Universidade Federal do Rio Grande do Sul

Especiação e diversidade genética no subgênero *Ortgiesia* (*Aechmea*, Bromeliaceae)

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Tese de Doutorado

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Sumário

Resumo

Aechmea subgênero *Ortgiesia* apresenta 17 espécies, distribuídas no sul e sudeste do Brasil, Argentina, Paraguai e Uruguai. São espécies epífitas, rupícolas ou terrestres, ocorrendo desde o nível do mar até altas altitudes. *Ortgiesia* é um grupo característico da Mata Atlântica, região considerada um dos centros de diversidade para a família Bromeliaceae. O relacionamento interespecífico em *Ortgiesia* é pouco compreendido, assim como a distribuição da diversidade genética entre e dentro das espécies. Com o objetivo de contribuir para o entendimento dos processos responsáveis pela diversificação em *Ortgiesia* e pelo padrão de distribuição da variação genética, o presente estudo foi realizado, sendo dividido em quatro capítulos. No **capítulo II** o relacionamento interespecífico de *Ortgiesia* foi investigado utilizando marcadores moleculares AFLP ("Amplified Fragment Length Polymorphisms"). A evolução de caracteres morfológicos-chave foi acessada, e padrões biogeográficos e a importância da hibridação para a evolução do subgênero foram discutidos. Três grupos genéticos principais de espécies proximamente relacionadas foram detectados, que de uma maneira geral puderam ser caracterizados pela cor das flores. Cor de pétala foi confirmada como um bom caratere taxonômico a ser usado na distinção de espécies dentro de *Ortgiesia*, enquanto tipo e forma da inflorescência mostrou-se homoplásico. O sul da Mata Atlântica foi considerado o centro de diversidade para *Ortgiesia* enquanto hibridação parece ser um fator importante na evolução do grupo. No **capítulo III** a diferenciação genética entre espécies de *Ortgiesia* com pétalas amarelas foi investigada, usando marcadores plastidiais e o gene nuclear *phyC*. Compartilhamento de haplótipos foi observado tanto no genoma nuclear como no plastidial. Os resultados sugerem que eventos de hibridação e separação incompleta das linhagens (polimorfismo ancestral) podem ser responsáveis pelo compartilhamento de haplótipos entre as linhagens de *Ortgiesia*. Ainda, nesse capítulo, áreas com maior diversidade genética foram identificadas no norte do estado de Santa Catarina, que podem ser consideradas como de grande valor evolutivo e conservacionista. Locos de microssatélites para o gênero *Aechmea* foram isolados pela primeira vez, conforme artigo no **capítulo IV**, com o objetivo de investigar aspectos da diversidade e estruturação genética de *A. caudata* e espécies relacionadas. Dez locos foram caracterizados, apresentando alta variação genética. Esses locos também amplificaram com sucesso em outras 21 espécies de bromélias, pertencentes

a 12 gêneros, mostrando que serão úteis em estudos com inúmeros membros de Bromeliaceae. No **capítulo V** foi investigada a estruturação genética em populações de *A. calyculata* usando marcadores plastidiais, o gene nuclear *phyC* e microssatélites nucleares. Os resultados detectaram forte estruturação genética entre populações localizadas na Floresta Ombrófila Densa e na Semidescídua. A Mata de Araucária, que separa essas duas formações florestais no sul do Brasil, foi considerada como barreira ao fluxo gênico entre as populações de *A. calyculata*. A partir dos resultados encontrados na presente tese, foi observada, de uma maneira geral, baixa diferenciação genética interespecífica no subgênero *Ortgiesia*, apesar do uso de diferentes marcadores moleculares. Esse padrão pode ser explicado pela recente diversificação das linhagens do subgênero, aliado a retenção de polimorfismo ancestral e a ocorrência localizada de eventos de hibridação. O isolamento geográfico e de habitat foram identificados como os principais fatores da diversificação em *Aechmea* subgênero *Ortgiesia*.

Abstract

Aechmea subgenus *Ortgiesia* presents 17 species distributed in south and southeastern Brazil, Argentina, Paraguay, and Uruguay. They are epiphytes, lithophytes, or terrestrials, occurring from sea level to high altitudes. *Ortgiesia* is a characteristic group from the Brazilian Atlantic rainforest, which is considered one of the diversity centers of family Bromeliaceae. The interspecific relationships in *Ortgiesia* are poorly understood, as well as the distribution of genetic diversity within and among species. With the objective to contribute to the understanding of the processes underlying the diversification in *Ortgiesia* and the patterns of genetic diversity distribution, the presented study was conducted and divided in four chapters. In **chapter II** the interspecific relationships in *Ortgiesia* were investigated using AFLP (Amplified Fragment Length Polymorphisms) molecular markers. The evolution of key-morphological characters was examined and their taxonomic value discussed. We also discussed biogeographical patterns as well as the importance of hybridization for *Ortgiesia* evolution. Three main genetic groups were recovered, which could at broad scale be characterized by flower color. Petal color seems to be a good taxonomic character to be used to distinguish *Ortgiesia* species, while inflorescence type and shape were homoplasic. The southern region of Atlantic rainforest was considered the center of diversity for *Ortgiesia* and hybridization may have played an important role during the diversification of this group. At **chapter III** genetic differentiation of yellow flowered *Ortgiesia* species was investigated by using plastid markers and the nuclear gene *phyC*. The sharing of haplotypes was observed in both plastid and nuclear genomes. The results suggest that hybridization events and incomplete lineage sorting may be responsible for the haplotype sharing between *Ortgiesia* lineages. Still, in this chapter we identified areas with high genetic diversity as located at northern Santa Catarina state, which may be considered of conservation and evolutionary value. Microsatellite loci for genus *Aechmea* were isolated for the first time as described in the **chapter IV**, aiming to investigate genetic diversity and structure of *A. caudata* and related species. Ten loci were characterized, showing high genetic variation. These loci also amplified in other 21 bromeliads species, belonging to 12 genera, showing that they can be useful as well in other taxa of Bromeliaceae. In the **chapter V** the genetic structure of *A. calyculata* populations was assessed using plastid markers, *phyC* gene, and nuclear

