### **3. *Dyckia distachya*, uma bromélia ameaçada**

*Dyckia distachya* Hassler é uma bromélia rara que pertence à subfamília Pitcairnioideae, exclusiva de ambientes reofíticos, ocorrendo nas margens e ilhas rochosas de rios, estando sujeita às forças das corredeiras nos períodos de cheia e a secas extremas sobre as rochas nos períodos de vazante (Reitz, 1983). No entanto, espécies restritas a esses ambientes encontram-se muitas vezes ameaçadas, principalmente, devido à construção de barragens (Wiesbauer, 2008; Hmeljevski *et al.*, 2011).

A espécie consegue sobreviver a essa alternância ambiental através de alguns mecanismos especiais. Quando as plantas ficam submersas, uma membrana cuticular espessa e impermeabilizante, localizada na superfície da folha, impede a entrada de água. Além disso, na parte interna da folha, existe o aerênquima, atuando como uma reserva de ar entre as células e contribuindo para a sobrevivência nessa fase. Por outro lado, nos períodos secos, os estômatos encobertos por tricomas, associados à membrana cuticular e a corpos silicosos, auxiliam na redução da perda excessiva de água. O hidrênquima, localizado na parte interna da folha, atua como uma reserva de água, impedindo que a planta murche nesses períodos (Wiesbauer e Zimmermann, 2010).

*Dyckia distachya* é uma espécie rupestre, formando densas touceiras arredondadas, composta por vários indivíduos, capazes de diminuir o atrito com a água e favorecer a retenção de matéria orgânica carregada pelo rio (Wiesbauer e Zimmermann, 2010). Ela apresenta folhas rígidas, dispostas em forma de uma bela roseta, com cerca de dez a doze

centímetros de comprimento, coloração verde clara, nervuras visíveis na face abaxial, espinhos nas margens e ápice pontiagudo. As inflorescências são emitidas lateralmente em um escapo tênue, com aproximadamente 70 a 130 centímetros de comprimento, geralmente formando uma panícula terminal, com dois a oito ramos e flores amarelo-alaranjadas (Reitz, 1983).

A reprodução dessa bromélia ocorre através de propagação clonal por afillhos laterais, divisão do meristema apical e produção de sementes. A espécie é policárpica, apresenta floração anual, com flores completas e hermafroditas. Ainda é capaz de emitir mais de um escapo floral por indivíduo, aumentando assim o seu período de floração. A antese é sequencial, ocorrendo à abertura das flores da base para o ápice das inflorescências. Acontecem menos de três anteses por indivíduo por dia, sendo que as flores apresentam baixa durabilidade, cerca de um dia e meio. Esses fatores diminuem as chances de ocorrência de geitonogamia e aumentam a probabilidade de ocorrência de polinização cruzada. A planta possui ainda auto-incompatibilidade pós-zigótica podendo ocorrer à formação de frutos, porém, a maioria com sementes inviáveis (Wiesbauer, 2008).

O florescimento ocorre entre os meses de setembro e janeiro e a maturação dos frutos de outubro a fevereiro (Wiesbauer, 2008). O néctar dessa bromélia possui características de espécies meliófilas e ornitófilas, sendo observado pelo menos vinte espécies de visitantes florais, incluindo abelhas, beija-flores, borboletas e moscas, destacando-se como principais polinizadores as abelhas (*Bombus atratus* e *Xilocopa* spp.) e o beija-flor (*Chlorostilbon lucidus*) (Wiesbauer, 2008). Os frutos são cápsulas com coloração escura, tripartidas e multisseminadas (Reitz, 1983), produzindo em média cem sementes aladas (Wiesbauer, 2008). A dispersão das sementes ocorre através de anemocoria e hidrocoria. A semente é fotoblástica positiva, capaz de germinar quando está submersa, ocorrendo a sobrevivência das plântulas durante algumas semanas nessas condições, possibilitando a dispersão através de sementes e plântulas (Wiesbauer, 2008).

*Dyckia distachya* é uma bromélia endêmica que ocorria originalmente em ilhas e margens rochosas da bacia do rio Uruguai, possuindo distribuição disjunta ao longo de 617 km, nos rios Pelotas e Uruguai, na divisa dos estados do Rio Grande do Sul (RS) e de Santa Catarina (SC) (Reis *et al.*, 2005). Devido ao aproveitamento do potencial hidrelétrico dessa bacia hidrográfica, três usinas hidrelétricas foram construídas em apenas cinco anos (2000, 2003 e 2005), levando a extinção de sete das oito populações conhecidas de *D.*

*distachya* na natureza (Reis *et al.*, 2005). Atualmente, é conhecida apenas uma população natural, localizada no Salto do Yucumã, divisa entre Brasil e Argentina, dentro de áreas protegidas. No Brasil, encontra-se no Parque Florestal Estadual do Turvo, no estado do Rio Grande do Sul e na Argentina, no Parque Provincial Moconá, na província de Misiones (Reis *et al.*, 2005).

A partir de 1992, *D. distachya* passou a integrar a Lista Oficial de Espécies da Flora Brasileira Ameaçada de Extinção na categoria *em perigo* (Portaria do IBAMA nº 37-N/3 de abril de 1992). Hoje, encontra-se na Lista Nacional das Espécies da Flora Brasileira Ameaçada de Extinção publicada em setembro de 2008 pelo Ministério do Meio Ambiente (Instrução normativa nº 6/23 de setembro de 2008). Nessa lista, são classificadas como espécies ameaçadas de extinção aquelas com alto risco de desaparecimento na natureza em um futuro próximo (Ministério do Meio Ambiente, 2008).

#### **4. Introdução de *Dyckia distachya* na natureza**

A necessidade de conservar *D. distachya* surgiu durante a construção da usina hidrelétrica de Itá (SC, 2000), pois na ocasião, acreditava-se que essa espécie era endêmica daquela região. Com o intuito de preservá-la houve o resgate de vários indivíduos, inclusive por moradores da região, os quais foram mantidos em coleções *ex situ*. Posteriormente, durante a implantação das usinas hidrelétricas de Machadinho (RS, 2003) e Barra Grande (SC, 2005) novas populações dessa bromélia foram descobertas. Parte dessas populações também foi coletada e destinada à conservação *ex situ* (Reis *et al.*, 2005).

Visando a preservação *inter situ*, parte dos indivíduos que haviam sido resgatados e mantidos em viveiros e coleções *ex situ* foram introduzidos em locais semelhantes aos de ocorrência natural. As introduções iniciais ocorreram nas áreas de influência das usinas hidrelétricas de Itá e Machadinho, tendo sido relocadas quatro e duas populações, respectivamente. Porém, essas introduções obtiveram pouco êxito, principalmente por esses locais não apresentarem características de habitat reofítico, dificuldade de fixação dos indivíduos nas rochas e predação das bromélias por capivaras (*Hydrochaeris hydrochaeris* Linnaeus) (Reis *et al.*, 2005; Wiesbauer *et al.*, 2009).



A usina hidrelétrica Energética Barra Grande S/A estabeleceu o *Projeto de Resgate e Relocação de Reófitas*, como condicionante da licença de operação (LO 447/2005) expedida pelo IBAMA. A empresa ficou responsável pelo programa que tentava impedir a extinção de *D. distachya* através do resgate e introdução de indivíduos na natureza (Meio Biótico Consultoria, 2009).

O programa foi dividido em quatro etapas. Na primeira etapa, também chamada de *Resgate de reófitas*, foram salvos cerca de 18.700 exemplares de *D. distachya* antes e durante o processo de enchimento do reservatório, ocorrido entre junho a setembro de 2005. Os indivíduos foram resgatados das populações Pedras Brancas/Gateados, Encanados e Encanadinhos (Figura 1). Foram recuperadas 3.977 rosetas procedentes de 48 touceiras da população Pedras Brancas/Gateados, 14.063 rosetas de 22 touceiras da população Encanados e 683 rosetas de cinco touceiras da população Encanadinhos (Meio Biótico Consultoria, 2009). Na segunda etapa do projeto, *Taxonomia e Seleção de Áreas para Relocação*, foram pré-selecionadas 23 áreas potenciais para a introdução da espécie, das quais foram selecionadas sete com maior potencial (Meio Biótico Consultoria, 2009). Na terceira etapa, *Relocação dos Exemplares Resgatados*, foram transferidos 14.738 indivíduos para uma área piloto e sete áreas de relocação. A segunda e a terceira etapa foram realizadas no ano de 2006 (Meio Biótico Consultoria, 2009).



**Figura 1.** Localização das populações naturais extintas e populações introduzidas existentes atualmente de *Dyckia distachya* na área de influência da usina hidrelétrica Energética Barra Grande S/A. Fonte: Meio Biótico Consultoria, 2011.

Na área piloto, localizada em Lajeado dos Portões (SC), foram relocadas 164 rosetas provenientes da população Pedras Brancas. Na área de relocação AR01, situada no reservatório da usina hidrelétrica Barra Grande (SC), foram transferidas 1.230 rosetas provenientes da população Pedras Brancas. Na área AR02, no reservatório da usina (RS), foram relocados 4.448 indivíduos da população Encanados. Na área AR05, no reservatório da usina (SC), foram transferidas 257 rosetas da população Encanadinhos. Na área AR23, a jusante do reservatório (RS), foram relocados 2.162 indivíduos da população Encanados. Na área AR15, em Lajeado dos Tijolos (SC), foram relocadas 2.010 rosetas da população Pedras Brancas. Na área AR16, no rio Vacas Gordas (SC), foram relocadas 4.316 rosetas da população Encanados e na área AR17, em Lajeado Limitão (SC), foram transferidos 315 indivíduos da população Encanadinhos (Meio Biótico Consultoria, 2009).

A quarta etapa do programa, *Manutenção e Monitoramento de Reófitas Relocadas*, diz respeito à manutenção das áreas de relocação e das plantas abrigadas em viveiros, bem como, o monitoramento dos experimentos a campo, com o objetivo de avaliar o grau de efetividade das estratégias de relocação e plantio utilizado (Meio Biótico Consultoria, 2009). Nesta fase, que ocorreu entre agosto de 2006 e agosto de 2013, foram realizadas avaliações periódicas, o manejo das áreas e algumas alterações, visando o restabelecimento das plantas na natureza.

As áreas de relocação foram avaliadas a partir de 2009, sendo os indivíduos remanejados para outros locais quando do não sucesso das regiões iniciais. A área AR17 foi substituída pela área piloto, pois apresentou grande perda de indivíduos pela correnteza e baixo potencial para manutenção da espécie. A área piloto passou a ser denominada de AR14. Também houve a transferência de rosetas das áreas AR01 e AR05 para novas áreas, devido à falta de condições reofíticas e ao excesso de competição com outras plantas. Esses indivíduos foram transferidos para duas áreas do rio Vacas Gordas e afluentes, onde já existia a área de relocação AR 16. As áreas passaram a ser chamadas de AR 16.1 (Ponte Velha/SC), antiga AR 16, implantada com indivíduos provenientes da população Encanados, e AR 16.2 (Cachoeira dos Varões/SC), estabelecida a partir das plantas transferidas da área AR05 e AR 16.3 (Miniencanadinhos/SC), instituída a partir das plantas transferidas da área AR01 (Meio Biótico Consultoria, 2011).

Além das áreas de relocação, foi mantida na usina hidrelétrica Energética Barra Grande S/A uma coleção *ex situ* com o objetivo de preservar o material genético resgatado

das três populações originais, visando a relocação de plantas adultas e também a produção de sementes. Uma parte das sementes obtidas através do cruzamento das três populações foi germinada e cerca de 5.000 plântulas foram introduzidas nas áreas AR14, AR16.2, AR16.3 e AR23 no ano de 2009. As plantas obtidas através dos cruzamentos foram denominadas de *Megapopulação* (Meio Biótico Consultoria, 2011).

A área de relocação AR15 apresentou perda significativa de indivíduos devido ao ataque da lagarta *Strymon rufofusca* Hewitson, sendo criada uma nova área para substituí-la, no rio Vacas Gordas, a qual recebeu uma mistura de plantas adultas das três populações, sendo denominada de AR16.4 (Ponte Nova). As áreas AR02 e AR23 foram desativadas no ano de 2010 devido ao excesso de competição com outras espécies e transferidas para as áreas AR21 (Rio do Frade) e AR16.3 (Miniencanadinhos), respectivamente (Meio Biótico Consultoria, 2011).

Atualmente existem oito áreas de relocação (AR14, AR15, AR16.1, AR16.2, AR16.3, AR16.4, AR21 e AR23), estando três áreas (AR16.1, AR16.2 e AR16.4) localizadas dentro de uma unidade de conservação, na Reserva Particular de Patrimônio Natural Emílio Einsfeld Filho, no município de Campo Belo do Sul (SC) (Figura 1). Essas áreas estão apresentando melhores resultados do que os locais de relocação próximos às usinas de Itá e Machadinho. Inclusive tendo sido observado o recrutamento de novos indivíduos por sementes em duas áreas (AR16.2 e AR 16.3) (Meio Biótico Consultoria, 2011). Porém, a predação pela lagarta *S. rufofuscus* e a competição com outras espécies ainda prejudica o sucesso das introduções de *D. distachya* na natureza.

## **5. Genética e conservação**

A informação genética é essencial para o planejamento de ações de conservação de espécies raras e ameaçadas de extinção, principalmente para as que possuem poucas populações ou um número reduzido de indivíduos em cada população (Gitzendanner *et al.*, 2012). Os marcadores moleculares são ferramentas valiosas muito úteis nas ações de conservação, possuindo várias aplicações importantes, destacando-se o acesso à variabilidade genética, a análise do sistema de cruzamento e a estimativa do fluxo gênico nas plantas (Faleiro, 2007; Kalia *et al.*, 2011).

Os microssatélites ou *Simple Sequence Repeats* (SSR) são marcadores moleculares que possuem considerável importância em estudos genéticos, devido à existência de várias características desejáveis, incluindo expressão codominante, hipervariabilidade, natureza multialélica, reprodutibilidade, relativa abundância, localização específica nos cromossomos e ampla cobertura do genoma (Faleiro, 2007; Kalia *et al.*, 2011). Os SSR são pequenas sequências de um a seis nucleotídeos, repetidos em *tandem* ao longo do DNA, presente no genoma de eucariotos e procariotos (Tóth *et al.*, 2000; Zane *et al.*, 2002), sendo percebidos através de alterações no número de repetições das sequências motivo dos SSR (Li *et al.*, 2002). As mutações ocorrem principalmente devido ao escorregamento da fita de DNA durante a replicação (*slippage*) e a recombinação (*crossing over*) (Li *et al.*, 2002). Nas plantas ocorre em maior frequência uma sequência motivo de SSR perfeita, sendo o elemento repetido com maior assiduidade o di-nucleotídeo AT (Kalia *et al.*, 2011).

A principal limitação dos SSR está na necessidade da utilização de sequências de nucleotídeos que flanqueiam os SSR (*primers*) específicos para cada espécie, sendo o seu desenvolvimento muito oneroso e laborioso. A elaboração dos marcadores envolve, basicamente, a construção de uma biblioteca genômica de pequenos fragmentos (300 a 700 pares de bases) para a espécie de interesse, gerando clones. Esses clones são selecionados para a presença de SSR através de sondas sintéticas complementares aos elementos repetidos mais comuns no organismo. Os clones positivos são sequenciados e pares de *primers* específicos são construídos para sequências únicas selecionadas com o auxílio de programas computacionais (Ferreira e Gratapaglia, 1998; Zane *et al.*, 2002; Kalia *et al.*, 2011).

A transferência de marcadores entre espécies ou gêneros também é possível, quando ocorre à conservação de sítios de microssatélites entre espécies relacionadas (Ferreira e Gratapaglia, 1998; Kalia *et al.*, 2011), tendo sido demonstrada com sucesso em algumas espécies de bromélias (Sarhou *et al.*, 2003; Barbará *et al.*, 2007; Paggi *et al.*, 2008; Wöhrmann e Weising, 2011; Miranda *et al.*, 2012; Zanella *et al.*, 2011; Büttow, 2012; Zanella *et al.*, 2012b; Goetze *et al.*, 2013; Lavor *et al.*, 2013). Assim, a transferibilidade poderia ser vista como uma alternativa, reduzindo consideravelmente os custos (Ferreira e Gratapaglia, 1998) e também permitindo a utilização dos SSR nas pesquisas com maior rapidez (Barbará *et al.*, 2007).

Apesar da relevância da utilização dos SSR poucos marcadores foram publicados até agora para espécies de plantas ameaçadas (White e Powell, 1997; Collevatti *et al.*, 1999; Kang *et al.*, 2006; Fan *et al.*, 2009; Csencsics *et al.*, 2010; Micheneau *et al.*, 2011; Tew *et al.*, 2011; Wadl *et al.*, 2011; Hou *et al.*, 2012; Li *et al.*, 2012; Zanella *et al.*, 2012b; Angrizani *et al.*, 2013; Elliot *et al.*, 2013; Gajurel *et al.*, 2013; Ismail *et al.*, 2013; Mansour *et al.*, 2013) e de bromélias (Boneh *et al.*, 2003; Sarthou *et al.*, 2003; Palma-Silva *et al.*, 2007; Kinsuat e Kumar, 2007; Paggi *et al.*, 2008; Wöhrmann e Weising, 2011; Krapp *et al.*, 2012; Wöhrmann *et al.*, 2012; Zanella *et al.*, 2011; Zanella *et al.*, 2012b; Feng *et al.*, 2013; Goetze *et al.*, 2013; Hmeljevski *et al.*, 2013; Rodríguez *et al.*, 2013; Wöhrmann *et al.*, 2013).