microsatellites. The results detected strong genetic differentiation between populations located at the ombrophilous and semi-deciduous forest. *Araucaria* forest, which separates these two forest formations in south Brazil, was considered as a barrier to gene flow between *A. calyculata* populations. The results obtained in the presented thesis showed shallow genetic interspecific differentiation in subgenus *Ortgiesia*, despite the use of different molecular markers. This pattern could be explained by the recent divergence of the lineages, allied to the persistence of ancestral polymorphism and localized hybridization events. Geographical and habitat isolation were identified as the main factors triggering the diversification process in *Aechmea* subgenus *Ortgiesia*.

Capítulo I

Introdução geral

Introdução geral

1 Família Bromeliaceae

Bromeliaceae Juss. é a segunda família mais importante entre as epífitas dos Neotrópicos, atrás apenas de Orchidaceae Juss. (Cascante-Marín e Nivia-Ruíz, 2013). Bromélias ou gravatás como são popularmente conhecidas, são ervas perenes, morfológica e ecologicamente diversas, apresentando uma grande variação de formas, cores e tamanhos (Coffani-Nunes, 2002). Cerca de 3140 espécies são atualmente conhecidas (Luther, 2008), sendo a família um exemplo de radiação adaptativa (Benzing, 2000). Elas ocorrem da região sul dos Estados Unidos até o norte da Argentina com uma única espécie no oeste da África (*Pitcairnia feliciana* (A. Chev.) Harms & Mildbraed), o que parece ser decorrente de um evento recente de dispersão a longa distância (Benzing, 2000; Givnish *et al*., 2004). São encontradas em praticamente todos os ambientes, desde o nível do mar aos elevados altiplanos da cordilheira dos Andes, em locais úmidos como a Mata Atlântica ou regiões áridas como a Caatinga, bem como em solos sujeitos a inundações regulares (espécies reofíticas) (Benzing, 2000). Podem ser terrestres, terrestres ocasionais, rupícolas, saxícolas ou epífitas, mas nunca parasitas (Coffani-Nunes, 2002).

O sucesso das bromélias pode ser atribuído à evolução de três principais características-chave (Silvestro *et al*., 2014): (1) a presença de tricomas foliares que permitem a absorção de água e nutrientes através da superfície das folhas; (2) a presença de cisterna ou tanque (estrutura formada pela inserção das folhas em forma de roseta) o que permite o acúmulo de água e detritos orgânicos, facilitando a independência do substrato; (3) o metabolismo ácido das crassuláceas (CAM), rota fisiológica que reduz a perda de água durante a fotossíntese (Benzing, 2000; Crayn *et al*., 2004). Essas características têm sido vistas como as responsáveis pela grande diversificação de linhagens que ocorreu dentro de Bromeliaceae (Crayn *et al*. 2004; Schulte *et al*. 2009; Givnish *et al*. 2011).

A família Bromeliaceae era tradicionalmente subdividida em três subfamílias: Bromelioideae Burnett, Pitcairnioideae Harms e Tillandsioideae Burnett (Smith e Downs, 1974). No entanto, estudos filogenéticos moleculares recentes sugerem que Bromeliaceae pode ser melhor dividida em oito linhagens monofiléticas, subdividindo Pitcairnioideae em

seis subfamílias (Givnish *et al*., 2007, 2011). Sendo assim, Bromeliaceae apresenta oito subfamílias com o seguinte relacionamento entre elas: (Brocchinioideae, (Lindmanioideae, (Tillandsioideae, (Hechtiooideae, (Navioideae, (Pitcarnioideae, (Puyoideae, Bromelioideae))))))) (Givnish *et al*., 2011). Três centros de diversidade são considerados para a família Bromeliaceae: do norte dos Andes até o México e as Antilhas, o Planalto das Guianas e o leste do Brasil (Smith e Downs, 1974). O leste brasileiro, representado pela Mata Atlântica, se destaca por apresentar um grande número de espécies endêmicas (Leme e Marigo, 1993; Martinelli *et al*., 2008). Informações oriundas de macro e microfósseis indicam a existência de representantes de Bromeliaceae a partir do Terciário médio (Benzing, 2000). De acordo com Givnish *et al*. (2011) as bromélias surgiram no escudo das Guianas a cerca de 100 milhões de anos atrás (Ma). Porém, a diversificação entre as atuais subfamílias teria começado a apenas cerca de 19 Ma.

Bromeliaceae contêm espécies que são economicamente importantes, como o *Ananas comosus* (L.) Merr. (abacaxi), a quarta fruta tropical mais importante para a produção comercial, atrás da melancia, banana e manga (Chwee e Ahmad, 2008). Algumas são utilizadas na medicina popular, como *Bromelia antiacantha* Bertol. (Zanella *et al*., 2011), assim como muitas outras espécies são localmente valiosas como fonte de fibras (Sass e Specht, 2010). Ecologicamente, as bromélias desempenham um importante papel no ambiente onde ocorrem, devido à interação com a fauna, por serem fonte de frutos carnosos, néctar, pólen, água (acumulada nos tanques formados pelas folhas) e por servirem de abrigo para animais associados (mamíferos, anfíbios, pássaros e insetos) (Benzing, 2000).