O conhecimento da diversidade genética é fundamental em programas de conservação, sendo a sua preservação extremamente importante para a manutenção do potencial evolutivo e necessária para as populações se adaptarem as alterações no ambiente (Frankham *et al.*, 2008). Bottin *et al.* (2007) destacam a importância do conhecimento da variabilidade genética de populações ameaçadas que fazem parte de programas de introdução, visando evitar problemas futuros devido a deriva genética e a depressão endogâmica.

Recentemente estão sendo realizados estudos com o objetivo de acessar a variabilidade genética de populações ameaçadas para serem utilizadas em futuros programas de reintrodução na natureza. Shapcott e Powell (2011) avaliaram a diversidade genética da única população existente de *Macadamia janseni* C.L.Gross e P.H.Weston, uma árvore ameaçada da Austrália, com o auxílio de cinco pares de *primers* heterólogos desenvolvidos para duas espécies cultivadas de macadâmia. Hou *et al.* (2012) avaliaram a eficiência de uma coleção *ex situ* de *Dendrobium officinale* Kimura et Migo, uma orquídea ameaçada utilizada como planta medicinal na China, com 15 marcadores SSR desenvolvidos para a espécie.

Conhecer o sistema de cruzamento é imprescindível para o planejamento de ações relacionadas à conservação (Godefroid *et al.*, 2011), pois ele determina como a informação genética é transferida entre gerações e como a diversidade é distribuída entre indivíduos de uma espécie (Zhang *et al.*, 2010). As variações existentes no sistema de cruzamento, tais como, a taxa de polinização cruzada e o grau de parentesco entre plântulas de um mesmo

indivíduo podem ser acessadas facilmente através da análise de progênies com o auxílio de marcadores moleculares (Ritland, 2002).

Os sistemas de cruzamento das plantas são baseados principalmente no modelo de sistemas mistos de cruzamentos, como o estimado pelo programa MLTR, onde uma parte da progênie é derivada de autofecundação e o restante de cruzamentos aleatórios (Ritland, 2002). Esses cruzamentos podem ser caracterizados através da estimativa da taxa de polinização cruzada, da taxa de cruzamento entre parentais e da probabilidade de dois irmãos terem o mesmo pai (Ritland e Jain, 1981). As estimativas da probabilidade de dois irmãos terem o mesmo pai podem evidenciar a dimensão da diversidade do *pool* de pólen dos doadores e também o número efetivo de doadores de pólen por planta mãe (Ritland, 1989).

Outro fator importante relacionado ao manejo de espécies ameaçadas é a compreensão do fluxo gênico dentro e entre populações (Frankham *et al.*, 2008). Nas plantas que se reproduzem sexuadamente, o pólen e a semente são os principais componentes do fluxo gênico, sendo que a capacidade de dispersão deles está relacionada com a habilidade das espécies em manter a diversidade genética e colonizar novos locais (Nathan *et al.*, 2008; Chybicki e Burczyk, 2010). A dispersão do pólen e das sementes pode ocorrer a curtas distâncias, influenciando principalmente os padrões de recrutamento e a utilização de recursos locais e também a longas distâncias, afetando a disseminação e as taxas de colonização (Nathan *et al.*, 2008).

A análise TwoGener é um método indireto utilizado para estimar o fluxo gênico contemporâneo, contando apenas com os genótipos maternos e suas coordenadas geográficas e os genótipos de suas progênies. Essa análise nos permite quantificar a heterogeneidade existente no *pool* de pólen amostrado através das plantas mães distribuídas ao longo da paisagem e também estimar a distância média e a área efetiva de polinização. Portanto, essa técnica não requer o mapeamento e a genotipagem de todos os potenciais doadores de pólen, diminuindo assim os custos com a pesquisa (Austerlitz e Smouse, 2001; Smouse *et al.*, 2001).

Mesmo sendo de grande importância, poucos trabalhos foram realizados para determinar o sistema de cruzamento e o fluxo gênico de espécies raras e ameaçadas com o auxílio de marcadores moleculares (Coates e Hamley, 1999; Hoebee e Young, 2001; González-Astorga *et al.*, 2004; Bottin *et al.*, 2007; Grivet *et al.*, 2009; Zhang *et al.*, 2010;

Wang *et al.*, 2010; Hmeljevski *et al.*, 2011; Rosas *et al.*, 2011; Büttow, 2012). Em relação a espécies de bromélias ameaçadas, Hmeljevski *et al.* (2011) caracterizaram o sistema de cruzamento de *Dyckia ibiramensis* Reitz como misto e Büttow (2012) determinou o sistema de cruzamento de polinização cruzada e fluxo gênico restrito para *Aechmea winkleri* Reitz.

## **6. Objetivos**

O conhecimento de características genéticas de espécies de plantas que fazem parte de programas de introdução na natureza é escasso. Sendo assim, a presente tese tem como objetivo geral investigar o sucesso reprodutivo e aspectos genéticos em populações introduzidas e na única população natural conhecida de *D. distachya*, visando obter informações para o estabelecimento de estratégias de conservação para essa bromélia e outras espécies relacionadas.

### **6.1 Objetivos específicos**

- Avaliar o sucesso reprodutivo e a viabilidade de populações introduzidas em comparação com uma população natural de *D. distachya*;
- Estimar a diversidade genética, bem como a sua manutenção entre gerações, utilizando marcadores moleculares microssatélites;
- Estudar os efeitos da introdução nos padrões do sistema de cruzamento através da análise de progênies;
- Inferir o fluxo gênico contemporâneo, a estrutura genética do *pool* de pólen e a área de vizinhança efetiva de polinização nas populações estudadas;
- Contribuir para estratégias de conservação da espécie.





## Capítulo II

*Reproductive success and viability of introduced and natural populations of Dyckia distachya Hassler (Bromeliaceae)*

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1 **Original article**

2 Reproductive success and viability of introduced and natural populations of *Dyckia*  
3 *distachya* Hassler (Bromeliaceae)

4

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18

19 **ABSTRACT**

20 *Dyckia distachya* is a rare and endangered bromeliad. Due to destruction of its  
21 habitat, almost all populations were extinct remaining only one in nature. Attempting to  
22 prevent the complete disappearance, populations were introduced in similar places to their  
23 original occurrence. We evaluated the reproductive success of four introduced populations  
24 and the only know natural population. We analyzed the average production of flowers,  
25 fruits and seeds; the average number of vegetative and reproductive individuals; seed  
26 germination and pollen viability. Since the species reproduces sexual and asexually, the  
27 average area of the genet and the average number of ramets per genet were also evaluated.  
28 The results showed significant differences between introduced and natural populations in  
29 all variables studied. Introduced populations have greater investment in sexual  
30 reproduction than the natural, with high production of flowers, fruits and seeds. On the  
31 other hand, the natural population seems to have increased investment in clonal  
32 propagation. Despite the differences found between introduced and natural populations, it

33 appears that the introduced populations are obtaining reproductive success, being reported  
34 the recruitment of new individuals from seeds.

35

36 **Key words:** conservation, endangered, introduction, pollen viability, rare, seed  
37 viability.

38

39 The extinction is related to the permanent disappearance of a species as part of the  
40 natural process of evolution. However, when there is no balance between extinction and  
41 emergence of new species, the loss of biodiversity occurs (Frankham et al., 2010). A large  
42 number of plants has already been extinct (Pitman and Jorgensen, 2002; Millennium  
43 Ecosystem Assessment, 2005) and many could disappear before they are even described,  
44 particularly those with a small geographical distribution and occur in less explored sites  
45 (Mora et al., 2011). Human activities that produce environmental impacts are leading to  
46 progressive degradation of the habitat of various plants (Heywood and Iriondo, 2003),  
47 being able to cause the loss of a population or even a species (Gauthier et al., 2013). The  
48 construction of dams, for example, has caused fragmentation and habitat loss through the  
49 flood areas and the transformation of rivers of free flow in reservoirs, leading to decrease  
50 of the communication of species between different points of a river basin (Secretariat of  
51 the Convention on Biological Diversity, 2010).

52 Plants that have lost their natural habitat can be conserved in nature and thus have  
53 reduced your chances of extinction through the introduction. The introduction is the  
54 establishment of populations in a new ecosystem, similar to naturally occurring  
55 environment (IUCN, 2013), determining the method of inter situ conservation (Guerrant et  
56 al., 2004). The success of an introduction can be assessed by the ability to realize the basic  
57 processes of the plant life cycle, such as, establishment, reproduction, dispersal and  
58 recruitment of new individuals from seeds (Pavlik, 1996; Frankham et al., 2010).

59 The place where introductions are carried out deserves special attention. Introduced  
60 populations have to adapt to ambient conditions and are susceptible to herbivory and  
61 competition with other species, as well as other important ecological factors (Menges,  
62 2008). Individuals poorly adapted to the new environment may have reduced their rate of  
63 survival, which may compromise the successful introduction (Thornton et al., 2008). The  
64 monitoring and management of new populations are needed to ensure the successful

65 establishment and persistence of these individuals, especially in species that are slow to  
66 reach reproductive maturity (Cochrane, 2004). Studies involving fertility, viability and  
67 reproductive success serve as an important tool in the process of conservation of  
68 endangered species (Bottin et al., 2007).

69 *Dyckia distachya* is an endemic and endangered bromeliad (Ministério do Meio  
70 Ambiente, 2008), that is classified as rupicolous, heliophyte and rheophyte (Reitz, 1983;  
71 Klein 1990). It occurred on islands and rocky shores of the Uruguay River Basin, having  
72 disjunct distribution over 617 km in Pelotas and Uruguay rivers, bordering the states of Rio  
73 Grande do Sul and Santa Catarina, Brazil (Reis et al., 2005). Due to the exploitation of the  
74 hydroelectric potential of this basin, three hydroelectric plants were built in just five years,  
75 leading to extinction of seven of the eight known populations of *D. distachya* in nature,  
76 being known currently only one natural population (Reis et al., 2005). However, during the  
77 construction of hydroelectric plants several individuals of this species were rescued from  
78 nature, with the intention of preserving them. These specimens were kept in collections ex  
79 situ and part of them was used later on introduction programs (Reis et al., 2005).

80 The main objective of this study was to evaluate comparatively the reproductive  
81 success and the viability of four introduced populations and the only known natural  
82 population of *D. distachya*. Our aims were (1) to assess the investment in sexual and  
83 vegetative reproduction; (2) register the production of flowers, fruits and seeds; (3)  
84 determine the germination rate and the pollen viability; and (4) discuss the viability of the  
85 studied populations. The results obtained with this investigation will help us to propose  
86 some recommendations for conservation management of this highly endangered species.

87

88

## 89 MATERIALS AND METHODS

90

### 91 *Study species*

92 *Dyckia distachya* Hassler is a rare bromeliad that belongs to the subfamily  
93 Pitcairnioideae, being exclusive to rheophytic environments and occurring on the banks of  
94 rivers and rocky islands. In these environments they are subject to the forces of the rapids  
95 in the flood season and extreme droughts over the rocks during ebb periods (Reitz, 1983).  
96 The species is rupestrian, forming dense clumps (Figure 1A) able to reduce friction with

97 the water and favor the retention of organic matter carried by the river. Each individual has  
98 many rigid sheets, with thorns on the margins, arranged in a beautiful rosette (Reitz, 1983).  
99 This bromeliad is pollinated mainly by bees and hummingbirds. It can also reproduce by  
100 clonal propagation by side tillers, division of the apical meristem and seed production  
101 (Wiebauer, 2008). *Dyckia distachya* is polycarpic, has annual flowering and complete and  
102 hermaphrodite flowers. The flowering occurs between September and January and fruit  
103 maturation from October to February (Wiesbauer, 2008).

104

#### 105 ***Study sites and populations***

106 The only known natural population of *D. distachya* is located in Salto do Yucumã,  
107 boundary between Brazil and Argentina (Figure 2). In both places, the plants are located in  
108 protected natural areas. In Brazil, is found in the Parque Florestal Estadual do Turvo, Rio  
109 Grande do Sul state and in Argentina, they are located in the Parque Provincial Moconá,  
110 Misiones province (Table 1). The average temperature for this area is around 19.1°C, and  
111 the annual rainfall averages is 1,800 mm/year.

112 Four introduced populations were studied (Table 1), they are located near the area of  
113 influence of the Barra Grande hydroelectric power plant: Cachoeira dos Varões (Figure  
114 1B), Miniencanadinhos, Ponte Velha e Ponte Nova. Introduced populations are placed near  
115 to the Hydrographic Basin of the Uruguay River, border between the states of Rio Grande  
116 do Sul and Santa Catarina (Figure 2). The introduced populations were formed from  
117 individuals collected from extinct populations: Cachoeira dos Varões was formed from  
118 Encanadinhos, Miniencanadinhos was formed from Pedras Brancas/Gateados, Ponte Velha  
119 from Encanados and Ponte Nova from a mixture of the three extinct populations.

120 Three populations (Cachoeira dos Varões, Ponte Nova and Ponte Velha) were  
121 allocated within a Reserva Particular do Patrimônio Natural – RPPN Emílio Einsfeld Filho,  
122 Campo Belo do Sul city, Santa Catarina state. The four populations are 3 to 17 km of  
123 distance from each other and around 330 km distant from the natural population. The  
124 average temperature for Campo Belo do Sul is around 16.0°C, and the annual rainfall  
125 averages is 1,850 mm/year.

126 This study was carried out in the reproductive season of 2010 in the natural  
127 population and in the years 2010 and 2011 in the introduced populations. We evaluated the

128 reproduction investment and the reproductive success in two years and the seed viability,  
129 germination rate and pollen viability in 2010.

130

131 ***Reproduction investment***

132 The reproduction investment was evaluated using the area of the genet (m<sup>2</sup>), the  
133 number of ramets per genet and the reproductive ramets per genet in fifteen genets per  
134 studied population. According to Harper et al. (1977) individuals formed by clonal  
135 propagation are referred to as ramets and all ramets produced by a genotype are referred to  
136 as genet. In this work, we consider each group of plants as a genet (Figure 1A) and each  
137 rosette that are part of a group as a ramet. The rate of investment in vegetative  
138 reproduction was calculated by the proportion of ramets per genet and the rate of  
139 investment in sexual reproduction was considered as the proportion of vegetative and  
140 reproductive individuals per genet. Here we consider vegetative individuals as those with  
141 rosette shape which had no signs or emission of the inflorescence, and reproductive ones  
142 those that had shaped rosette and had signal or emission of inflorescence.

143

144 ***Reproductive success***

145 *Production of flowers* – It was counted the total number of flowers produced by  
146 plant, in the different racemes of the inflorescence, in fifteen plants of each studied  
147 population.

148 *Fruit production* – It was calculated the percentage of fruits formed, in fifteen plants  
149 of each population, by the following formula: % fruits = total number of fruits per  
150 plant/total number of flowers per plant X 100 (Burne et al., 2003).

151 *Seed production* – We randomly collected four capsules per inflorescence, from ten  
152 plants per population, and counted the number of seeds formed in each capsule.

153

154 ***Seed viability and germination rate***

155 We randomly collected four capsules per plant of ten mother-plants per studied  
156 population. After, we germinate 25 seeds per capsule, totaling 100 seeds per plant. Seeds  
157 were disinfected and placed in petri dishes with a culture medium, containing medium MS  
158 salts (Murashige and Skoog, 1962), B5 vitamins (Gamborg et al., 1968), 3% sucrose and  
159 0.3% Phytigel (Sigma, Saint Louis, MO, USA) with pH 6.4. The dishes were incubated in

160 a climate-control chamber with relative humidity near 100% and photoperiod of 16 h light  
161 at 25 °C and 8 hours dark at 22 °C. Germination was monitored daily for 30 days. The  
162 germination rates were determined as the proportion of seeds that had germinated, by  
163 counting the number of seedlings that presented two fully expanded leaves.

164

#### 165 *Pollen viability*

166 Floral buds of ten individuals per population studied were collected and fixed for 24  
167 hours in absolute ethanol: glacial acetic acid (3:1) at room temperature. After fixation, the  
168 buds were transferred to 70% alcohol and stored in a freezer at -20°C. Squash preparations  
169 were stained following Alexander's (1980) method, in which empty unviable pollen grains  
170 stain green and full viable pollen grains stain purple. Two flowers per individual were  
171 analyzed, being sampled 1.000 pollen grains per flower. It was also measured the size of  
172 pollen grains. Slides were examined and documented using a Zeiss Axioplan Universal  
173 photomicroscope (Carl Zeiss, New York, NY, USA).

174

#### 175 *Statistical analyses*

176 All parameters evaluated above were analyzed by the nested analysis of variance  
177 followed by the Tukey test to determine if the differences among plants or populations  
178 were significant ( $P < 0.05$ ). The analyses were performed using the SAS software package  
179 (Version 9.3, SAS Institute, Cary, North Carolina, USA).

180

181

## 182 **RESULTS**

183

#### 184 *Reproduction investment*

185 The overall mean area of genets of the four introduced populations, considering all  
186 years, was 0.122 m<sup>2</sup> and in the natural population was 2,172 m<sup>2</sup>, with significant  
187 differences among populations ( $P < 0.001$ ) and without significant differences between  
188 years ( $P = 0.1634$ ) (Table 2). The mean number of ramets per genet ranged from 1.9 to  
189 12.5 in the introduced populations, with an overall mean of 5.9 ( $s \pm 5.9$ ) and was of 297.6  
190 ( $s \pm 226.9$ ) in the natural population. Were observed significant differences among  
191 populations ( $P < 0.001$ ) and between years ( $P = 0.0025$ ) (Table 2). The percentage of



192 reproductive individuals per genet, considering all years in each introduced population,  
193 ranged from 41.6% to 78.9%, being observed only significant differences ( $P<0.001$ )  
194 among populations. The natural population was statistically different from all the  
195 introduced populations in all parameters analyzed above (Table 2).

196

### 197 ***Reproductive success***

198 Considering all introduced populations and all years of evaluation, the overall mean  
199 of flowers per inflorescence was 34.1 ( $s \pm 11.6$ ), being observed significant differences  
200 ( $P<0.001$ ) among populations. The average flower production per inflorescence ranged  
201 from 26.7 to 41.3 among introduced populations, being higher in PV and lowest in PN  
202 population. The natural population differed significantly ( $P<0.001$ ) from all the introduced  
203 populations studied, presenting lower average production of flowers (2.7;  $s \pm 4.2$ ) per  
204 inflorescence (Table 3). The mean fruit set per inflorescence ranged from 17.1% to 74.3%,  
205 with an overall mean of 37.5% ( $s \pm 23.1$ ) in the introduced populations and was 5.9% ( $s \pm$   
206 8.0) in the natural population. Significant differences ( $P<0.001$ ) were observed between  
207 years and among populations ( $P=0.0075$ ), being the fruit set higher in ME and lower in SY  
208 (Table 3).

209 The mean number of seeds per capsule ranged from 31 to 90, with significant  
210 differences ( $P<0.001$ ) between introduced and natural populations (Figure 3). The  
211 recruiting of new individuals by seeds was observed in the field in the populations CV and  
212 SY in a small amount and in the population ME (Figure 1E) in a slightly greater amount  
213 (A. Janke, personal observation). Furthermore, it was observed a high infestation of  
214 *Strymon rufofuscus* caterpillars in the areas of introduction of populations CV and PN,  
215 causing severe damage in the leaves and fruits of *D. distachya* (A. Janke, personal  
216 observation).

217

### 218 ***Seed viability and germination rate***

219 The seeds were evaluated at seven and 30 days after placed in culture medium. The  
220 overall mean of the introduced populations at seven days was 60% and at 30 days was  
221 69%, showing significant differences ( $P<0.001$ ) among these populations (Figure 3). The  
222 germination rate of the natural population was very low, being of 16% at seven days and

223 20% at 30 days. In the natural population it was observed the presence of lethal alleles  
224 through the albino phenotypes, which were responsible for 6% of the seedlings germinated.

225

#### 226 *Pollen viability*

227 A total of 60,000 pollen grains from 30 plants from three introduced populations  
228 (CV, ME e PV) and 20,000 pollen grains from 10 plants from the natural population were  
229 analyzed. The pollen viability of the introduced populations ranged from 84.8% to 95.6%,  
230 with an overall mean of 92% ( $s \pm 24.0$ ), and was 52% ( $s \pm 47.9$ ) in the natural population.  
231 Significant difference ( $P < 0.001$ ) were found only among introduced and natural  
232 populations (data not shown). Much of pollen grains from introduced populations showed  
233 normal morphology and an average size of 2.5x3.0 mm, while the natural population  
234 presented an average size of 2.6x3.2 mm (Figure 1C-D).

235

236

## 237 **DISCUSSION**

238

#### 239 *Reproduction investment*

240 Sexual and vegetative reproduction may occur simultaneously in the same genet of  
241 natural and introduced populations of *D. distachya*. This combination of the reproductive  
242 systems, by seed production and clonal propagation, is common in the family  
243 Bromeliaceae (Benzing, 2000; Paggi et al., 2007; Hmeljevski et al., 2011; Büttow, 2012)  
244 and happens in many other plant species (Schmid et al., 1995; Silvertown, 2008; Eckert et  
245 al., 2009; Vallejo-Marín et al., 2010; Jacquemyn et al., 2012; Meiado, 2012; Ortega-Baes  
246 and Gorostiague, 2013). Sexual reproduction allows the maintenance of genetic variability  
247 over time by genic recombination, making possible the evolution of species and also  
248 generating individuals capable of developing into different environments (Richards, 1997;  
249 Ortega-Baes and Gorostiague, 2013).

250

251 The vegetative propagation through the clonal reproduction occurs in many species  
252 of bromeliads (Sarhou et al., 2001; Wendt et al., 2002; Canela and Sazima, 2003;  
253 Gonzalez-Astorga, 2004; Lenzi et al., 2006; Barbará et al.; 2009; Paggi et al., 2010; Wang  
254 et al., 2010; Hmeljevski et al., 2011; Zanella et al., 2011, Büttow, 2012). This reproductive  
system occurs with less energy loss, allowing the maintenance of genotypes in nature when

255 environmental conditions are unfavorable for sexual reproduction. However, this type of  
256 propagation decreases the genetic variability, which may interfere in the genetic structure  
257 of populations (Vallejo-Marín et al., 2010; Meiado, 2012; Ortega-Baes and Gorostiague,  
258 2013).

259 The genets of the introduced populations, that are being formed, seem to invest more  
260 in sexual reproduction when compared with natural population, although the natural  
261 population has been evaluated in only one year (Table 2). Comparing the natural with the  
262 introduced populations of *D. distachya*, appears to be a greater investment in vegetative  
263 reproduction in the established population than in the younger populations (Table 2). The  
264 clonal reproduction may be favored in older populations than in younger populations of the  
265 same species, suggesting that the absence of perturbations favors asexual reproduction  
266 (Silvertown, 2008). On the other hand, extreme situations, mainly related to habitat  
267 fragmentation, may affect negatively the sexual reproduction, favoring the species' clonal  
268 propagation (Vandepitte et al., 2009; Jacquemyn et al., 2012). Even, the propagation form  
269 can vary in different populations of the same species, depending on the environmental  
270 conditions of each site (Honnay and Jacquemyn, 2008; Vandepitte et al., 2009).

271 Although populations have been introduced just a few years, being the PV population  
272 introduced in 2006 and CV, PN and ME populations in 2009, in two years of observation  
273 they showed a slight trend in increasing the clonal investment over time, presenting the  
274 oldest introduced population (PV) the higher number of ramets per genet between years.

275

### 276 ***Reproductive success***

277 The overall mean production of flowers per inflorescence in the introduced  
278 populations was 30.7 ( $s \pm 14.9$ ), being considered low when compared to other rare species  
279 of bromeliads of the same genus and the same habitat rheophytic (Hmeljevski, 2007;  
280 Rogalski et al., 2009). This is in agreement with Wiesbauer (2008) who reported an  
281 average production of flowers per inflorescence of 41.3 in three populations of *D.*  
282 *distachya*, conserved *ex situ*, from the same region of origin of the introduced populations  
283 analyzed in this study. The flower production of the plants maintained in *ex situ* conditions  
284 was slightly higher, probably because they are kept in controlled environmental conditions.

285 A few rosettes of the populations studied, natural and introduced, presented more  
286 than one inflorescence in the flowering period (A. Janke, personal observation). This was

287 also observed in *ex situ* collections of the same species (Wiesbauer, 2008). Furthermore,  
288 the species is polycarpic (iteroparous), allowing an individual survives after a reproductive  
289 event, and may reproduce many times during their life cycle (Benzing, 2000). These  
290 elements may represent an advantage in sexual reproduction of *D. distachya* when  
291 favorable conditions occur.

292 The environment of the introduced populations differs from natural due to  
293 management performed by man. In the areas of introduction periodic maintenance are  
294 made through cleaning and fertilization practices. This enables a reduction in the  
295 competition between species and also an increase in the availability of nutrients. These  
296 factors may have favored increased investment in sexual reproduction in the introduced  
297 populations.

298 The average fruit set per inflorescence differed among studied populations, with  
299 lower fruit formation in the natural population SY, and the introduced populations CV and  
300 PV (Table 3). The introduced population ME presented the highest mean fruit set (60.9%),  
301 resembling the values obtained from Wiesbauer (2008) when studied populations of the  
302 same species in *ex situ* conditions (mean fruit set of 66.2%). Our results may be related to  
303 pollination. In the populations that showed lower fruit formation may be exhibiting  
304 deficiency pollinators. Also, the differences observed between populations may be due to  
305 habitat quality, related the capability of the environment to provide appropriate conditions  
306 for the persistence of the population (Hall et al., 1997; Uriarte et al., 2010), directly  
307 influencing the establishment and the reproductive success of the plants (Kéry and  
308 Matthies, 2004; Leimu et al., 2006; Paggi et al., 2007; Uriarte et al., 2010; De Crop et al.,  
309 2012; Hiebeler et al., 2013). Although all populations of our work have in common the  
310 origin of rheophytic environments, they showed differences in other environmental  
311 aspects, for example, the occurrence of insect predators and the period in which the plants  
312 remained submerged, which may have contributed for our results.

313 The seedling recruitment, which includes germination, emergence and establishment,  
314 is very important for plant population dynamics and for persistence of new populations,  
315 being a fundamental issue in the matter of conservation genetics (Amm et al., 2012;  
316 Wagenius et al., 2012). The low recruitment observed in the populations studied here was  
317 also observed for other bromeliads species in natural conditions (Marín et al., 2008).  
318 Abiotic factors seem to influence the establishment of seedlings, mainly the light

319 availability and habitat quality (Uriarte et al., 2010). Low levels of seedling recruitment  
320 may positively affect the amount of clonal diversity within populations, because in the  
321 sexual reproduction occurs the genetic recombination, and consequently the emergence of  
322 new genotypes (Watkinson and Powell, 1993).

323 The plants from introduced populations CV and PN had their leaves, flowers and  
324 capsules preyed by the caterpillar *S. rufofuscus*. Also, the area of the introduced population  
325 PV was propitious for flooding, probably hindering the pollinators foraging and the  
326 recruitment of new individuals, because the plants spent long periods submerged. On the  
327 other hand, the introduced population ME produced the greatest number of flowers and  
328 fruits per inflorescence, as well as it was observed the higher recruitment of new  
329 individuals from seeds. Probably the site of the ME population is that the most resembles  
330 to the natural rheophytic environment of this species.

331 The natural population SY showed lower results in all parameters analyzed for  
332 reproductive success when compared with the introduced populations. In this area may be  
333 occurring a greater investment in clonally for being an established population and also by a  
334 consequence of low habitat quality. The disturbances in this environment may be occurring  
335 due to changes in the hydrological regime of the region caused by the construction of  
336 hydroelectric power, having no more periods of flood and ebb defined, leading to a  
337 lowering in habitat quality and reduced pollination.

338 Despite having only one year of observation for the natural population, the  
339 differences found above must be repeated, since it were observed for all parameters  
340 studied.

341

#### 342 ***Seed viability and germination rate***

343 The seeds of the introduced populations analyzed were considered moderately viable,  
344 with an overall mean germination rate of 69% while the natural population was considered  
345 slightly viable, with a germination rate of 19.7%. The results obtained in the introduced  
346 populations are higher than the reported by Wiesbauer (2008) for populations of *D.*  
347 *distachya* with open pollination conserved *ex situ* (42.75%). In natural populations of *D.*  
348 *ibiramensis* the average germination rate reported was 94% (Hmeljevski, 2007) and *D.*  
349 *brevifolia* was 95.85% (Rogalski et al., 2009), being considered the seeds of these natural  
350 populations highly viable.

351 The presence of completely albino seedlings obtained from the germination of  
352 natural population SY may have occurred due to the existence of inbred crosses among  
353 individuals with the same genotype. This phenomenon was also observed in tree species of  
354 cross-pollination when occurs endogamic crosses, attributed to the presence of a single  
355 recessive gene (Squillace and Kraus, 1963).

356 Conversely, the chloroplast deficiency has also been observed in progenies obtained  
357 by crosses between hybrids, being caused by plastome-genome incompatibility. This fact  
358 has been studied in interspecific hybrids of azalea, where albino seedlings occurred due to  
359 plastome-genome incompatibility between the plastid genome from evergreen azalea  
360 (*Rhododendron kiusianum* x *Rhododendron eriocarpum*, maternal plastome) and the  
361 nuclear genome from deciduous azalea (*Rhododendron japonicum* f. *flavum*) (Ureshino et  
362 al., 1999; Kobayashi et al., 2013).

363 The occurrence of interspecific crosses in natural population SY can not be fully  
364 discharged since occur other bromeliads' species near that location, for example *Dyckia*  
365 *tuberosa*. This bromeliad has the same flowering period and possesses at least one  
366 common pollinator, the hummingbird (Vosgueritchian and Buzato, 2006). The interspecific  
367 crosses could also have contributed to a decrease in seed viability of SY population,  
368 although this hypothesis had not been tested yet.

369

### 370 ***Pollen viability***

371 The pollen grains of all introduced populations studied were highly viable,  
372 presenting an average viability of 92%. Similar results were reported for *Vriesea gigantea*  
373 and *Aechmea winkleri*, other bromeliads species (Palma-Silva et al., 2008; Büttow, 2012).

374 The natural population SY showed the lowest pollen viability (52%), with some  
375 plants that produced pollen grains that failed to stain (Figure 1). The inbreeding depression  
376 may contribute to an increase in pollen infertility (Goodwillie, 2000; Goodwillie and  
377 Knight, 2006). Abiotic factors may also be related to pollen inviability, including pollen  
378 age, temperature and humidity (Kelly et al., 2002; Zhou, 2013).

379 In addition, an inadequate quality or quantity of pollen can decrease the  
380 reproductive success of a species. The term used to describe this event is known as pollen  
381 limitation (Ashman et al., 2004). The pollen quality can be reduced if self-fertilization and  
382 matings between related plants occurs, and pollen quantity may be reduced as a result of

383 fewer pollinator visits or less pollen delivered per visit (Ashman et al., 2004; Aizen and  
384 Harder, 2007; Harder and Aizen, 2010).

385 The pollen quality may also hamper the seed production, mainly in outcrossing  
386 species, because during seed development they can exhibit strong inbreeding depression,  
387 what must frequently decrease seed set. Probably this occurs because embryos  
388 homozygous for deleterious alleles die during development (Aizen and Harder, 2007). This  
389 question may have influenced the low viability of seeds found in SY population.

390

#### 391 ***Conservation of Dyckia distachya in nature***

392 Our findings suggest that the natural population SY is investing more in clonal  
393 reproduction than in sexual, leading to a decrease in flowers, fruits and seed production  
394 and lower viability of seeds and pollen. This reduction in fitness traits can be related to  
395 lower habitat quality or inbreeding depression (Kéry and Matthies, 2004; Silvertown,  
396 2008). There are situations where plants are able to grow and reproduce clonally but not  
397 sexually. This occurs due the vegetative progeny tend to be better established in nature  
398 than seeds, because they start their lives as miniatures of their mothers, already complete  
399 with their own roots (Silvertown, 2008).

400 In addition, isolated populations, like SY, generally have a higher risk of extinction  
401 because of reduced reproduction and increased crosses between related individuals, what  
402 may result in inbreeding depression and decreased vigor of plants (Kéry and Matthies,  
403 2004). The presence of albino seedlings may be an indication that inbreeding depression is  
404 occurring in the natural population of *D. distachya*. The inbreeding depression may be one  
405 of the major problems currently faced by populations of plants (Angeloni et al., 2011).

406 Nonetheless, the natural population SY might be considered viable, having been  
407 observed a small recruitment of seedlings and occurring the maintenance of the genets by  
408 clonal reproduction. However, this population can be undergoing a disturbance period,  
409 being necessary more studies to verify the viability of this population in the long term. If  
410 natural regeneration is not sufficiently effective for the conservation of genetic diversity  
411 and the maintenance of natural population the introducing of genetically compatible  
412 individuals should be considered.

413 The introduction of species in nature is an important method of preservation, and  
414 should be taken into account when there are favorable conditions for the reestablishment of

415 the plant in natural conditions (Thorton et al., 2008; Godefroid et al., 2011). The main  
416 objective is to establish self-sustaining populations, with wide genetic diversity and high  
417 reproductive fitness in their natural habitat (Frankham et al., 2009).

418 The success of an introduction may be assessed by the ability to realize the basic  
419 processes of the plant life cycle, like establishment, reproduction and dissemination.  
420 Moreover, the recruitment of new individuals by seeds should also be noted (Pavlik, 1996).  
421 Our results indicate that the introduced populations of *D. distachya* are viable, showing  
422 higher values of reproductive success and more recruitment of new individuals by seeds  
423 when compared to the natural population. The sexual reproduction is crucial for long term  
424 persistence of populations, because it represents the opportunity to increase or maintain the  
425 genetic diversity (Aguilar et al., 2006; Silvertown, 2008).