O interesse pelo cultivo de bromélias para comercialização como plantas ornamentais é considerado recente, do início dos anos 1990 (Coffani-Nunes, 2002). A crescente demanda de mercado tem sido responsável pelo aumento da produção e comercialização de bromélias. No entanto, um considerável aumento no extrativismo ilegal, especialmente de espécies com ciclos de vida longos, vem reduzindo muitas populações (Coffani-Nunes, 2002). Além disso, a coleta predatória e a perda de habitat devido à ação antrópica vêm contribuindo para o aumento do número de plantas vulneráveis, ameaçadas de extinção ou mesmo em extinção (Bered *et al*., 2008). Apesar de um crescente aumento de estudos com espécies desta família e de sua importância

ecológica e econômica, a bibliografia científica ainda é consideravelmente restrita (Zanella *et al*., 2012a).

1.1 Gênero *Aechmea*

O gênero *Aechmea* Ruiz & Pav. (do grego: folhas ponta de lança) ocorre desde o México e Antilhas até o Uruguai e Norte da Argentina (Reitz, 1983; Benzing, 2000). Apresenta cerca de 240 espécies, sendo o gênero mais amplo e diverso da subfamília Bromelioideae (Faria *et al*., 2004; Luther, 2008). Cerca de 70% das espécies do gênero estão distribuídas no Brasil (Smith e Downs, 1979) sendo a Mata Atlântica o centro de diversidade para o grupo (Smith, 1934). **N**a última monografia da subfamília Bromelioideae (Smith e Downs, 1979) oito subgêneros foram reconhecidos para *Aechmea*: *Aechmea*, *Chevaliera* (Gaudich. ex. Beer) Baker, *Lamprococcus* Beer (Baker), *Macrochordion* de Vriese (Baker), *Ortgiesia* (Regel) Mez, *Platyaechmea* (Baker) Baker, *Podaechmea* Mez e *Pothuava* (Baker) Baker.

O gênero *Aechmea* é caracterizado por apresentar sépalas assimétricas, porém, muitas das características utilizadas para designar as espécies como integrantes dos oito subgêneros ou mesmo para identificar espécies pertencentes ao gênero são as mesmas utilizadas para classificar espécies que pertencem a outros gêneros, confundindo as delimitações taxonômicas (Faria *et al*., 2004). A enorme diversidade estrutural e morfológica junto com a má compreensão da delimitação natural dessas espécies, e, portanto, dos subgêneros, faz de *Aechmea* um dos maiores desafios taxonômicos de hoje em Bromeliaceae (Leme *et al*., 2010).

Diversos estudos têm sido direcionados para a revisão de gêneros, subgêneros e complexos de espécies da família Bromeliaceae. Porém, ainda persistem lacunas de conhecimento, especialmente nos gêneros mais ricos (*Aechmea*, *Vriesea* Lindl., *Tillandsia* L., *Neoregelia* L.B. Sm.) (Martinelli *et al*., 2008). Estudos envolvendo revisão taxonômica de alguns subgêneros de *Aechmea* já foram realizados, como para *Chevaliera* (Canela *et al*., 2003; Sousa *et al*., 2005, 2008), *Lamprococcus* (Aoyama e Sajo, 2003), *Macrochordion* (Faria *et al*., 2010, 2012), *Podaechmea* (Izquierdo e Piñero, 1998) e *Pothuava* (Wendt, 1997), porém para os subgêneros *Aechmea* e *Platyaechmea* ainda não

há trabalhos realizados. Para o subgênero *Ortgiesia* somente um trabalho, envolvendo aspectos genéticos e taxonomia para duas espécies do grupo, foi realizado (Goetze, 2010).

1.2 Subgênero *Ortgiesia*

O subgênero *Ortgiesia* tem distribuição geográfica restrita a América do Sul. Segundo Smith e Downs (1979) o subgênero apresenta 17 espécies que são encontradas no sul e sudeste do Brasil, sendo que duas delas também ocorrem no leste da Argentina (*A. calyculata* (E. Morren) Baker) e no noroeste do Uruguai e região leste da Argentina e Paraguai (*A. recurvata* (Klotzsch) L.B. Sm.). *Ortgiesia* é caracterizado por apresentar brácteas florais decorrentes, flores sésseis, sépalas conadas de 1/3 até metade de seu comprimento, com mucron tão longo quanto o lobo livre e pétalas com apêndices distintos (Smith e Downs, 1979). Características como padrão da inflorescência (simples ou composta), cor das pétalas (amarela, azul, rosa e branca) e sépalas, cor e tamanho de ovário, tamanho das flores e época de florescimento são utilizadas para a delimitação taxonômica entre espécies deste subgênero. Entretanto, ocorre uma intensa variação morfológica nessas características, tornando a distinção das espécies em muitos casos difícil, especialmente em regiões onde as espécies ocorrem em simpatria (Smith e Downs, 1979; Reitz, 1983; Faria *et al*., 2004; Wanderley e Martins, 2007). Além disso, as informações das fichas de exsicatas nos herbários geralmente não trazem informações a respeito da coloração das peças florais, gerando confusão também na delimitação geográfica das espécies.

Treze das 17 espécies do subgênero *Ortgiesia* foram amostradas e incluídas na presente tese, sendo elas: *A. blumenavii* Reitz, *A. calyculata*, *A. candida* E. Morren ex Baker, *A. caudata* Lindm., *A. coelestis* (K. Koch) E. Morren, *A. comata* Baker, *A. cylindrata* Lindm., *A. gamosepala* Wittm., *A. gracilis* Lindm., *A. kertesziae* Reitz, *A. organensis* Wawra, *A. recurvata* e *A. winkleri* Reitz (Fig. 1). Para alguns desses *taxa* o local do tipo é desconhecido, e a descrição da espécie foi realizada a partir de material cultivado (*A. candida*, *A. calyculata*, *A. coelestis*, *A. comata* e *A. recurvata*, Smith e Donws, 1979). Para as espécies *A. blumenavii*, *A. caudata*, *A. gamosepala*, *A. kertesziae*, *A. organensis* e *A. winkleri* foram realizadas coletas nos locais dos tipos. *Aechmea kleinii* Reitz, descrita originalmente como pertencente ao subgênero *Ortgiesia* (Reitz, 1953), e um

possível *taxa* novo em processo de descrição (Wanderley e Martins, 2007), aqui denominado *Aechmea* sp, também foram amostrados e incluídos no presente estudo (Fig. 1).