426 We also showed that there are differences among introduced populations, probably  
427 due to habitat quality of each place. Some areas still need human intervention to continue  
428 existing, like the areas PN and CV. Thus, continuous monitoring and management of  
429 recently created populations are needed to ensure the successful establishment and  
430 persistence of these populations, especially for species that are slow to reach reproductive  
431 maturity (Cochrane, 2004).

432 Finally, our study suggests that the introduced populations of this bromeliad can play  
433 an important role in the conservation and should be considered in management strategies  
434 for this endangered species. Future studies should be carried out to verify the performance  
435 of these populations in long term, as well as the establishment of seedlings.

436  
437

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- 662  
663
- 664 **BIOSKETCH** This study is part of Aline Janke's doctoral research on the conservation of  
665 *Dyckia distachya*, carried out at the Universidade Federal do Rio Grande do Sul. The

666 authors of this paper share interests in the genetics and conservation of the Brazilian  
667 Atlantic Forest plant species, with main emphasis on the family Bromeliaceae. We use  
668 different approaches and tools to resolve issues related to biology, ecology, genetics and  
669 evolution of species of this taxonomic group (<http://www.ufrgs.br/ngcp/>).  
670



671 **List of Tables**

672 **Table 1** Location of the five studied populations of *Dyckia distachya*.

673

Population	Location
Cachoeira dos Varões (CV) <sup>*</sup>	28°00'01.8" S, 50°46'38.1" W
Ponte Nova (PN) <sup>*</sup>	28°01'12.5" S, 50°47'21.5" W
Ponte Velha (PV) <sup>*</sup>	28°02'30.5" S, 50°48'18.5" W
Miniencanadinhos (ME) <sup>*</sup>	27°58'48.2" S, 50°39'06.1" W
Salto do Yucumã (SY) <sup>**</sup>	27°9'37.3" S, 53°53'27.2" W

674 <sup>\*</sup>Introduced population

675 <sup>\*\*</sup>Natural population

676

677 **Table 2** Reproduction investment: Mean size of the genet (m<sup>2</sup>), mean number of ramets per  
678 genet and percentage of reproductive individuals per genet evaluated in two years (2010  
679 and 2011) in four introduced populations and in one year (2010) in the only know natural  
680 population of *Dyckia distachya*. The means are shown with standard deviation ( $\pm$  s).

681

Population	Year	Mean size of the genet (m <sup>2</sup> )	Mean number of ramets/genet	Percentage of reproductive individuals/genet
CV <sup>†</sup>	2010	0.083 <sup>a*C**</sup>	7.0 $\pm$ 6.0 <sup>aB</sup>	54.0 $\pm$ 23.6 <sup>aBC</sup>
	2011	0.097 <sup>aC</sup>	8.0 $\pm$ 6.0 <sup>aB</sup>	49.8 $\pm$ 23.5 <sup>aBC</sup>
PN	2010	0.064 <sup>aC</sup>	1.9 $\pm$ 1.0 <sup>aD</sup>	76.3 $\pm$ 29.8 <sup>aA</sup>
	2011	0.084 <sup>aC</sup>	2.5 $\pm$ 3.0 <sup>aD</sup>	78.9 $\pm$ 30.8 <sup>aA</sup>
PV	2010	0.191 <sup>aB</sup>	6.2 $\pm$ 5.8 <sup>aB</sup>	48.7 $\pm$ 29.9 <sup>aC</sup>
	2011	0.236 <sup>aB</sup>	12.5 $\pm$ 8.7 <sup>bB</sup>	41.6 $\pm$ 25.6 <sup>aC</sup>
ME	2010	0.055 <sup>aC</sup>	2.7 $\pm$ 1.6 <sup>aC</sup>	69.6 $\pm$ 30.6 <sup>aAB</sup>
	2011	0.067 <sup>aC</sup>	5.3 $\pm$ 4.0 <sup>aC</sup>	61.9 $\pm$ 32.0 <sup>aAB</sup>
SY	2010	2.172 <sup>A</sup>	297.6 $\pm$ 226.9 <sup>A</sup>	10.4 $\pm$ 11.3 <sup>D</sup>

682 <sup>†</sup>For locality abbreviations see Table 1.

683 \* Means followed by the same letter in the column do not differ by Tukey test (P>0.05) between the years  
684 within the population.

685 \*\* Means followed by the same capital letter in the column do not differ by Tukey (P>0.05) among  
686 populations.

687

688 **Table 3** Reproductive success: Mean number of flowers per inflorescence and percentage  
689 of fruit set per inflorescence evaluated in two years (2010 and 2011) in four introduced  
690 populations and in one year (2010) in the only know natural population of *Dyckia*  
691 *distachya*. The means are shown with standard deviation ( $\pm$  s).  
692

Population	Year	Mean number of flowers/ inflorescence	Percentage of fruit set/ inflorescence
CV <sup>†</sup>	2010	32.1 $\pm$ 10.2 <sup>aBC</sup>	21.7 $\pm$ 6.2 <sup>aC</sup>
	2011	29.8 $\pm$ 10.4 <sup>aBC</sup>	17.1 $\pm$ 7.5 <sup>aC</sup>
PN	2010	26.7 $\pm$ 10.2 <sup>aC</sup>	48.7 $\pm$ 20.6 <sup>aB</sup>
	2011	29.7 $\pm$ 7.9 <sup>aC</sup>	52.2 $\pm$ 16.0 <sup>aB</sup>
PV	2010	38.8 $\pm$ 15.2 <sup>aA</sup>	18.9 $\pm$ 7.0 <sup>aC</sup>
	2011	39.3 $\pm$ 5.8 <sup>aA</sup>	20.9 $\pm$ 5.0 <sup>aC</sup>
ME	2010	41.3 $\pm$ 12.9 <sup>aAB</sup>	74.3 $\pm$ 13.6 <sup>aA</sup>
	2011	32.9 $\pm$ 10.6 <sup>aAB</sup>	50.7 $\pm$ 10.3 <sup>bA</sup>
SY	2010	2.7 $\pm$ 4.2 <sup>D</sup>	5.9 $\pm$ 8.0 <sup>D</sup>

693 <sup>†</sup>For locality abbreviations see Table 1.

694 \* Means followed by the same letter in the column do not differ by Tukey test ( $P > 0.05$ ) between the years  
695 within the population.

696 \*\* Means followed by the same capital letter in the column do not differ by Tukey ( $P > 0.05$ ) among  
697 populations.

698

699 **Figure legends**

700 **Figure 1** *Dyckia distachya*: (A) clump in natural population, SY; (B) introduced  
701 population, CV; (C) pollen grains of natural population, SY; (D) pollen grains of  
702 introduced population, ME; (E) seedling, ME. Scale bar = 10  $\mu$ m.

703

704 **Figure 2** Study region: (A) Map of Latin America including Brazil. Showing in detail the  
705 location of natural and introduced populations of *Dyckia distachya* studied; (B) Detail of  
706 the location of the introduced populations.

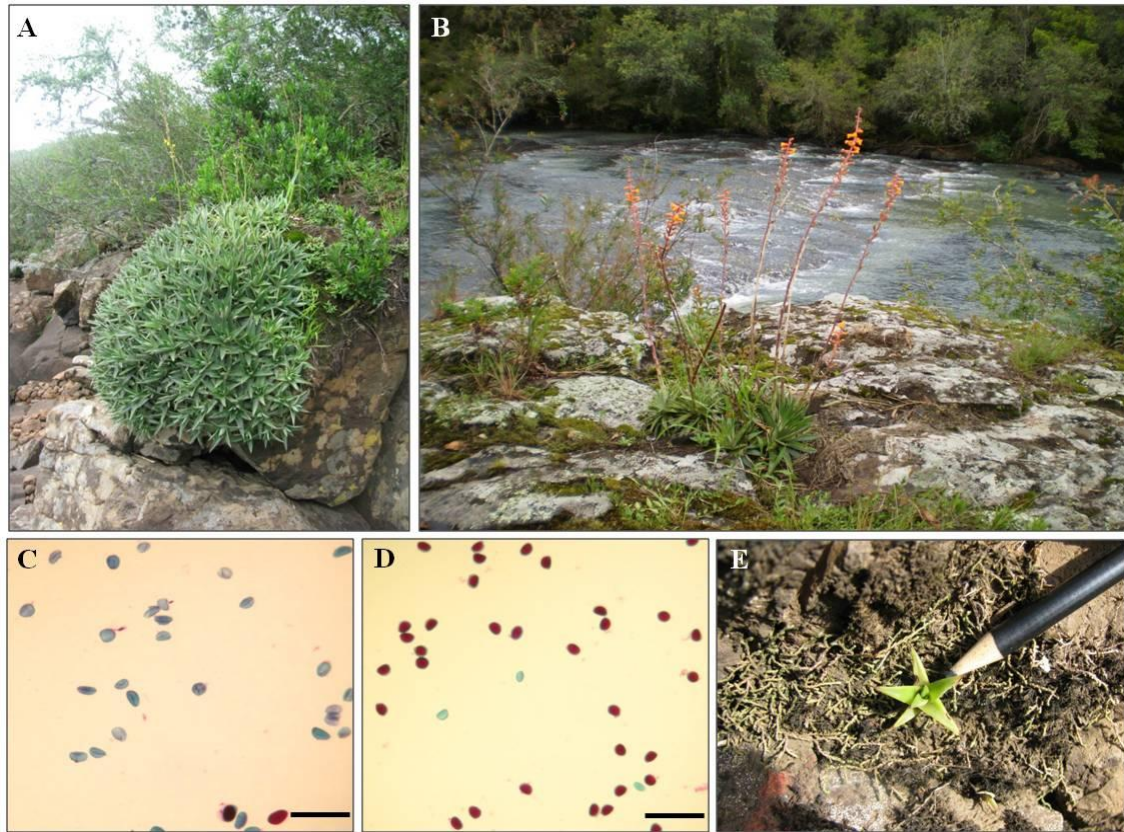
707

708 **Figure 3** Mean number of seeds per capsule (above) and germination rate (below) of seeds  
709 of *Dyckia distachya*, estimated for 200 capsules from four introduced and one natural  
710 population in the reproductive season of the year of 2010. The means are shown with  
711 standard deviation. Columns with the same letter are not significantly different by the  
712 Tukey test ( $P>0.05$ ).

713

714

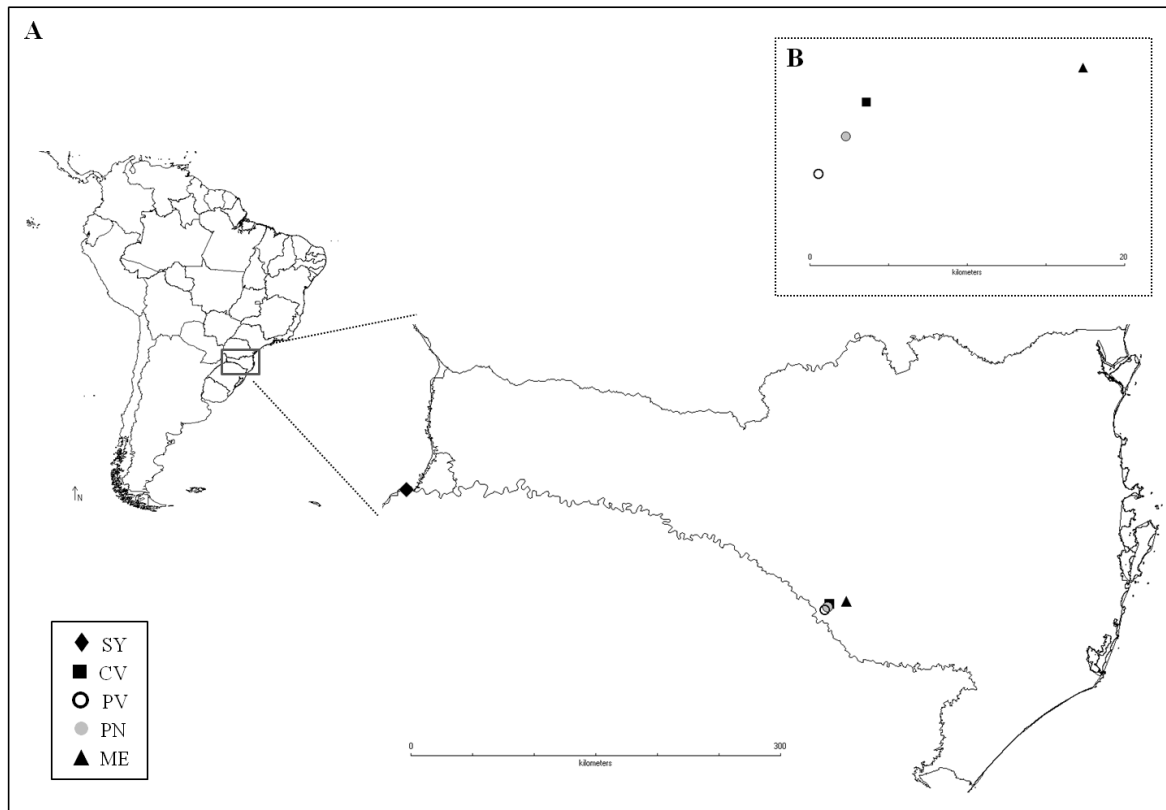
715 **Figure 1**



716

717

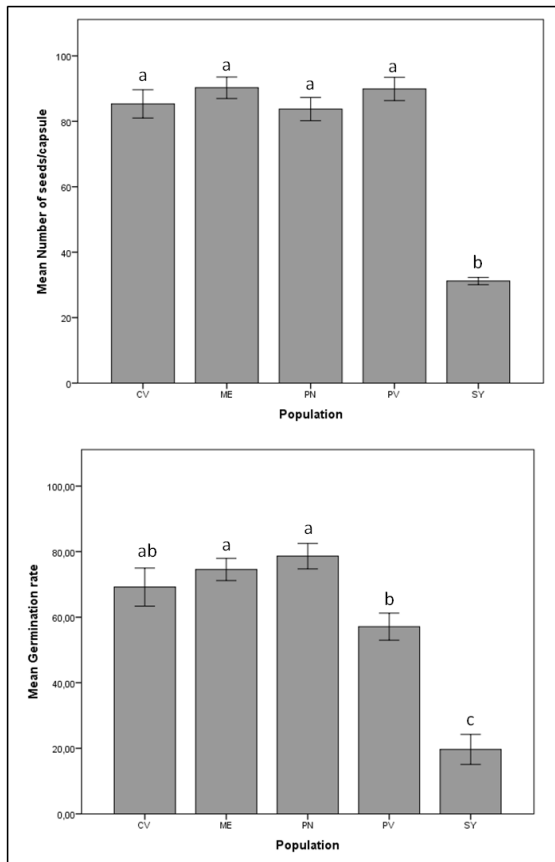
718 **Figure 2**



719 For locality abbreviations see Table 1.

720

721 **Figure 3**



722



### **Capítulo III**

*Conservation genetics of natural and introduced populations of  
Dyckia distachya, an endangered bromeliad*

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Artigo a ser submetido para o periódico *Conservation Genetics*



1 **Original article**

2 Conservation genetics of natural and introduced populations of *Dyckia distachya*, an  
3 endangered bromeliad

4

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16

17 **ABSTRACT**

18 Knowledge of parameters related to genetic variability of endangered and introduced  
19 populations is essential for species conservation programs. We used microsatellites to  
20 investigate the genetic diversity, the mating system and the pollen flow in four introduced  
21 populations and in the only known wild population of *Dyckia distachya*. Introduced  
22 populations showed high levels of genetic diversity compared to the natural population.  
23 The outcrossing rate was higher in introduced populations ( $\hat{t}_m = 0.927$  to  $1.007$ ), indicating  
24 that *D. distachya* is alogamous, and lowest in the natural ( $\hat{t}_m = 0.520$ ), suggesting the  
25 occurrence of some disturbance in that population. The estimate of genetic structure of  
26 pollen pool was higher in the natural ( $\hat{\phi}_{FT} = 0.296$ ) than in the introduced ( $\hat{\phi}_{FT}$  = average  
27 of  $0.089$ ) populations, indicating that pollen flow is more restricted in the wild. The  
28 effective number of pollen donors in introduced populations ( $\hat{N}_{ep} = 5.5$ ) was higher than  
29 that found in the wild population ( $\hat{N}_{ep} = 1.7$ ). Our study also shows that pollen of this  
30 bromeliad is dispersed in short distances and seems to be influenced by the combination of  
31 density and spatial distribution of reproductive individuals. Based on these results, the

32 introduced populations seem to be successful, while the natural seems to be undergoing  
33 some kind of threat.

34

35 **Key words:** *endemic plant, genetic diversity, introduction, mating system, pollen flow*

36

37 *Dyckia distachya* is classified as endangered according to the Official List of Brazilian  
38 Endangered Species (Ministério do Meio Ambiente, 2008), in consequence of its peculiar  
39 environment, that have been exploited for hydroelectric construction and energy  
40 production. This bromeliad takes place exclusively in rheophytic environments, being  
41 subject to the strong river currents and submerged in the flood season and severe droughts  
42 and sun exposure during ebb periods (Reitz, 1983). In an attempt to prevent the  
43 disappearance of this species, introductions are being carried out on similar places to its  
44 natural occurrence, since it is currently known only one population of this bromeliad in the  
45 wild (Meio Biótico Consultoria, 2009).

46 Introduction is the establishment of a species or a population in a new ecosystem,  
47 similar to its naturally occurring environment, and can be considered a possibility for the  
48 conservation of a species when there are no more suitable sites for reintroduction into their  
49 natural distribution area (Armstrong and Seddon, 2008; IUCN, 2013). The main goal of  
50 rare plant introductions is to establish viable and self-sustaining populations that have  
51 sufficient genetic resources necessary to undergo adaptive evolutionary changes  
52 (Frankham et al., 2009). The results of this conservation strategy are positively influenced  
53 when individuals with high genetic diversity, low coefficient of inbreeding and good  
54 reproductive potential are used (Frankham et al., 2009; Godefroid et al., 2011).

55 The genetic monitoring of introduced populations is an essential method for  
56 assessment the success of the establishment of new populations (McGlaughlin et al., 2002).  
57 Molecular markers, particularly those that employ the polymerase chain reaction, are  
58 powerful tools that mainly allow access the genetic variability, analyze the mating system  
59 and estimate the gene flow (Faleiro, 2007). Microsatellite markers have proved to be a  
60 powerful tool for estimation the genetic diversity (Kalia et al., 2011), being used in studies  
61 of endangered populations (Shapcott and Powell, 2011; Hou et al.; 2012).

62 The mating system is a key factor that influences the degree of inbreeding and the  
63 spatial genetic structure within and among populations (Wang et al., 2010), being its

64 knowledge essential in introduction programs (Godefroid et al., 2011). It is widely  
65 examined based on the mixed mating system model, as implemented in MLTR software,  
66 where a portion of the progeny is derived from selfing and the remainder of random mating  
67 (Ritland, 2002). These crosses may be assigned by estimating the rate of cross-pollination,  
68 the outcrossing rate between parenting and the likelihood of two siblings have the same  
69 father (Ritland and Jain, 1981). The estimates of the probability of two siblings have the  
70 same father can highlight the extent of diversity of the pool of pollen donors and also the  
71 effective number of pollen donors per mother plant (Ritland, 1989).

72         The gene flow is the total gene movement among individuals and populations, being  
73 mainly determined by pollen dispersal in many plant species (Storfer, 1999). It determines  
74 the genetic structure of populations, prevents population isolation, while maintaining the  
75 genetic variation and avoiding the inbreeding depression (Storfer, 1999; Frankham et al.,  
76 2009). The TwoGener analysis is an indirect method to estimate contemporary gene flow  
77 using only the maternal and offspring genotypes. This analysis allows us to quantify the  
78 heterogeneity in the pollen pool sampled by mother plants distributed across the landscape  
79 and also estimate the average distance and the effective area of pollination (Austerlitz and  
80 Smouse, 2001; Smouse et al., 2001).

81         In this study we evaluated the genetic diversity between generations, the matting  
82 patterns, and the contemporary pollen dispersal of four introduced populations and in the  
83 only known natural population of *Dyckia distachya*, using eight nuclear microsatellite loci.  
84 The specific aims were: (1) to estimate the genetic diversity of adult individuals and  
85 seedlings from the five studied populations; (2) evaluate the mating patterns comparing  
86 introduced and the natural populations; (3) assess the contemporary pollen flow; and (4)  
87 recommend effective strategies for conservation of this endangered species. The results  
88 achieved here will provide a better understand regarding introductions and may help in  
89 future conservation programs.

90

91

## 92         **MATERIALS AND METHODS**

93

### 94         *Study species and study area*

95 *Dyckia distachya* Hassler (Bromeliaceae) originally occurred on islands and rocky  
96 shores of the Uruguay River Basin, having disjunct distribution over 617 km in Pelotas and  
97 Uruguay rivers, bordering the states of Rio Grande do Sul and Santa Catarina, Southern  
98 Brazil (Reis et al, 2005). However, due to the exploitation of the hydroelectric potential of  
99 this basin, three hydroelectric plants were built in just five years, leading to extinction of  
100 seven of the eight known populations of *D. distachya* in nature.

101 Before the flooding of the hydroelectric lakes occurred the rescue of several  
102 specimens of *D. distachya*. This action was taken with the purpose of preserve the species  
103 in *ex situ* collections and also use them in later introductions in nature (Reis et al., 2005).  
104 Some introductions have already been carried out in similar areas of naturally occurring,  
105 but, not all the populations were successful (Meio Biótico Consultoria, 2009).

106 During the construction of the last hydroelectric plant (Energética Barra Grande  
107 S/A), several individuals of *D. distachya* were rescued and conserved *ex situ*. On that  
108 occasion it was thought that those were the only places of occurrence, being unknown the  
109 population located in Salto do Yucumã, boundary between Brazil and Argentina. Around  
110 18,700 individuals from 75 clumps were rescued in the influence area of this hydroelectric,  
111 belonging to three populations: Pedras Brancas/Gateados (3,977 specimens - 48 clumps),  
112 Encanados (14,063 specimens - 22 clumps) and Encanadinhos (683 specimens - 5 clumps).  
113 These bromeliads were kept in nurseries and later, part of them was introduced into nature  
114 (Meio Biótico Consultoria, 2009).

115 *Dyckia distachya* is cross pollinated, self-incompatible and polycarpic, showing  
116 annual flowering between September and January and fruit maturation from October to  
117 February (Wiesbauer, 2008). The mainly pollinators are bees (*Bombus atratus* and  
118 *Xilocopa* spp.) and hummingbirds (*Chlorostilbon lucidus*) (Wiesbauer, 2008). Seeds are  
119 dispersed by anemochory and hydrochory (Wiesbauer, 2008), also presenting clonal  
120 propagation, resulting in the formation of dense clumps (Reitz, 1983).

121 This study was conducted in four introduced areas located in Campo Belo do Sul  
122 county, Santa Catarina state, in the following populations: Cachoeira dos Varões (CV;  
123 28°00'01,8" S, 50°46'38.1" W), with individuals from the extinct population Encanadinhos  
124 (ES; 28°10'24.2" S, 50°47'3.5" W); Miniencanadinhos (ME; 27°58'48.2" S, 50°39'06.1"  
125 W), with individuals from the extinct Pedras Brancas/Gateados (PB; 28°0'27.6" S,  
126 50°55'24.5" W); Ponte Velha (PV; 28°02'30.5" S, 50°48'18.5" W), with individuals from

127 extinct Encanados population (EN; 28°6'34.3" S, 50°52'3.7" W); and Ponte Nova (PN;  
128 28°01'12.5" S, 50°47'21.5" W) consisting of a mixture of the three extinct populations  
129 (Figure 1).

130 For a comparative matter the only known natural population was also included  
131 (Figure 1). Salto do Yucumã (SY; 27°9'37.3" S, 53°53'27.2" W), is located in protected  
132 natural areas, being situated in Brazil in the Parque Florestal Estadual do Turvo, Rio  
133 Grande do Sul state and in Argentina it is located in Parque Provincial Moconá, Misiones  
134 province. Geographical distances between the four introduced populations are three to 17  
135 km and around 330 km distant from the natural population.

136

### 137 *Sample collection*

138 Leaves from all reproductive individuals found of each studied population, were  
139 collected, totaling 104 individuals from four introduced populations and 26 from natural  
140 population. The sampling strategy used was to collect only one individual per clump, in an  
141 attempt to avoid sampling the same genet, because the genetic similarity within each clump  
142 is unknown. The leaves collected were dried and stored in silica gel before DNA  
143 extraction.

144 Four mature capsules were also collected randomly from five to 15 mother plants  
145 among the reproductive individuals from each population, totaling 40 mother plants (MP)  
146 for the introduced populations and ten for the natural population (Table 1). We define the  
147 reproductive individuals as those who emitted at least one inflorescence and mother plants  
148 as those who provided the seeds to generate the offspring.

149 In order to obtain the seedlings, the seeds were disinfected with hypochlorite and  
150 placed in petri dishes containing ½ MS medium salts (Murashige and Skoog, 1962), B5  
151 vitamins (Gamborg et al., 1968), 3% sucrose and 0.3% Phytigel (Sigma, Saint Louis, MO,  
152 USA) with pH 6.4. The petri dishes were incubated in a climate-control chamber with  
153 relative humidity near 100% and photoperiod of 16 hours light at 25°C and 8 hours dark at  
154 22°C for six months. For the analysis we used 15 seedlings for each mother plant from the  
155 introduced populations, and ten seedlings from the natural, totaling 700 seedlings.

156 The spatial positions (Figure 1) of all sampled individuals in the five areas studied  
157 were recorded. The density of reproductive individuals was estimated by the spatial  
158 distribution of the reproductive rosettes in each studied area.

159

160 ***DNA extraction and microsatellite analysis***

161 Total genomic DNA was extracted from 130 reproductive individuals and 700  
162 seedlings using a cetyl trimethylammonium bromide protocol based on the method of  
163 Doyle & Doyle (1990). Three microsatellite loci isolated for *D. distachya* (loci Dd07,  
164 Dd10 and Dd20; Zanella et al., 2012) and five loci previously characterized for other  
165 bromeliad species that were successfully amplified in *D. distachya* (*Ananas comosus*: loci  
166 Acom\_119.1 and Acom\_71.3; Wöhrmann and Weising, 2011; *Aechmea caudata*: loci  
167 Ac11 and Ac25; Goetze et al., 2013; *Dyckia marnier-lapostollei*: loci ngDy\_24;  
168 Wöhrmann et al., 2013) were used in this study. For the eight nuclear microsatellites, the  
169 forward primers were synthesized with a 19-bp M13 tail (5'-  
170 CACGACGTTGTAACGAC-3') at the 5' end to allow labeling with a tailed fluorescent  
171 dye M13 primer during genotyping procedures, following the method of Schuelke (2000).

172 The polymerase chain reaction (PCR) amplifications were conducted according to  
173 Zanella et al. (2012) and were performed in a TC-412 thermal cycler (Techne, Burlington,  
174 NJ, USA). The microsatellite alleles were resolved on a ABI 3100 DNA Analyzer  
175 Sequencer (Applied Biosystems, Foster City, CA, USA) and sized against the GS500 LIZ  
176 molecular size standard (Applied Biosystems, Foster City, CA, USA) using GeneMarker  
177 Demo version 2.4.0 (SoftGenetics, State College, PA, USA).

178

179 ***Genetic diversity analyses***

180 The genetic diversity indices: mean number of alleles ( $\hat{A}$ ), allelic richness ( $\hat{A}_r$ ),  
181 expected heterozygosity ( $\hat{H}_E$ ), observed heterozygosity ( $\hat{H}_O$ ) and the fixation index ( $\hat{F}_{IS}$ )  
182 (Weir and Cockerham, 1984), were calculated for reproductive individuals and seedlings  
183 from each population, using the programs MSA 4.00 (Dieringer and Schlötterer, 2003) and  
184 FSTAT version 2.9.3.2 (Goudet, 1995). Exact tests for deviation of  $\hat{F}_{IS}$  from Hardy–  
185 Weinberg Equilibrium (HWE) expectations was performed with the software Genepop 4.2  
186 (Rousset, 2008). The following indices:  $\hat{A}$ ,  $\hat{A}_r$ ,  $\hat{H}_E$  and  $\hat{H}_O$  were analyzed by the nested  
187 analysis of variance followed by the Tukey test to determine if the differences among  
188 populations, adults and seedlings were significant ( $P < 0.01$ ), using SAS software package  
189 (Version 9.3, SAS Institute, Cary, North Carolina, USA).

190 Genetic differentiation was investigated separately among all adults and seedlings  
191 populations by the standardized  $G'_{ST}$  statistic (Hedrick, 2005) with the program GenAIEx  
192 6.5 (Peakall and Smouse, 2006, 2012). Pairwise comparisons of  $\hat{F}'_{ST}$  between populations  
193 were estimated using the program GenAIEx 6.5 (Peakall and Smouse, 2006, 2012).  
194 Partitioning of genetic diversity within and among populations was examined by analysis  
195 of molecular variance (AMOVA) implemented in the software GenAIEx 6.5 (Peakall and  
196 Smouse, 2006, 2012). The hypothesis that populations are differentiated because of  
197 isolation-by-distance (Wright, 1965) was tested by calculating the correlation between  
198 geographic and genetic distance matrices ( $F_{ST}$ ) with a standardized Mantel test using  
199 Genepop 4.2 (Rousset, 2008). The significance was assessed through a randomization test  
200 using 10,000 Monte Carlo simulations.

201

### 202 ***Mating system***

203 Estimates of outcrossing rate and inbreeding were calculated for all populations with  
204 the program MLTR 3.4 (Ritland, 2002), based on mixed and correlated mating model. The  
205 model assumes that (a) each mating represents a random event of an outcross or a self-  
206 fertilization, with probabilities equal to  $t$  and  $(1-t)$ , respectively; (b) no selection and no  
207 mutation following fertilization may occur; (3) there is no assortative mating or variability  
208 in pollen pool frequencies (Ritland and Jain, 1981). The estimated parameters were the  
209 multilocus outcrossing rate ( $\hat{t}_m$ ), single-locus outcrossing rate ( $\hat{t}_s$ ), biparental inbreeding  
210 rate ( $\hat{t}_m - \hat{t}_s$ ), multilocus correlated paternity ( $\hat{r}_{p(m)}$ ) and the single-locus correlated  
211 paternity ( $\hat{r}_{p(s)}$ ). The effective number of pollen donors ( $1/\hat{r}_{p(m)}$ ), full ( $\hat{t}_m * \hat{r}_{p(m)}$ ) and half  
212 sibs [ $\hat{t}_m (1 - \hat{r}_{p(m)})$ ] were also estimated (Ritland and Jain 1981; Ritland 2002). Standard  
213 errors were estimated based on 1.000 bootstraps between individuals within a progeny  
214 array. We also tested the significance level of  $\hat{t}_m$ ,  $\hat{t}_m - \hat{t}_s$  and  $\hat{r}_p$  by a one-tailed Student's  $T$ -  
215 test based on the null hypothesis that  $\hat{t}_m = 1$ ,  $\hat{t}_m - \hat{t}_s > 0$  and  $\hat{r}_p = 0$ . Statistical analyzes  
216 were performed using the SAS software package (Version 9.3, SAS Institute, Cary, North  
217 Carolina, USA). The coefficient of correlation among offspring within progenies was  
218 estimated as  $\hat{r}_{xy} = 0.25 (1 + \hat{F}_p)[4s + (\hat{t}_m^2 + s\hat{t}_m\hat{r}_s)(1 + \hat{r}_{p(m)})]$ , being  $\hat{F}_p$  the coefficient of  
219 inbreeding in parental generation and  $s$  the selfing rate ( $s = 1 - \hat{t}_m$ ) (Ritland, 1989). The  
220 coancestry coefficient within progenies was estimated from  $\hat{r}_{xy}$  as  $\hat{\theta}_{xy} = \hat{r}_{xy}/2$  (Sebbenn,  
221 2002). Lastly, we estimated the variance effective size as  $\hat{N}_{ev} = 0.5/\hat{\theta}_{xy}$  (Cockerham, 1969).

222

223 ***Pollen gene pool analysis***

224 The TwoGener analysis (Smouse et al., 2001) was carried out for the progeny of all  
225 populations with the software POLDISP 1.0c (Robledo-Arnuncio et al., 2007) in order to  
226 estimate the contemporary pollen flow. This method is based on the genetic structure ( $\hat{\phi}_{FT}$ )  
227 of the pollen pool of all mother plants spaced across each population, providing estimates  
228 of dispersion related to an event of pollination (Smouse et al., 2001). The pollen dispersal  
229 distance was estimated assuming a normal dispersal curve ( $\hat{\delta}$ ) and using the density of  
230 reproductive individuals based on field observations. The number of pollen donors [ $\hat{N}_{ep} =$   
231  $1/(2*\hat{\phi}_{FT})$ ], the effective neighborhood pollination area ( $\hat{A}_{ep} = 4.\pi.\hat{\delta}^2$ ) and the radius ( $\hat{r}_{ep} =$   
232  $\sqrt{\hat{A}_{ep}/\pi}$ ) were also inferred from the TwoGener analysis (Smouse et al., 2001, Fernández-  
233 Manjarrés et al., 2006). We also tested the significance level of  $\hat{\phi}_{FT}$  by a one-tailed  
234 Student's *T*-test based on the null hypothesis that  $\hat{\phi}_{FT} = 0$  (SAS, version 9.3).