Figura 1. Espécies do subgênero *Ortgiesia* incluídas no presente trabalho. A) *Aechmea blumenavii*; B) *A. calyculata*; C) *A. candida*; D) *A. caudata*; E) *A. coelestis*; F) *A. comata*; G) *A. cylindrata*; H) *A. gamosepala*; I) *A. gracilis*; J) *A. kertesziae*; K) *A. kleinii*; L) *A. organensis*; M) *A. recurvata*; N) *A. winkleri*; O) *A.* sp. Fotos: Núcleo de Genética e Conservação de Plantas – UFRGS.

Dados referentes à biologia reprodutiva para o subgênero *Ortgiesia* estão disponíveis para poucas espécies. Os principais polinizadores para *A. comata*, *A. caudata*, *A. cylindrata*, *A. gamosepala*, *A. organensis* e *A. winkleri* são beija flores, abelhas e borboletas (Araújo *et al*., 2004; Kaehler *et al*., 2005; Lenzi *et al*., 2006; Machado e Semir,

2006; Piacentini e Varassin, 2007; Dorneles *et al*., 2011; Kamke *et al*., 2011; Büttow, 2012). A maioria dessas espécies floresce durante o outono (março a julho), com exceção de *A. cylindrata*, observada com flores durante o verão (dezembro a fevereiro; Kaehler *et al*., 2005). A maioria das demais espécies de *Ortgiesia* é descrita como florescendo durante o outono (Reitz, 1983; Wanderley e Martins, 2007), sendo *A. kleinii* a única do grupo com antese noturna (Reitz, 1983). Passeriformes generalistas foram observados como dispersores de sementes em *A. gamosepala* e *A. organensis* (Fischer e Araujo, 1995) e *A. comata* (Lenzi *et al*., 2006). *Aechmea winkleri* e *A. kertesziae* são descritas como autoincompatíveis (Büttow, 2012; Capra, 2012), enquanto *A. comata* é uma espécie autocompatível (Lenzi *et al*., 2006).

Espécies do subgênero com flores de pétalas amarelas (*A. blumenavii*, *A. calyculata*, *A. caudata*, *A. comata*, *A. kleinii*, *A. kertesziae* e *A. winkleri*) apresentam uma distribuição geográfica restrita a região sul do Brasil, com exceção de *A. caudata* que também ocorre no sudeste. Todas as seis espécies com pétalas azuis (*A. coelestis*, *A. cylindrata*, *A. gamosepala*, *A. gracilis*, *A. organenis* e *A.* sp) são encontradas no Paraná e São Paulo, com somente uma ocorrendo no Espírito Santo (*A. coelestis*) e uma no Rio Grande do Sul (*A. gamosepala*). *Aechmea recurvata* é a única espécie com pétalas rosa de *Ortgiesia* e também a única que não ocorre exclusivamente na Mata Atlântica, sendo encontrada também no bioma Pampa. Com pétalas brancas, *A. candida* é encontrada nos estados do Paraná e Santa Catarina (Smith e Donws, 1979; Reitz, 1983; Wanderley e Martins, 2007).

2 Estudos filogenéticos na família Bromeliaceae

Em Bromeliaceae os estudos moleculares iniciais abordaram principalmente o posicionamento filogenético da família, as relações entre as subfamílias e entre os seus respectivos gêneros (por exemplo, Gilmartin e Brown, 1986; Ranker *et al*., 1990; Brown, 2000; Horres *et al*., 2000; Barfuss *et al*., 2005). Entretanto, o progresso para obtenção de uma filogenia bem resolvida foi inicialmente lento, parcialmente devido ao posicionamento isolado de Bromeliaceae, sem um grupo externo claro; parcialmente porque o DNA de cloroplasto de bromélias evolui a uma taxa extremamente baixa, ou devido a problemas de amostragem (Givnish *et al*., 2011). A última filogenia para a

família, utilizando oito marcadores plastidiais conseguiu obter avanços significativos, elucidando o relacionamente das oito subfamílias de Bromeliaceae (Givnish *et al*., 2011).

A monofilia da subfamília Bromelioideae é suportada por dados morfológicos e moleculares, com *Puya* Molina como grupo irmão (Terry *et al*., 1997; Horres *et al*., 2000; Crayn *et al*., 2004; Givnish *et al*., 2004, 2007, 2011; Schulte *et al*., 2005, 2009; Horres *et al*., 2007; Schulte e Zizka, 2008; Sass e Specht, 2010; Silvestro *et al*., 2014). Entretanto, o relacionamento inter e intraespecífico dentro de Bromelioideae é pouco compreendido, e somente recentemente estudos moleculares, baseados em marcadores plastidiais e nucleares, identificaram algumas linhagens basais dentro da subfamília (*Greigia* Regel, *Ochagavia* Phil., *Fascicularia* Mez, *Deinacanthon* Mez, *Bromelia* Juss.) (Schulte *et al*., 2005, 2009; Schulte e Zizka, 2008; Silvestro *et al*., 2014). *Fernseea* Baker foi recuperado como grupo irmão do clado chamado Eu-Bromelioideae, o qual compreendeu os demais representantes da subfamília (Schulte e Zizka, 2008; Schulte *et al*., 2009; Silvestro *et al*., 2014). Dentro de Eu-Bromelioideae os gêneros *Orthophytum* Beer, *Cryptanthus* Otto & A. Dietr., *Ananas* Mill., *Neoglaziovia* Mez, e *Acanthostachys* Klotzsch foram identificados como linhagens basais enquanto que gêneros derivados, caracterizados pelo hábito de tanque, formaram um clado com suporte moderado e foram denominadas "core bromelioids" (Schulte e Zizka, 2008; Schulte *et al*., 2009; Sass e Specht, 2010; Givnish *et al*., 2011). No entanto, segundo a mais recente filogenia para Bromelioideae os gêneros *Neoglaziovia* e *Acanthostachys* pertencem ao clado "core bromelioids" (Silvestro *et al*., 2014).