235

236

237 **RESULTS**

238

239 ***Genetic diversity***

240 The number of alleles per locus for adult populations ranged from 3 to 7 (average of  
241 5.125 alleles), with a total of 41 alleles and for offspring the number of alleles per locus  
242 ranged from 4 to 7 (average of 5.5 alleles), with a total of 44 alleles (Supplemental Table  
243 1). The number of alleles and the allelic richness were similar between adults and offspring  
244 considering each population (Table 2). The observed heterozigosity ranged from 0.331 to  
245 0.525 (average of 0.466) in the adults of the introduced populations and was of 0.167 in the  
246 natural population. For the offspring this parameter varied from 0.371 to 0.573 (average of  
247 0.458) in the introduced populations and was of 0.147 in the natural population. For the  
248 adults of the introduced populations the expected heterozigosity ranged from 0.416 to  
249 0.591 (average of 0.523) and was of 0.169 in the natural. For seedlings this value ranged  
250 from 0.396 to 0.591 (average of 0.494) in the introduced and was of 0.183 in the natural  
251 population (Table 2). No significant differences were observed for the number of alleles,  
252 allelic richness, observed and expected heterozigosity between generations, only between  
253 introduced and natural populations ( $P < 0.01$ ). The fixation index was low and ranged from



254 -0.049 to 0.182 in adult populations and ranged from 0.031 to 0.196 in offspring. Almost  
255 all populations departed significantly from HWE, except the SY adult population (Table  
256 2). Comparing adult and offspring populations, the estimates of fixation index showed  
257 higher inbreeding in progeny of PN and SY populations and in the adult populations CV,  
258 ME and PV (Table 2). The estimate of genetic differentiation ( $\hat{G}'_{ST}$ ) among all adults from  
259 the different populations analyzed was 0.395 and for all offspring populations  $\hat{G}'_{ST}$  was  
260 0.436. The pairwise  $\hat{F}'_{ST}$  values also revealed high genetic structure, ranging from 0.104 to  
261 0.554 (Supplemental Table 2). No correlation between genetic and geographic distances  
262 was detected in the Mantel test considering all populations ( $r^2 = 0.7013$ ,  $P = 0.130$ ),  
263 indicating the absence of isolation by distance among them. AMOVA results indicate that  
264 the majority of the genetic variation resides within populations (62%,  $P < 0.0001$ ), and  
265 38% is found among populations.

266

### 267 *Mating system*

268 Mating system parameters estimated for all populations studied are summarized in  
269 Table 3. The multilocus outcrossing rate was high in the four introduced populations ( $\hat{t}_m =$   
270 0.927 – 1.007), and did not differ significantly from 1. Moreover, in the SY population,  
271 this value was lower ( $\hat{t}_m = 0.520$ ), being significantly different from 1 ( $P < 0.01$ ).  
272 Estimates of biparental inbreeding ( $\hat{t}_m - \hat{t}_s$ ) in the populations CV (0.075), ME (0.132), PV  
273 (0.092) and SY (0.065) were significantly greater than zero ( $P < 0.05$ ), indicating the  
274 presence of a moderate proportion of related mating. Paternity correlation was relatively  
275 high ( $\hat{r}_{p(m)} = 0.126 - 0.577$ ) and significant ( $P < 0.05$ ), indicating that some individuals have  
276 been performed better than others. The effective number of pollen donors ( $\hat{N}_{ep}$ ), estimated  
277 from paternity correlation ranged from 5.3 to 7.9 (average of 6.5) in introduced  
278 populations, and was 1.7 in natural population. The coancestry coefficient within progeny  
279 ranged from 0.151 to 0.312 among all populations and was higher than expected in half-sib  
280 families ( $\hat{\theta}_{xy} = 0.125$ ). As a result the effective size was lower than expected in panmitic  
281 populations ( $\hat{N}_{ev} = 4$ ).

282

### 283 *Pollen pool analysis*

284 The genetic structure ( $\hat{\phi}_{FT}$ ) of the pollen pools of the studied populations was  
285 relatively high (Table 4) and significantly different from zero ( $P < 0.01$ ), being greater in

286 the natural population (0.296) compared to the introduced (average of 0.089). The  
287 recommended sample for a good estimate of the mean level of differentiation among  
288 maternal plants is  $K = (\hat{\phi}_{FT})^{-1}$  for within family replications, where K is the number of  
289 seeds sampled per maternal plant (Smouse et al., 2001). Overall, our results suggest that K  
290 must be 10.6 and in average our K is 14, providing a good estimate for  $\hat{\phi}_{FT}$ . Pollen was  
291 dispersed at moderately short distances, with an estimate of mean pollen dispersal  
292 distances ( $\hat{\delta}$ ) in introduced populations of 80.0 m and of 72.3 m in the SY population. The  
293 effective neighborhood pollination area ( $\hat{A}_{ep}$ ) was on average of 59.9 m<sup>2</sup> in the introduced  
294 populations and was of 41.8 m<sup>2</sup> in the natural ones (Table 4). The average of the effective  
295 number of pollen donors ( $\hat{N}_{ep}$ ), estimated from TwoGener analysis was 6.1 in the  
296 introduced populations and was of 1.7 in the natural population. The density of  
297 reproductive individuals ranged from 0.003 to 0.100 plants/m<sup>2</sup> in the introduced  
298 populations and was of 0.012 plants/m<sup>2</sup> in the natural population (Table 4).

299

300

## 301 **DISCUSSION**

302

### 303 *Genetic differences between natural and introduced populations*

304 Our results did not show significant differences in measures of genetic diversity  
305 between offspring and adult, only between natural and introduced populations (Table 2).  
306 The SY population has the expected heterozygosity ( $\hat{H}_E = 0.169$ ) similar to what was  
307 found by Hmeljevski et al. (2011) in natural populations of *Dyckia ibiramensis* ( $\hat{H}_E =$   
308 0.219), another rheophytic and endemic bromeliad. The introduced populations exhibited  
309 high genetic diversity, which was also found for other species of bromeliads (Palma-Silva  
310 et al., 2009; Büttow, 2012).

311 The differences observed between natural and introduced populations regarding the  
312 genetic diversity may be due to edge effect, since our natural population is marginal and  
313 this kind of population use to have less genetic variation, on average, than central  
314 populations (Eckert et al. 2008, Aitken and Whitlock, 2013). Palma-Silva et al. (2009)  
315 studying the distribution patterns of the bromeliad *Vriesea gigantea* observed a decrease in  
316 diversity and increased differentiation in edge populations when compared to central  
317 populations. The differences may also be due to apparent change that is occurring in the

318 natural habitat that may be leading to a decline in genetic diversity of SY population.  
319 According to the report of some researchers and forest rangers of Parque Estadual do  
320 Turvo there was a greater number of individuals of *D. distachya* in the banks of the Salto  
321 do Yucumã (personal communication), however few specimens of this species were found  
322 in our field trips. In addition, many reproductive individuals had elevated formation of  
323 aborted fruits (Janke et al, *in prepare*). There are some assumptions about the population  
324 decrease of these bromeliads, such as illegal extraction of plants by tourists and the change  
325 in the water regime due to the presence of hydroelectric plant built in the area of influence  
326 of the Uruguay River basin. However, these are only assumptions, further research is  
327 necessary in this environment to know the real reason for the disappearance of individuals  
328 of this species and to avoid extinction of *D. distachya* in the wild.

329 The fixation index observed in SY offspring ( $\hat{F}_{IS} = 0.196$ ) suggests that in SY  
330 population may be occurring crosses between related individuals, probably due to some  
331 environmental disturbance, what is preventing the occurrence of random mating. In the  
332 absence of selection against homozygotes inbreeding depression can occur leading to a  
333 vigor and fertility reduction in plants (Frankham et al., 2008). Also, low genetic diversity  
334 in progeny may create a risk factor for the long-term persistence and viability of  
335 populations (Rosas et al., 2011). The  $\hat{F}_{IS}$  values reported for the progenies of introduced  
336 populations appear to be related to the formation of these new populations, which may  
337 have been formed from a small number of reproductive individuals. Furthermore, crosses  
338 between inbred individuals can generate an increase in heterozygosity (Gusson et al.,  
339 2006), reflecting the lower  $\hat{F}_{IS}$  observed in seedlings of CV, ME and PV.

340 The differences observed between natural and introduced populations may be due to  
341 the introduction process that occurred rapidly, without an appropriate planning regarding  
342 the genetic structure of the populations. When the bromeliads were rescued in some  
343 populations were collected few clumps, in other words, few genets (Pedras  
344 Brancas/Gateados: 48 genets; Encanados: 22 genets and Encanadinhos: 5 genets). The CV  
345 introduced population presented greater excess of homozygotes ( $\hat{F}_{IS} = 0.182$ ), having been  
346 derived from the population with the lowest number of families collected in nature  
347 (Encanadinhos). On the other hand, the PN population formed from a mixture of three  
348 populations (Encanados, Encanadinhos and Pedras Brancas/Gateados) exhibited excess of  
349 heterozygotes ( $\hat{F}_{IS} = - 0.049$ ).

350 Also, the estimate of differentiation among adult populations ( $\hat{G}'_{ST} = 0.395$ )  
351 suggests that 60% of the genetic diversity is within these populations and approximately  
352 40% is among the populations, indicating high structuring of these populations. The  
353 offspring showed an increase in the  $\hat{G}'_{ST}$  (0.436), indicating an increase in the divergence  
354 of these populations. This may be due to the absence of pollen flow between populations,  
355 but further studies are needed to elucidate these results.

356

### 357 ***Mating system and gene flow in natural and introduced populations***

358 The mating system determines the spatial distribution of genetic variation within and  
359 among populations (Aguilar et al., 2008). Our results showed high values of outcrossing  
360 rates ( $\hat{t}_m$ ) in introduced populations (Table 3), indicating that *D. distachya* is alogamous, as  
361 expected for a post-zygotic self-incompatible species (Wiesbauer, 2008). On the other  
362 hand, the natural population showed lower value of  $\hat{t}_m$  (0.520), probably due to some  
363 disturbance that is occurring in that population. Changes in mating patterns, related to  
364 lower outcrossing rates, may occur in populations whose habitat is suffering some kind of  
365 disruption (Aguilar et al., 2008; Rosas et al., 2011).

366 The values found for  $\hat{t}_m$  in natural and introduced populations reflect the results  
367 obtained for biparental inbreeding rate ( $\hat{t}_m - \hat{t}_s$ ), correlated paternity ( $\hat{r}_{p(m)}$ ), full and half  
368 sibs (Table 3). The biparental inbreeding rate can cause apparent selfing or increased the  
369 homozygosity, regarding these random mating (Ritland, 2002).

370 The introduced populations have obtained low values of biparental inbreeding rate  
371 (average of 0.060) and moderate values of multilocus correlated paternity (average of  
372 0.158), indicating the existence of crosses between related individuals. Probably these  
373 results are a consequence of the introduction process of these populations, since the rescue  
374 of few genets in extinct areas until the formation of the new populations from a few  
375 reproductive individuals and also the spatial distribution of these bromeliads. Nevertheless,  
376 the introduced populations had an average 81.2% (Table 3) of its progeny originated  
377 through random matings, consistent with the  $\hat{t}_m$  values found and for a cross-pollinated  
378 species.

379 The SY population showed low values for biparental inbreeding rate (0.065) and  
380 higher values for multilocus correlated paternity (0.577), suggesting that most of the  
381 progenies of crosses were generated by mating between the same paternal and maternal

382 parental. The environmental disturbance that is occurring in this area seems to be  
383 preventing the achievement of crosses between unrelated individuals, resulting in a higher  
384 degree of kinship of the progenies, being composed in greater proportion by full sibs  
385 (Table 3).

386 The average coancestry coefficient ( $\hat{\theta}$ ) within progenies indicates the existence of  
387 mixed parentage, confirming what had already been observed by the correlation of  
388 paternity, with the introduced populations (average of 0.171) higher than expected for half  
389 sibs (0.125) and in the natural population (0.312) higher than expected for full sibs (0.250).  
390 Possibly, the values obtained here for the  $\hat{\theta}$  were higher than expected due to the existence  
391 of crosses between related individuals due the proximity between them.

392 The estimate of the variance effective size ( $\hat{N}_{ev}$ ) measures the genetic  
393 representativeness of a population due to changes in allele frequencies between generations  
394 owing to genetic drift (Caballero, 1994). The  $\hat{N}_{ev}$  estimated here for natural and introduced  
395 populations (Table 3) are smaller than expected in random mating ( $\hat{N}_{ev} = 4$ ), indicating the  
396 occurrence of genetic drift.

397 The estimate of genetic structure of pollen pool ( $\hat{\phi}_{FT}$ ) was high in the natural  
398 population (0.296), indicating that pollen flow is more restricted in the SY population. This  
399 value is similar to that found for *Aechmea winkleri* (0.256), a narrow endemic bromeliad  
400 (Büttow, 2012). In contrast, the estimate of genetic structure of pollen pool showed greater  
401 heterogeneity (average of 0.089) in introduced populations, which may be due to the origin  
402 of the materials that formed these new populations.

403 The effective number of pollen donors ( $\hat{N}_{ep}$ ) estimated from progeny array analysis  
404 (average of 5.5) and from the contemporary gene flow analysis (average of 5.2) have  
405 similar results. The greater effective number of pollinator donors was observed in  
406 introduced populations (Tables 3 and 4) and in the natural population the effective number  
407 of pollen donors was only 1.7. The difference found relative to  $\hat{N}_{ep}$  between introduced and  
408 natural populations may be related to clustering of reproductive individuals genetically  
409 distinct and foraging behavior of pollinators. In the introduced populations reproductive  
410 individuals are closer, while in the natural population they are more distant from each  
411 other. Higher density of inflorescences can influence the behavior of pollinators, occurring  
412 the displacement mainly between the nearby inflorescences due to greater availability of  
413 resources, with at least half of the pollen deposited on the first flower after collection, thus

414 affecting the effective number of pollen donors and also the average distance of pollen  
415 dispersal (Richards, 1997).

416 Our study indicates that pollen of *D. distachya* is dispersed in short distances and  
417 seems to be influenced by the combination of density and spatial distribution of  
418 reproductive individuals. Populations with higher density (Table 4) and higher clustering  
419 among individuals (Figure 1) showed the lowest distance dispersal ( $\hat{\delta}$ ). The PN population  
420 with higher density and grouping of individuals per area had the lowest dispersion distance  
421 (25.0 m), on the other hand, the CV population with low density and moderate grouping of  
422 bromeliads showed the greatest distance of dispersion (111.8 m). Still, the average  
423 dispersal distance of introduced populations (80.0 m) resembled the dispersal distance in  
424 the natural population (72.3 m). The effective pollination neighborhood ( $\hat{A}_{ep}$ ) around  
425 mother plants in the introduced populations is less than 60 m<sup>2</sup> and in the natural, is around  
426 42 m<sup>2</sup>, being the pollen dispersal very restrict in the populations studied.

427

#### 428 ***Conservation implications***

429 *Dyckia distachya* is a rare bromeliad which is facing severe threats imposed mainly  
430 by humans of disappearing in the wild. The only known natural population seems to be  
431 experiencing some kind of disturbance that is preventing the realization of random mating,  
432 leading to an increase of inbreeding in the progeny. Further study should be carried out in  
433 this population to really know what is going on, in order to be performed effective  
434 conservation measures to protect the wild population.

435 Introduced populations seem to being successful because they are managing to  
436 establish, with good levels of genetic diversity, similar to other species of bromeliads and  
437 also not showing an increase of inbreeding in progeny. However, this bromeliad has short  
438 distance of pollen dispersal, and gene flow has not being observed between populations,  
439 which could lead to a reduction of the genetic diversity, local selection and loses by genetic  
440 drift. These factors can endanger the long-term maintenance and the viability of the  
441 introduced populations.

442 We suggest that a long-term assessment could be realized to monitor the indices of  
443 genetic diversity and pollen flow, because the pollen dispersal may vary due to changes in  
444 pollinator composition and foraging behavior in response to weather, the grouping and the  
445 density of individual reproductive. Also, it should be compared the potential pollen flow

446 based on seed and the realized pollen-mediated gene flow based on the establishing  
447 seedlings. If significant decreases in genetic diversity were observed, new individuals  
448 should be introduced from the *ex situ* collections.

449 To prevent the inbreeding and the increase in genetic divergence among populations  
450 we also propose the creation of new areas of introduction intermediate of those already  
451 existing, promoting the formation of metapopulations. These new populations could count  
452 on little clumps, serving as a connection to gene flow among populations, avoiding the  
453 genetic differentiation between them.

454

455

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457

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600

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603 authors of this paper share interests in the genetics and conservation of the Brazilian  
604 Atlantic Forest plant species, with main emphasis on the family Bromeliaceae. We use  
605 different approaches and tools to resolve issues related to biology, ecology, genetics and  
606 evolution of species of this taxonomic group (<http://www.ufrgs.br/ngcp/>).  
607

608 **List of Tables**

609 **Table 1** Number of reproductive individuals, mother plants and seedlings per mother plant  
610 evaluated in five populations of *Dyckia distachya*.

611

<b>Population</b>	<b>Reproductive individuals</b>	<b>Mother plants</b>	<b>Seedlings/ mother plant</b>
CV*	22	5	15
ME	40	15	15
PV	29	10	15
PN	13	10	15
SY	26	10	10

612 \*For locality abbreviations see Materials and Methods.