Dentro do clado "core bromelioids" o relacionamento intergenérico é pouco compreendido, assim como vários gêneros são recuperados como polifiléticos, entre eles, *Aechmea* (Schulte *et al*., 2005, 2009; Schulte e Zizka, 2008; Sass e Specht, 2010; Silvestro *et al*., 2014). Representantes de *Aechmea* são encontrados em vários clados e agrupados com espécies de outros gêneros e a monofilia da maioria dos subgêneros não é suportada (Faria *et al*., 2004; Schulte *et al*., 2005, 2009; Horres *et al*., 2007; Schulte e Zizka, 2008; Sass e Specht, 2010; Silvestro *et al*., 2014). No entanto, os subgêneros *Ortgiesia* e *Podaechmea* vêm sendo recuperados como linhagens monofiléticas com alto suporte estatístico (Schulte *et al*., 2005, 2009; Schulte e Zizka, 2008; Silvestro *et al*., 2014) assim como um clado agrupando os gêneros *Nidularium* Lem., *Neoregelia* L.B. Sm., *Wittrockia* Lindm., *Canistropsis* (Mez) Leme e *Edmundoa* Leme (Silvestro *et al*., 2014). Apesar disso,

nenhum estudo envolvendo ampla amostragem de espécies desses grupos recuperados como monofiléticos foram realizados até o momento. No que diz respeito a representantes do subgênero *Ortgiesia*, somente cinco espécies foram incluídas na última filogenia para Bromelioideae (Silvestro *et al*., 2014).

3 Especiação

A especiação é um processo evolutivo fundamental e de crucial importância para o entendimento da origem da biodiversidade. Pode ser definida como a origem de barreiras reprodutivas entre populações, que permitem a manutenção da distinção genética e morfológica, em proximidades geográficas (Seehausen, 2014). As barreiras reprodutivas podem ser desencadeadas por isolamento geográfico, alterações ecológicas, morfológicas ou comportamentais. A divergência das populações isoladas poderá ser acelerada pela ação de seleção e deriva genética, principais forças evolutivas atuantes na especiação (Turelli *et al*., 2001).

A especiação alopátrica ocorre quando uma barreira física, como topografia, rios ou habitat desfavorável isolam populações, diminuindo ou impedindo o fluxo gênico entre elas. Ocorre diferenciação genética entre essas populações, devido principalmente a seleção natural divergente e quando entram em contato novamente, após várias gerações, podem não ser mais capazes de intercruzarem. A especiação peripátrica ocorre quando populações periféricas dispersam para um novo habitat, oriundas de uma população original maior. Esse novo habitat na maioria das vezes é periférico em comparação ao habitat das espécies "parentais". Mudanças genéticas seriam muito rápidas nessas populações periféricas, fundadas por poucos indivíduos e o fluxo gênico com a população original maior também seria reduzido. Pelo fato de se tratarem de populações pequenas, a principal força evolutiva atuante seria a deriva genética (Futuyma, 2005).

A especiação parapátrica pode ocorrer se o fluxo gênico entre populações adjacentes é menor do que a seleção divergente para diferentes combinações de genes. Uma pressão de seleção forte irá criar uma barreira ao fluxo gênico entre populações em habitats diferentes. Consequentemente clinas nas frequências alélicas em vários locos poderão estabelecer-se e levar ao surgimento de isolamento reprodutivo entre alguns indivíduos. Na

especiação simpátrica ocorre a evolução de barreiras de isolamento reprodutivo dentro dos limites de uma população, sem separação espacial (Turelli *et al*., 2001; Futuyma, 2005).

A rápida diferenciação de um ancestral em várias espécies que habitam uma grande variedade de ambientes, e que apresentam características que as permitem explorar esses ambientes é o que define o conceito de adaptação radiativa. Adaptação radiativa é o surgimento de novas espécies e a evolução de diferenças ecológicas entre elas, podendo ser considerada a síndrome mais comum na origem e proliferação de *taxa* (Schluter, 2000). Apesar de frequentemente ocorrer em regiões geográficas confinadas, como lagos ou ilhas, a maior parte da diversidade funcional observada atualmente deve ter surgido de episódios de adaptação radiativa (Seehausen, 2004).

A família Bromeliaceae é considerada um exemplo de radiação adaptativa (Benzing, 2000) e, portanto, torna-se um bom modelo para estudos de especiação. Entretanto, alguns grupos dentro da família são considerados de origem recente (representantes da subfamília Bromelioideae, por exemplo; Silvestro *et al*., 2014), com espécies não completamente definidas. Isso pode levar a ocorrência de hibridação, uma vez que não existe um isolamento reprodutivo completo entre as espécies, o que, consequentemente, pode tanto acelerar como impedir a completa diferenciação entre os *taxa* envolvidos (Abbott *et al*., 2013). Ainda, em espécies ou linhagens de divergência recente, algumas partes do genoma podem já estar diferenciadas, enquanto outras ainda são compartilhadas com espécies ancestrais. A especiação teria início em alguns poucos locos adaptativos, com a diferenciação completa somente em fases mais tardias da divergência (Wu *et al*., 2001). Assim, haveria compartilhamento de polimorfismos pelo menos nos estágios iniciais do estabelecimento das espécies.

4 Marcadores moleculares

O advento dos marcadores moleculares de DNA, principalmente aqueles baseados na reação em cadeia da polimerase (PCR), oportunizou a caracterização genética de diferentes espécies (Reif *et al*., 2004). O sequenciamento de regiões plastidiais e nucleares está entre as técnicas mais utilizadas em estudos populacionais e de filogenia. Entre as regiões plastidias merecem destaque os espaçadores intergênicos (Lorenz-Lemke *et al*., 2010; Turchetto-Zolet *et al*., 2012; Novaes *et al*., 2013, Silvestro *et al*., 2014). O DNA plastidial

(cpDNA) é uniparental (herdado maternalmente na maioria das angiospermas; Ennos, 1994), permitindo a diferenciação entre fluxo gênico via pólen e semente (Petit *et al*., 2005) e a detecção de eventos antigos de hibridação (Scotti-Saintagne *et al*., 2013).

Entre as regiões nucleares, os espaçadores internos transcritos do DNA ribossomal (ITS) são geralmente utilizados. Entretanto, em estudos envolvendo espécies da família Bromeliaceae esse marcador apresentou baixa ou nenhuma variação genética (Schulte *et al.*, 2009). Outras regiões nucleares vêm sendo utilizadas em estudos filogéticos com bromélias (Schulte *et al*., 2009; Sass e Specht, 2010), com destaque para o gene "Phytocrome C" (Jabaily e Systma, 2010; Krapp *et al*., 2014; Louzada *et al*., 2014; Silvestro *et al*., 2014).

Marcadores AFLP ("Amplified Fragment Length Polymorphism") também podem ser utilizados em estudos filogenéticos, particularmente em grupos de espécies proximamente relacionadas, como em recentes adaptações radiativas. O fato dos fragmentos obtidos estarem distribuídos ao longo do genoma faz com que diferenças genéticas raras sejam descobertas entre taxa que apresentam, por exemplo, baixa variação genética em sequências plastidiais (Meudt e Clarke, 2008). Além disso, esses marcadores apresentam altas taxas de variabilidade e não requerem conhecimento prévio do genoma da espécie a ser analisada. Por todas essas características os AFLPs são muito utilizados em estudos de grupos que apresentam uma história evolutiva complexa, envolvendo a ocorrência de hibridação, introgressão e poliploidização (revisado em Gaudeul *et al*., 2012).

Os marcadores microssatélites também conhecidos como "simple sequence repeats" (SSR) ou "short tandem repeats" (STR) são regiões repetitivas do DNA dispostas lado a lado, onde pequenos motivos (1 a 6 pares de base) são repetidos *n* vezes (Oliveira *et al*., 2006). Os microssatélites representam regiões instáveis do genoma, que estão sob alterações mutacionais, geralmente adições ou deleções de um número integral de repetições, com taxa muito mais elevada do que o observado nas demais regiões do genoma (Jarne e Lagoda, 1996). Estão distribuídos ao longo de sequências codificantes e não codificantes do DNA (Schlötterer e Tautz, 1992) e têm sido identificados nos genomas de procariotos e eucariotos. Em eucariotos são encontrados nos três genomas: nuclear e plastidial (Powell *et al*., 1996) e mitocondrial (Soranzo *et al*., 1999).

Um loco homozigoto de microssatélite tem o mesmo número de repetições em ambos os cromossomos homólogos, enquanto que um loco heterozigoto tem um número diferente de repetições para cada alelo, por exemplo, um alelo pode conter nove repetições e o outro dez. Entretanto, em um mesmo loco, uma população geralmente contém muitos alelos, cada um com um número diferente de repetições, o que significa que os marcadores microssatélites são úteis para discriminar diferentes indivíduos (Oliveira *et al*., 2006).

A natureza codominante dos SSR faz deles uma ferramenta muito útil em estudos para resolver problemas que variam desde a taxonomia, questões relacionadas à paternidade, à estrutura genética de populações, padrões de hibridação e sistema de cruzamento (Parker *et al*., 1998; Boneh *et al*., 2003).

Microssatélites são espécie-específicos, sendo necessário o isolamento desses marcadores para cada espécie. Porém, a presença de regiões flanqueadoras conservadas permite a amplificação desses locos em espécies próximas. Entretanto, o alto grau de polimorfismo observado na espécie em que os locos foram descritos pode ocasionalmente não ser observado em espécies relacionadas, especialmente quando aumenta a distância evolutiva entre as mesmas (Rubinsztein *et al*., 1995; Morin *et al*., 1998).

Para a família Bromeliaceae bibliotecas de microssatélites nucleares já foram isoladas e caracterizadas para diversas espécies (p.e. Boneh *et al*., 2003; Sarthou *et al*., 2003; Palma-Silva *et al*., 2007; Paggi *et al*., 2008; Wöhrmann *et al*., 2012a, 2012b; Zanella *et al*., 2012b; Hmeljevski *et al*., 2013) mas para a subfamília Bromelioideae, SSR estão disponíveis para poucas espécies: *Ananas comosus* (Wöhrmann e Weising, 2011) e *Orthophytum ophiuroides* Louzada e Wand. (Aoki-Gonçalves *et al*., 2014). Para o gênero *Aechmea* dez SSR foram isolados e caracterizados recentemente para a espécie *A. caudata* (Goetze *et al*., 2013).