613  $n = 830$

614

615 **Table 2** Genetic diversity averaged over eight microsatellite loci for adults and offsprings of five populations of *Dyckia distachya*. Sample  
 616 size ( $n$ ), number of alleles ( $\hat{A}$ ), allelic richness ( $\hat{A}_r$ ), observed heterozygosity ( $\hat{H}_O$ ), expected heterozygosity ( $\hat{H}_E$ ) and fixation index ( $\hat{F}_{IS}$ ).  
 617

Population/ Parameters	Adults						Offsprings					
	$n$	$\hat{A}$	$\hat{A}_r$	$\hat{H}_O$	$\hat{H}_E$	$\hat{F}_{IS}$	$n$	$\hat{A}$	$\hat{A}_r$	$\hat{H}_O$	$\hat{H}_E$	$\hat{F}_{IS}$
CV <sup>†</sup>	22	3.750	3.388	0.331	0.416	0.182*	75	4.000	3.635	0.371	0.396	0.062*
ME	40	3.875	3.481	0.525	0.591	0.113*	225	3.750	3.600	0.573	0.591	0.031*
PV	29	3.625	3.504	0.487	0.588	0.175*	150	3.875	3.559	0.497	0.554	0.104*
PN	13	3.625	3.625	0.519	0.496	-0.049	150	3.875	3.391	0.390	0.434	0.102*
SY	26	2.375**	2.050**	0.167**	0.169**	0.014	100	2.125**	2.121**	0.147**	0.183**	0.196*
Introduced populations	-	3.719	3.450	0.466	0.523	0.109	-	3.875	3.546	0.458	0.494	0.073
Mean												
Overall Mean	-	3.450	3.210	0.406	0.452	0.102	-	3.525	3.261	0.396	0.432	0.083

618 <sup>†</sup> For locality abbreviations see Materials and Methods.

619 \* $\hat{F}_{IS}$  values that deviated significantly from Hardy-Weinberg equilibrium ( $P < 0.01$ ).

620 \*\*Means that differed significantly among populations by Tukey test ( $P < 0.01$ ).

621 No significant differences between adults and offspring were observed by Tukey test ( $P > 0.01$ ).

622

623 **Table 3** Mating system parameters estimated for five populations of *Dyckia distachya*. Bootstrap-based standard deviations are in  
 624 parentheses.  
 625

Parameters	CV*	ME	PV	PN	SY	Introduced populations Mean	Overall Mean
Single-locus outcrossing rate: $t_s$	0.898 (0.144)	0.819 (0.040)	0.914 (0.035)	0.988 (0.045)	0.454 (0.229)	0.905	0.815
Multilocus outcrossing rate: $t_m$	0.974 (0.105)	0.951 (0.025)	1.007 (0.061)	0.927 (0.047)	0.520 (0.256)	0.965	0.876
Biparental inbreeding rate: $t_m - t_s$	0.075 (0.084)	0.132 (0.031)	0.092 (0.056)	-0.062 (0.030)	0.065 (0.073)	0.060	0.061
Single-locus correlated paternity: $r_{p(s)}$	0.118 (0.082)	0.205 (0.046)	0.253 (0.099)	0.092 (0.048)	0.539 (0.292)	0.167	0.241
Multilocus correlated paternity: $r_{p(m)}$	0.152 (0.044)	0.189 (0.037)	0.166 (0.033)	0.126 (0.043)	0.577 (0.306)	0.158	0.242
Effective number of pollen donors: $1/r_{p(m)}$	6.6	5.3	6.0	7.9	1.7	6.5	5.5
Full sibs: $t_m * r_{p(m)}$	0.148	0.180	0.167	0.117	0.300	0.153	0.182
Half sibs: $[t_m(1-r_{p(m)})]$	0.826	0.771	0.840	0.810	0.220	0.812	0.693
Coancestry coefficient within progeny ( $\theta_{xy} = r_{xy}/2$ )	0.185	0.178	0.171	0.151	0.312	0.171	0.199
Variance effective size ( $N_{ev} = 0.5 / \theta_{xy}$ )	2.701	2.802	2.920	3.321	1.603	2.936	2.667

626 \*For locality abbreviations see Materials and Methods.  
 627

628 **Table 4** Pollen pool structure parameters estimated for five populations of *Dyckia distachya* based on a normal distribution of the  
 629 TwoGener analysis.  
 630

Parameters	CV <sup>†</sup>	ME	PV	PN	SY	Introduced populations Mean	Overall Mean
Density: (m <sup>2</sup> ) <sup>*</sup>	0.005	0.003	0.007	0.100	0.012	0.029	0.052
Differentiation in pollen gene pool among mother plants: $\hat{\phi}_{FT}$	0.092	0.127	0.079	0.057	0.296	0.089	0.130
Number of pollen donors: $\hat{N}_{ep} = 1/(2*\hat{\phi}_{FT})$	5.4	3.9	6.3	8.8	1.7	6.1	5.2
Average dispersal distance (m): $\hat{\delta}$	111.8	88.8	94.5	25.0	72.3	80.0	78.5
Effective pollination neighborhood: $\hat{A}_{ep} = 4.\pi.\sigma^2$ (m <sup>2</sup> )	100.0	63.1	71.4	5.0	41.8	59.9	56.3
Radius (m): $\hat{r}_{ep} = \sqrt{\hat{A}_{ep}/\pi}$	5.6	4.5	4.8	1.3	3.6	4.1	4.0
Error associated with the model	0.041	0.895	0.256	0.051	0.031	0.311	0.255

631 <sup>†</sup>For locality abbreviations see Materials and Methods.

632 <sup>\*</sup>Density of reproductive individuals is based on ecological observations.

633

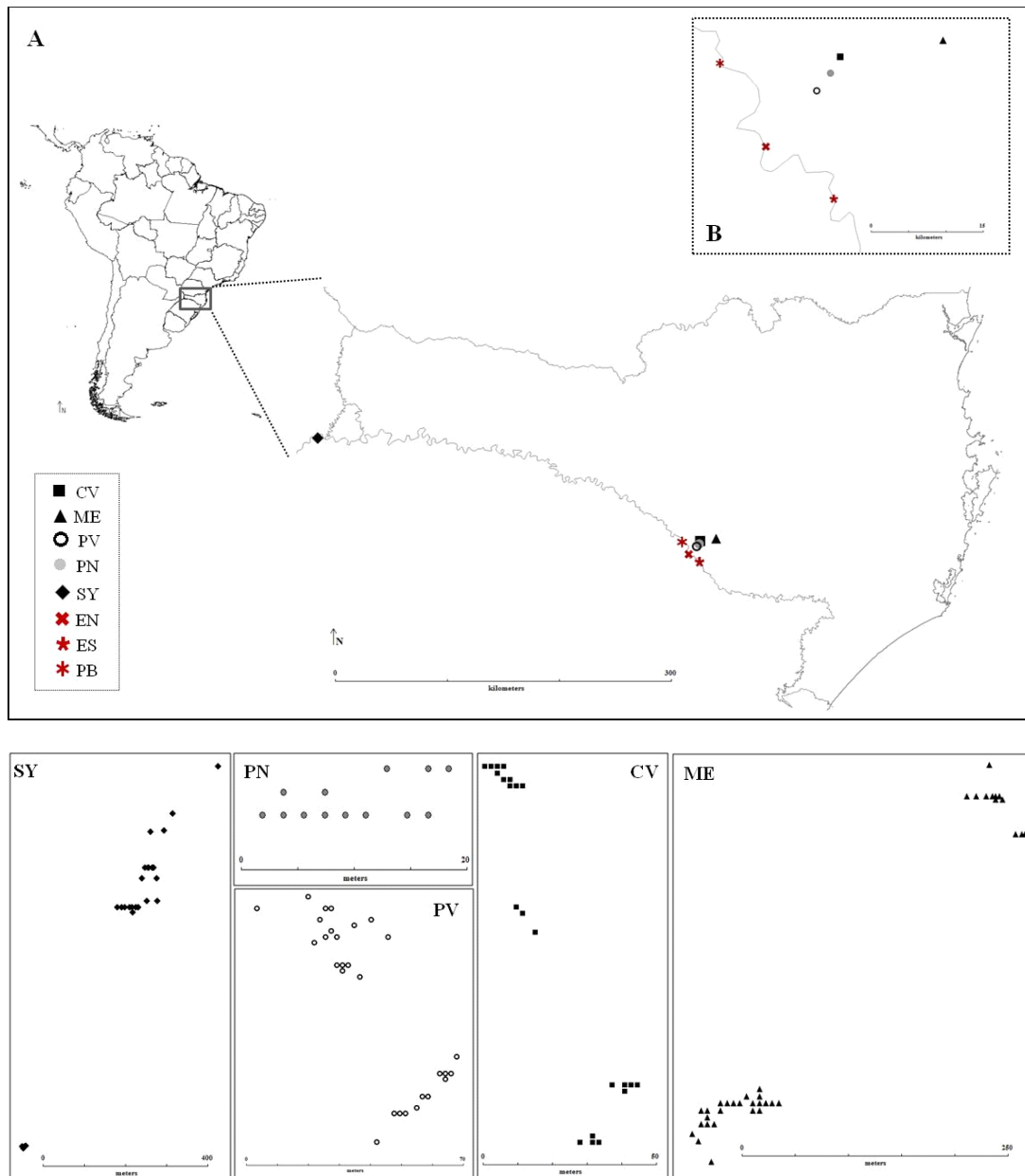


634 **Figure legend**

635 **Figure 1** Study region. Above: (A) Map of Latin America including Brazil. Showing in  
636 detail the location of extinct (EN, ES, PB), natural (SY) and introduced (CV, ME, PN, PV)  
637 populations of *Dyckia distachya* studied; (B) Detail of the location of the extinct and  
638 introduced populations. Below: Distribution of individuals in natural and the introduced  
639 populations.

640

641 **Figure 1**



642 For locality abbreviations see Materials and Methods.

643

644 **Supplemental Table 1** Characteristics of eight microsatellite loci of adults and offsprings of five populations of *Dyckia distachya*,  
 645 including locus name, number of alleles per locus ( $\hat{A}$ ), observed heterozygosity ( $\hat{H}_O$ ), expected heterozygosity ( $\hat{H}_E$ ) and fixation index  
 646 ( $\hat{F}_{IS}$ ).  
 647

Locus	Adults				Offspring			
	$\hat{A}$	$\hat{H}_O$	$\hat{H}_E$	$\hat{F}_{IS}$	$\hat{A}$	$\hat{H}_O$	$\hat{H}_E$	$\hat{F}_{IS}$
Dd07	3	0.423	0.477	0.113	4	0.291	0.432	0.326
Dd10	7	0.490	0.526	0.068	7	0.492	0.515	0.045
Dd20	6	0.310	0.501	0.381	6	0.403	0.432	0.067
Acom_119.1	4	0.267	0.292	0.086	4	0.358	0.337	-0.062
Acom_71.3	4	0.291	0.294	0.010	4	0.365	0.352	-0.037
Ac11	6	0.326	0.490	0.335	7	0.499	0.514	0.029
Ac25	5	0.619	0.606	-0.021	6	0.553	0.571	0.032
ngDy_24	6	0.528	0.470	-0.123	6	0.444	0.446	0.004
Mean	5.125	0.407	0.457	0.109	5.5	0.426	0.450	0.053

648

649 **Supplemental Table 2** Pairwise  $F'_{ST}$  among populations of *Dyckia distachya* based on 8  
650 microsatellite loci.  
651

Population	CV	ME	PV	PN
CV <sup>†</sup>				
ME	0.426			
PV	0.406	0.104		
PN	0.349	0.298	0.236	
SY	0.444	0.421	0.466	0.554

652 <sup>†</sup> For locality abbreviations see Materials and Methods.  
653 All values were significant at  $P < 0.0001$ .  
654



## **Capítulo IV**

### *Considerações Finais*

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## CONSIDERAÇÕES FINAIS

Este estudo foi realizado com o intuito de obter informações essenciais a respeito da introdução de plantas na natureza, as quais ainda são escassas. Foram investigados assuntos referentes ao sucesso reprodutivo e a questões genéticas relevantes para a conservação de *Dyckia distachya* na natureza, em populações introduzidas e na única população natural conhecida. Assim, as informações obtidas neste trabalho podem contribuir para um melhor entendimento dos aspectos envolvidos na introdução de espécies, bem como, para a elaboração de estratégias de preservação da bromélia em estudo.

As populações introduzidas investigadas apresentaram, em geral, viabilidade e altos índices de sucesso reprodutivo (Capítulo II). Inclusive, em duas populações foi observado o recrutamento de novos indivíduos através de sementes. Esses resultados são animadores, visto que o sucesso de uma introdução pode ser avaliado também pela habilidade das plantas em realizar os processos básicos do seu ciclo de vida, tais como, estabelecimento, reprodução e disseminação (Pavlik, 1996). Além disso, o recrutamento de plântulas é essencial para a persistência de novas populações, sendo um ponto fundamental em genética da conservação (Amm *et al.*, 2012; Wagenius *et al.*, 2012).

A qualidade do ambiente das áreas de introdução também refletiu nos resultados deste estudo (Capítulo II). Algumas populações tiveram mais dificuldades de estabelecimento devido à predação das bromélias (folhas, flores e frutos) pela lagarta *Strymon rufofuscus* e pela propensão da área ao alagamento por longos períodos. Esses fatores influenciaram no comportamento dos polinizadores, no recrutamento de novos indivíduos e na necessidade de maior intervenção humana. Por isso, ressaltamos a necessidade de um acompanhamento das populações introduzidas em médio e longo prazo, sendo necessários para garantir o sucesso das populações introduzidas (Cochrane, 2004).

O conhecimento e a manutenção da diversidade genética é outro fator essencial em programas de conservação (Frankham *et al.*, 2009). Sendo assim, a variabilidade genética das populações estudadas foi acessada com o auxílio de marcadores moleculares microsatélites (Capítulo III). Nossos resultados indicaram a ocorrência de níveis elevados de diversidade nas populações introduzidas em comparação com a população natural. Essas diferenças podem ter ocorrido devido ao modo como as introduções foram realizadas

(coleta de espécimes e formação das novas populações), bem como, devido ao efeito de borda (Eckert *et al.* 2008, Aitken e Whitlock, 2013), uma vez que a população natural encontra-se em uma localização marginal.

O sistema de cruzamento possui aplicação direta para a conservação, determinando a distribuição espacial da variação genética dentro e entre populações (Aguilar *et al.*, 2008), igualmente contribuindo para a manutenção da diversidade ao longo do tempo (Godefroid *et al.*, 2011). A taxa de polinização cruzada (Capítulo III) foi elevada nas populações introduzidas ( $\hat{t}_m = 0,965$ ), como era esperado por ser uma espécie alógama (Wiesbauer, 2008). Nessas populações também foram revelados valores baixos de endogamia biparental (0,060) e moderados em relação à correlação de paternidade (0,158), indicando a existência de cruzamentos entre indivíduos relacionados (Capítulo III). Mesmo assim, os resultados demonstraram que as introduções não estão interferindo significativamente no sistema de cruzamento dessa bromélia, sendo observado, em média, mais de 80% da progênie originada através de cruzamentos aleatórios.

Considerando os dados de fluxo gênico (Capítulo III) a estrutura genética do *pool* de pólen foi altamente heterogênea nas populações introduzidas ( $\hat{\phi}_{FT} = 0,089$ ), enquanto a população natural parece estar mais estruturada ( $\hat{\phi}_{FT} = 0,296$ ). O número efetivo de doadores de pólen nas populações introduzidas ( $\hat{N}_{ep} = 5,5$ ) também é superior ao encontrado na população natural ( $\hat{N}_{ep} = 1,7$ ). Esses resultados podem estar ligados ao processo de introdução como um todo, iniciando pelo resgate de poucos *genets* nas populações agora extintas até a formação das novas populações, envolvendo um pequeno número de indivíduos reprodutivos e a disposição espacial desses espécimes, podendo interferir inclusive no comportamento dos polinizadores.

A distância média de dispersão do pólen de *D. distachya* é um fator preocupante para o estabelecimento de populações dessa espécie (Capítulo III), visto que a distância média de polinização ( $\hat{\delta} = 78,5$  m) não ultrapassa a distância média entre todas as populações estudadas, inexistindo fluxo gênico entre esses indivíduos. Isso pode prejudicar a viabilidade das populações introduzidas em longo prazo, podendo ocorrer uma redução na diversidade genética, seleção local e avultamento das perdas por deriva genética. Por esses fatores, sugerimos a criação de novas áreas de introdução intermediárias as existentes visando ampliar a conectividade genética entre as populações estudadas.

A existência de apenas uma população natural de *D. distachya* torna essa espécie ainda mais frágil, pois populações isoladas possuem risco de extinção agravado devido à redução da reprodução e ao aumento de cruzamentos entre indivíduos aparentados (Kéry e Matthies, 2004). Os resultados deste estudo apontam para a existência de algum tipo de perturbação ambiental que pode estar ocorrendo na área da população natural (Capítulos II e III). Observamos em laboratório a presença de plântulas albinas, taxa de germinação reduzida e baixa viabilidade de pólen (Capítulo II). Além disso, também foi verificada uma alteração significativa na taxa de cruzamento ( $\hat{t}_m = 0,520$ ) associada a uma elevada correlação de paternidade (0,158) (Capítulo III).

Por outro lado, na população natural, observamos a campo um alto investimento em reprodução clonal e a ocorrência de plântulas geradas através de sementes (Capítulo II). Por isso, sugerimos uma investigação aprofundada dessa área, para verificar a viabilidade desta população em longo prazo e o que realmente está acontecendo. Caso a regeneração natural não seja suficientemente eficaz para a conservação da diversidade genética e a manutenção da população natural propomos a introdução de novos indivíduos geneticamente compatíveis nessa área.

Em síntese, levando em consideração as questões que foram investigadas neste estudo e a elevada vulnerabilidade desta bromélia propomos:

- ⊗ A introdução de populações de *D. distachya* ser considerada em programas de conservação, sendo acompanhadas em médio e longo prazo;
- ⊗ O monitoramento genético das populações introduzidas, avaliando os índices de diversidade genética e fluxo gênico;
- ⊗ Fazer a escolha das áreas de introdução baseada em ambientes que realmente possuam características reofíticas e com pouca ou nenhuma incidência da lagarta *S. rufofuscus*;
- ⊗ A distância entre as áreas de introdução ser baseada na distância média de polinização; e,
- ⊗ Um estudo aprofundado na população natural para averiguar as possíveis causas de perturbação nesse ambiente, visando à elaboração de estratégias de conservação específicas para esse ambiente único.





## Capítulo V

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

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## **Anexo**

*Microsatellites in the Endangered Species Dyckia distachya  
(Bromeliaceae) and Cross-Amplification in Other Bromeliads*

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Article

## Microsatellites in the Endangered Species *Dyckia distachya* (Bromeliaceae) and Cross-Amplification in Other Bromeliads

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**Abstract:** Microsatellite markers were isolated in *Dyckia distachya*, an endangered bromeliad from southern Brazil, which will be useful to assess the population genetic structure and reproductive success in introduced and natural populations of this species. Twenty microsatellite loci were developed from an enriched genomic library, and nine of these were amplified. The loci were characterized in 43 individuals from introduced and wild *D. distachya* populations. All nine loci were polymorphic, with four to ten alleles per locus. In an introduced population the observed and expected heterozygosities ranged from 0.136–0.667 and 0.543–0.877, respectively, while in a wild population it ranged from 0.000 to 0.895 and from 0.050 to 0.811, respectively. The development of these microsatellite markers will contribute to investigations of the reproductive potential and viability of introduced populations of *D. distachya* as well as the single known wild population. Cross-amplification in other Bromeliaceae species was successful, with high rates in four loci, demonstrating the applicability of these microsatellite markers in other taxa.

**Keywords:** Bromeliad; endemism; introduction; rheophytic species; SSR

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## 1. Introduction

*Dyckia distachya* Hassler is a rare, endemic bromeliad (subfamily Pitcairnioideae) with a rupicolous, rheophytic habit that lives on riverbanks along the rapids of the Pelotas and Uruguay Rivers in southern Brazil [1]. The species is adapted to extreme variations of low and high water periods, and is characterized by high clonal reproduction [2,3]. According to Reitz [1] and Reis *et al.* [2], *D. distachya* is facing extinction due to increased hydroelectric construction along the rivers. Originally, eight wild populations of *D. distachya* were known along the Uruguay and Pelotas Rivers. Currently, only one wild population is known, located in Salto do Yucumã in Turvo State Park, Derrubadas, Rio Grande do Sul State, which is on the border between Brazil and Argentina. Therefore, *D. distachya* is on the Official List of Brazilian Endangered Species [4]. During Barra Grande hydroelectric construction, some *D. distachya* populations were rescued and conserved *ex situ*. However, the process occurred rapidly, without appropriate planning regarding the genetic structure of the populations. Initially, the plants were kept in nurseries before being introduced in environments similar to their origin. In addition to the long-term monitoring of introduced populations, knowledge of the genetic diversity of these populations is needed so that they can be managed appropriately and the success of the introduction is ensured.

Here, we report the isolation and characterization of a set of nine microsatellite loci for *D. distachya* in one introduced population and in the only one known wild population. The cross-amplification of these loci was examined in 22 related species from three subfamilies of Bromeliaceae. The transferability of these microsatellites will be helpful in conservation genetics studies in many bromeliad taxa.

## 2. Results and Discussion

### 2.1. Isolation, Characterization and Cross-Amplification of Microsatellite Loci

Nine from 20 *D. distachya* loci amplified robustly and proved to be polymorphic. Markers isolation involved the development of a genomic enriched library. A total of 96 positive clones were captured and 48 colonies were sequenced, of which 24 clones showed simple sequence repeats (SSR) motifs. Twenty primer pairs were designed in which the product size ranged between 100 and 300 base pairs. Nine of these primer pairs generated consistent patterns of amplification and were used for further characterization of two *D. distachya* populations. The primer sequences and product sizes are shown in Table 1.

**Table 1.** Description and characterization of nine microsatellite loci of *Dyckia distachya*, including locus name, primer sequences, repeat motif, number of alleles (A), allele size range and GeneBank accession number.

Locus	Primer Sequences (5'-3')	Repeat motif	A	Size (bp)	GeneBank accession No
Dd03	F: TAGGCAGATGCGAATTGAGT R: CTCAGCATAGCTTTTCGATGG	(CA) <sub>10</sub>	6	202–212	JX290116
Dd04	F: CGCTTTTCTCGGAATCTTTG R: AGGGCTTCGTCCTCCTAAAA	(TG) <sub>8</sub>	6	227–257	JX290117
Dd07	F: GATTCGGAAGGATGACAAGA R: CGGCACAGGAATACAAAGAG	(GA) <sub>25</sub>	5	201–213	JX290118
Dd08	F: GATCGGTCTTTTACTCCTTGG R: CACGCTAGGATGATGTAGGC	(AC) <sub>6</sub> G(GA) <sub>9</sub>	10	192–232	JX845415
Dd09	F: ACTCTGCTGCCTCATTACACA R: CACAGCAAAGGCAAACCTTGA	(GA) <sub>10</sub>	10	176–228	JX845416
Dd10	F: CTATGGGACTGCTGGACACT R: CTTGCTGGTAATCGTGTTCC	(TG) <sub>7</sub>	4	248–254	JX290119
Dd16	F: AATTGCACCAAACAGGGATT R: GACACGACCCACATAGATGC	(GT) <sub>7</sub> (GC) <sub>4</sub>	7	170–190	JX290120
Dd19	F: GTGCCAAACATAAACCGATG R: AACGACCAAAAAGGGTGTTTC	(GT) <sub>5</sub>	6	239–278	JX290121
Dd20	F: GGTGGAAATGGTGGGTTACA R: GGCAGGCAAGGTATGAAGAA	(CA) <sub>7</sub>	6	227–253	JX290122

In the introduced population, the number of alleles per locus ranged from 4 to 10, with an average of 6.2, and the expected and observed heterozygosities ranged from 0.136 to 0.667 and from 0.543 to 0.877, respectively. In the wild population, the number of alleles per locus varied from 2 to 8, with an average of 3.8, and the expected and observed heterozygosities ranged from 0.000 to 0.895 and from 0.050 to 0.811, respectively (Table 2). Four loci showed significant departures from the Hardy-Weinberg equilibrium (HWE) due to heterozygote deficiency in both populations (Dd04, Dd08, Dd09 and Dd20; Table 2). Such significant deviation from HWE could have been caused by the involvement of null alleles. We tested the presence of null alleles and the four loci that showed significant departures from HWE in both populations exhibited high null allele frequencies (data not shown). Other three loci showed significant departures from HWE in only one population ( $p < 0.001$ ; Table 2) and did not show null alleles, being the departures probably due to inbreeding or Wahlund effects.

Cross-species amplification was successful in different degrees (Table 3). All loci amplified in species from same genus (except Dd19 for *D. maritima*). Locus Dd10 and Dd20 amplified in all species tested, and locus Dd07 and Dd16 were successful in 86.3% of the species evaluated. The 12 genus tested correspond to three representative Bromeliaceae subfamilies from Atlantic Rainforest, indicating high potential for use of these four loci in phylogeography and population genetic studies of many Bromeliaceae species, mainly for *Dyckia* genus.



**Table 2.** Results of nine microsatellite loci in two populations of *Dyckia distachya*, including number of individuals ( $N$ ), number of alleles ( $A$ ), observed ( $H_O$ ) and expected ( $H_E$ ) heterozygosities.

Locus	Introduced ( $N = 22$ )			Natural ( $N = 21$ )		
	$A$	$H_O$	$H_E$	$A$	$H_O$	$H_E$
Dd03	6	0.636	0.724 *	3	0.350	0.309
Dd04	6	0.136	0.636 *	3	0.214	0.659 *
Dd07	4	0.667	0.582	2	0.050	0.050
Dd08	10	0.300	0.877 *	7	0.300	0.720 *
Dd09	8	0.136	0.558 *	8	0.167	0.811 *
Dd10	4	0.545	0.589	3	0.143	0.257
Dd16	6	0.545	0.601	4	0.895	0.552 *
Dd19	6	0.182	0.543 *	2	0.048	0.136
Dd20	6	0.476	0.784 *	3	0.000	0.284 *
Mean	6.2	0.402	0.591	3.8	0.241	0.188

\* Indicates  $H_O$  departed significantly from  $H_E$  under HWE ( $p < 0.001$ ).

## 2.2. Genetic Diversity of *D. distachya*

In our study, introduced population showed, on average, a higher number of alleles per locus, and higher observed and expected heterozygosities compared with the wild population (Table 2). The results could indicate that the individuals rescued may be representative of the original population, retaining the genetic diversity, although further studies should be made for a more accurate conclusion. The introduced population presented 25 exclusive alleles of a total 60 alleles (data not show). The wild population showed only three exclusive alleles and low genetic diversity. The microsatellites characterized here will be used to assess more thoroughly the genetic diversity of natural and introduced populations. However, one hypothesis for lower genetic diversity found in natural population would be the edge effect [5], since this population occurs at the range edge of the original geographical distribution of the species. This phenomenon has also been observed in *Vriesea gigantea*, another species of bromeliad [6].

## 3. Experimental Section

### 3.1. Isolation of Microsatellite Markers

To identify and characterize microsatellites, genomic DNA was extracted from fresh leaves of one *D. distachya* individual following the protocol of Doyle and Doyle [7]. Microsatellite markers were developed as described by Kijas *et al.* [8] with modifications by Billote *et al.* [9], using an enriched library methodology. In brief, total DNA was cleaved with the restriction enzyme *RsaI* (Invitrogen, Carlsbad, CA, USA). The cleaved DNA was linked to two complementary oligo adapters. Polymerase chain reaction (PCR) was performed using one of the adapters as a primer. The PCR product was purified using a *QIAquick PCR Purification Kit* (Qiagen, Hilden, Germany). Fragments that contained microsatellite repeats were selected using biotinylated probes (dCT)<sub>8</sub> and (dGT)<sub>8</sub> and isolated with *Dynabeads M-280 Streptavidin* magnetic particles (Invitrogen, Carlsbad, CA, USA). An additional

PCR was performed using one of the adapters as a primer. The PCR products were inserted into the *pGEM-T Easy* vector (Promega, Madison, WI, USA) and used to transform XL1-Blue competent cells (*Escherichia coli*, Stratagene, La Jolla, CA, USA). Recombinant colonies were selected by blue/white screening. Ninety-six recombinant colonies were randomly selected, and 48 were double-sequenced using the *BigDye Terminator* version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an *ABI PRISM 377* DNA Sequencer (Applied Biosystems, Foster City, CA, USA). Every the forward and reverse sequences obtained were aligned and edited in *SeqMan* (DNASTAR package, Madison, WI, USA), and primers were designed using the *Primer3* program [10].

### 3.2. Primer Validation

To assess polymorphisms, 22 individuals from one *D. distachya* population introduced in Anita Garibaldi, Santa Catarina State, Brazil (27°58'81"S, 50°39'11"W) and 21 individuals from the wild population were genotyped. The wild population was collected in Salto do Yucumã, in Turvo State Park, Derrubadas, Rio Grande do Sul State (27°09'62"S, 53°53'45"W), southern edge of species original distribution and the introduced population came from the *ex situ* collection.

For each SSR, the forward primers were synthesized with a 19-bp M13 tail (5'-CACGACGTTGTAAAACGAC-3') following the method of Schuelke [11], which involved three primers: a forward SSR-specific primer with the M13 tail at its 5' end, a reverse locus-specific primer, and a universal M13 primer labeled with one of four fluorescent dyes (6-FAM, VIC, NED, and PET; Applied Biosystems, Foster City, CA, USA). All PCR amplifications were performed in a *TC-412* thermal cycler (Techne, Burlington, NJ, USA) in 10 µL reactions containing 10 ng of DNA template, 1× *Taq* buffer, 2 mM MgCl<sub>2</sub>, 200 µM of each dNTP, 1 pmol forward primer, 4 pmol reverse primer, 0.4 pmol universal M13 primer, and 0.5 U *Taq* DNA polymerase (*GoTaq*; Promega, Madison, WI, USA). The touchdown cycling program was used: 95 °C for 3 min, 10 cycles of 94 °C for 30 s, 58 °C decreasing to 48 °C at 1 °C per cycle for 30 s, 72 °C for 30 s, followed by 30 cycles of 94 °C for 30 s, 48 °C for 30 s, and 72 °C for 30 s, and concluding with a 10 min extension at 72 °C. The microsatellite alleles were resolved on a *ABI 3100* DNA Analyzer Sequencer (Applied Biosystems, Foster City, CA, USA) and sized against the *GS500 LIZ* molecular size standard (Applied Biosystems, Foster City, CA, USA) using GeneMarker DEMO version 1.97 (SoftGenetics, State College, PA, USA). The number of alleles, and expected and observed heterozygosities were analyzed with MSA 4.00 software [12], and Genepop 4.1 [13] was used to test for departure from the Hardy-Weinberg equilibrium (HWE). Null allele frequencies were calculated following [14] for each population, using the software Micro-Checker version 2.2.3 [15].

The polymorphic markers were also tested for cross-transferability in 22 related species from three subfamilies of Bromeliaceae. The PCR conditions were the same as described above. The amplification products were separated on 2% agarose gels (Agarose LE; USB, Cleveland, OH, USA) stained with *GelRed* (Biotium, Hayward, CA, USA) and visualized under ultraviolet light. A locus was considered to have been amplified successfully when at least one band of the expected size was visualized. A 50-bp *DNA* ladder (Ludwig Biotec, Alvorada, RS, Brazil) was used as a molecular size marker. All voucher specimens of the species investigated are identified in Table S1.

**Table 3.** Cross-amplification of nine microsatellite markers isolated from *Dyckia distachya* across three subfamilies of Bromeliaceae.

Species	Subfamily	Dd03	Dd04	Dd07	Dd08	Dd09	Dd10	Dd16	Dd19	Dd20
<i>Dyckia leptostachya</i>	Pitcairnioideae	+	+	+	+	+	+	+	+	+
<i>Dyckia maritima</i>	Pitcairnioideae	+	+	+	+	+	+	+	-	+
<i>Dyckia tuberosa</i>	Pitcairnioideae	+	+	+	+	+	+	+	+	+
<i>Acanthostachys strobilacea</i>	Bromelioideae	-	-	+	-	-	+	+	-	+
<i>Aechmea caudata</i>	Bromelioideae	-	-	+	-	-	+	+	-	+
<i>Aechmea coelestis</i>	Bromelioideae	-	-	-	-	-	+	+	-	+
<i>Aechmea comata</i>	Bromelioideae	w	-	+	-	-	+	+	-	+
<i>Aechmea gamosepala</i>	Bromelioideae	-	-	+	+	-	+	+	-	+
<i>Aechmea recurvata</i>	Bromelioideae	+	-	+	-	-	+	+	-	+
<i>Aechmea winkleri</i>	Bromelioideae	w	-	+	w	-	+	+	-	+
<i>Billbergia amoena</i>	Bromelioideae	-	-	w	-	+	+	+	-	+
<i>Bromelia antiacantha</i>	Bromelioideae	-	-	+	-	+	+	+	+	+
<i>Edmundoa lindenii</i>	Bromelioideae	-	-	-	-	-	+	+	-	+
<i>Hohenbergia augusta</i>	Bromelioideae	-	-	-	w	+	+	+	-	+
<i>Neoregelia guttata</i>	Bromelioideae	+	-	+	+	+	+	+	-	+
<i>Nidularium procerum</i>	Bromelioideae	+	-	+	+	-	+	+	w	+
<i>Quesnelia quesneliana</i>	Bromelioideae	-	-	+	w	-	+	+	-	+
<i>Alcantarea extensa</i>	Tillandsioideae	+	-	+	w	-	+	+	-	+
<i>Vriesea carinata</i>	Tillandsioideae	+	-	+	-	-	+	w	+	+
<i>Vriesea gigantea</i>	Tillandsioideae	+	-	+	-	-	+	-	-	+
<i>Vriesea incurvata</i>	Tillandsioideae	+	-	+	-	-	+	-	-	+
<i>Vriesea reitzii</i>	Tillandsioideae	+	-	+	-	-	+	-	-	+
Total = 22		13	3	19	10	7	22	19	5	22

Note: + = successful PCR amplification; W = weak PCR amplification; - = PCR failure.

#### 4. Conclusions

In the present study, microsatellite-enriched genomic library of *Dyckia distachya* was constructed and a total of nine polymorphic microsatellite DNA markers were characterized. The development of these markers will contribute to future population genetic studies in *D. distachya* and other bromeliad species. The nine loci will also be useful for identifying the effects of introduction on outcrossing and selfing rates, with a comparative analysis among introduced and wild populations of *D. distachya* using progeny arrays. The use of such markers will facilitate inferences about the reproductive potential and viability of the introduced *D. distachya* populations and will aid in the understanding of the role of genetics in plant species introduction.

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#### Conflict of Interest

The authors declare no conflict of interest.

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