5 Genética de populações e filogeografia em Bromeliaceae

A caracterização da variação genética dentro e entre populações naturais é de extrema importância para a conservação dos recursos genéticos e pode contribuir para o conhecimento acerca do polimorfismo de uma espécie, o que pode influenciar o seu potencial evolutivo (Hamrick e Godt, 1996). A diversidade genética é o material bruto sobre o qual a seleção natural atua para permitir a adaptação e evolução dos organismos e a

sua adequação às mudanças ambientais (Frankham *et al*., 2008). A estrutura genética das populações reflete a interação entre diferentes processos, incluindo a sua história evolutiva (distribuição, fragmentação de habitat, isolamento da população), mutações, deriva genética, sistema de cruzamento, fluxo gênico e seleção (Sales *et al*., 2001), as quais podem ajudar na compreensão dos processos de adaptação a circunstâncias ecológicas particulares (Parker *et al*., 1998).

Estudos de genética de populações estão disponíveis para algumas espécies de bromélias, como por exemplo, para o gênero *Aechmea* (Murawski e Hamrick, 1990; Izquierdo e Piñero, 2000), *Alcantarea* (E. Morren ex Mez) Harms (Barbará *et al*., 2007, 2008, 2009), *Bromelia* L. (Zanella *et al*., 2011), *Dyckia* Schult. f. (Hmeljevski *et al*., 2011), *Encholirium* Mart. ex Schult. (Cavallari *et al*., 2006), *Pitcairnia* L'Hér. (Sarthou *et al*., 2001; Boisselier-Dubayle *et al*., 2010; Palma-Silva *et al*., 2011), *Tillandsia* (Soltis *et al*., 1987; González-Astorga *et al*., 2004), *Puya* (Sgorbati *et al*., 2004) e *Vriesea* (Alves *et al*., 2004; Palma-Silva *et al*., 2009; Zanella, 2013). Em uma revisão sobre padrões de diversidade genética de espécies de bromélias, Zanella *et al*. (2012b) observaram que tais espécies apresentam grande variação nos índices de diversidade genética, dependendo do tipo de marcador molecular usado; alto coeficiente de endocruzamento em espécies autógamas ou com sistema misto de cruzamento, enquanto espécies alógamas apresentam um F_{IS} baixo. Já quanto à estruturação genética, os valores de F_{ST} variaram de 0,043 a 0,961.

A filogeografia é uma disciplina relativamente recente que trata dos princípios e processos que governam a distribuição geográfica de linhagens genealógicas, especialmente dentro e entre espécies proximamente relacionadas (Avise, 2009). A análise e interpretação da distribuição de linhagens usualmente requerem informações da genética molecular, genética de populações, filogenias, demografia, etologia e geografia histórica, sendo a filogeografia uma disciplina integrativa (Avise, 1998). Estudos filogeográficos têm sido utilizados para investigar os efeitos de mudanças climáticas do passado na estrutura genética de espécies animais e vegetais. Estes estudos permitem inferir sobre a evolução de espécies dentro de biomas, o que pode ser utilizado como subsídio para estratégias de conservação (Bermingham e Moritz, 1998; Ramos *et al*., 2007).

Estudos filogeográficos envolvendo espécies de bromélias são escassos, e foram realizados para espécies dos gêneros *Pitcairnia* e *Vriesea*. Para *Pitcairnia geyskesii* L.B.

Sm., uma espécie endêmica da Guiana Francesa, encontrada em afloramentos rochosos, o estudo identificou algumas barreiras ao fluxo gênico, rotas de expansão populacional e isolamento nas populações do sul (Boisselier-Dubayle *et al*., 2010). Já os resultados encontrados para *Vriesea gigantea* Mart. ex Schult. f., *V. carinata* Wawra e *V. incurvata* Gaudich., espécies endêmicas da Mata Atlântica, revelaram que as espécies devem ter se mantido em mais de um refúgio durante as oscilações climáticas do Pleistoceno, e que houve uma recente expansão populacional para o sul do bioma (Palma-Silva *et al*., 2009; Zanella, 2013).

Objetivos

Este estudo teve como objetivo geral investigar os processos evolutivos responsáveis pela especiação dentro de *Aechmea* subgênero *Ortgiesia*, visando contribuir para a delimitação taxonômica das espécies e para o entendimento da grande diversidade encontrada dentro da subfamília Bromelioideae. Também, objetivou examinar a diversidade genética e fluxo gênico de espécies de *Ortgiesia* endêmicas do Sul da Mata Atlântica para identificação de áreas com valor para a conservação e de importância evolutiva.

Objetivos específicos

a) Reconstruir o relacionamento interespecífico em *Ortgiesia* usando marcadores AFLP;
b) Examinar a evolução de caracteres morfológicos-chave dentro de *Ortgiesia* e discutir o seu valor taxonômico;
c) Explorar padrões biogeográficos e discutir o papel da hibridação para a evolução de *Ortgiesia*;
d) Examinar a diferenciação genética das espécies dentro do grupo com pétalas amarelas, usando marcadores plastidiais e um gene nuclear;
e) Quantificar a diversidade genética e sua distribuição entre populações do grupo de pétalas amarelas, visando identificar áreas com valor para conservação;
f) Isolar e caracterizar locos de microssatélites nucleares para o gênero *Aechmea*;

g) Investigar a estruturação e diversidade genética de populações de *Aechmea calyculata*, usando marcadores microssatélites, plastidiais e um gene nuclear;

Capítulo II

Diversification of Bromelioideae (Bromeliaceae) in the Brazilian Atlantic rainforest: a case study in *Aechmea* subgen. *Ortgiesia*

Manuscrito a ser submetido a Molecular Phylogenetics and Evolution

Diversification of Bromelioideae (Bromeliaceae) in the Brazilian Atlantic rainforest: a case study in *Aechmea* subgen. *Ortgiesia*

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ABSTRACT

Aechmea subgenus *Ortgiesia* encompasses ca. 17 species, which are endemic to South America and diversified in Brazilian Atlantic rainforest. Here we used 1032 polymorphic AFLP markers and performed neighbor-joining analysis on 96 samples representing fourteen *Ortgiesia* species. We investigated interspecific relationships in *Ortgiesia*, mapped key morphological characters evolution and discussed biogeographical patterns and the role of hybridization in the evolution of this group. Our results revealed that subgenus *Ortgiesia* is a natural group. Three main genetic groups were recovered, which at broad scale could be characterized by flower color. Morphological character state reconstruction revealed that yellow petal and simple inflorescence are ancestral within *Ortgiesia*. Hybridization seems to have played a role during the evolution of *Aechmea* subgenus *Ortgiesia* in Atlantic rainforest, which may have contributed to the conquest of a great variety of environments. The current geographical distribution highlighted south Brazilian Atlantic rainforest as the diversity center of *Ortgiesia.*

Keywords: AFLPs, bromeliads, interspecific relationships, hybridization, morphology, speciation.

1. Introduction

The Brazilian Atlantic rainforest represents one of the top five biodiversity hotspots on earth, with high levels of endemism (Myers et al., 2000). Despite its reduction to only 11 – 16 % of its original area (Ribeiro et al., 2009), it is home to more than 15,000 plants species of which 45 % are found nowhere else (Stehmann et al., 2009). Bromeliaceae, the second largest family among vascular epiphytes in the Neotropics, are an important and characteristic element of the Atlantic rainforest, where 816 bromeliad species occur, of which 651 are endemic (Stehmann et al., 2009). Bromeliads play an important ecological role due to their interaction with fauna, as sources for pollen, nectar, fruit, and water, as well as for providing microhabitats for many invertebrates and even vertebrates (Benzing, 2000). For subfamily Bromelioideae (800 species, Luther, 2008) the Atlantic rainforest is the center of diversity, where ca. 505 of its species are found (Martinelli et al., 2008), and ten of the 33 bromelioid genera are endemic to this biome (Smith and Downs, 1979;

Stehman et al., 2009). The patterns and processes that led to these high levels of endemism and species richness in the Atlantic rainforest are still poorly understood.

Subfamily Bromelioideae is morphologically and ecologically highly diverse, comprising terrestrials, lithophytes and epiphytes. Molecular phylogenetic studies revealed that the Bromelioideae comprises several smaller, early divergent lineages and the core bromelioid clade, which harbors the majority of species (Schulte et al., 2005, 2009; Schulte and Zizka, 2008). Recent molecular phylogenetic studies inferred an Andean origin of the subfamily in the late Miocene, at around 13 Ma, and an early migration to East Brazil via a central South American corridor, at around 10 Ma (Schulte et al., 2005; Silvestro et al., 2014). Whereas the early diverging Bromelioideae lineages were terrestrials and lithophytes, the acquisition of a central water impounding structure, the tank, facilitated the core bromeliads to conquer the epiphytic niche, especially within the Atlantic rainforest, where they diversified extensively (Schulte et al., 2009; Givnish et al., 2011; Silvestro et al., 2014).

Despite being recovered as a well-supported clade, intergeneric relationships of the core bromelioids still remain unclear, with the largest Bromelioideae genus, *Aechmea* (240 species), being the most problematic (Smith and Downs, 1979; Luther, 2008). In *Aechmea*, which has traditionally been subdivided into eight subgenera (Smith and Downs, 1979), many of the characters used to circumscribe subgenera or species are vague and often fail to delimit natural groups (Faria et al., 2004, 2010; Schulte and Zizka, 2008). Molecular phylogenetic studies demonstrated that *Aechmea* is highly polyphyletic (Faria et al., 2004; Schulte et al., 2005, 2009; Horres et al., 2007; Schulte and Zizka, 2008; Sass and Specht, 2010; Givnish et al., 2011; Silvestro et al., 2014). However, *Aechmea* subgenus *Ortgiesia* has been recovered as a highly supported and monophyletic lineage within the core bromelioids (Schulte et al., 2005, 2009; Schulte and Zizka, 2008; Silvestro et al., 2014). The subgenus comprises ca. 17 species, most of which are endemic to the Atlantic rainforest (Smith and Downs, 1979, Luther, 2008). Therefore, *Ortgiesia* constitutes a suitable model group to study and gain insights into the diversification of Bromelioideae in the Atlantic rainforest.

Subgenus *Ortgiesia* occurs in south and southeastern Brazil (Espírito Santo to Rio Grande do Sul states), mainly in the subtropical area, with several narrow endemic species, some only known from the type collection. Two more widespread species, *Aechmea*

calyculata and *A. recurvata*, also expand more to the west and reach into Argentina, Paraguay, and Uruguay (Smith and Downs, 1979). *Ortgiesia* species are encountered from sea level to 1,200 m elevation as epiphytes, lithophytes, or terrestrials (Smith and Downs, 1979; Reitz, 1983; Wanderley and Martins, 2007). The crown diversification of *Ortgiesia* was estimated to have started during the late Pliocene, at around 2.5 Ma (Silvestro et al., 2014). Nevertheless, in previous molecular studies sampling was restricted to few *Ortgiesia* species and therefore, interspecific relationships of the subgenus are still unclear.

In *Ortgiesia* taxonomy and systematics, great emphasis has placed on floral features, in particular the petal color, the inflorescence branching pattern and inflorescence shape (Smith and Downs, 1979). Petal color in *Ortgiesia* is mostly yellow or blue, sometimes pink or white, and rarely individuals are found that exhibit different intermediate colors (personal observation M. Goetze). However, a great variation is found in inflorescence morphology, which varies from simple to compound, dense to lax, few to many flowered, with many intermediate forms, thus rendering the delimitation of species boundaries very difficult (Smith and Downs, 1979; Faria et al., 2004; Wanderley and Martins, 2007). Sympatric occurrence of species and the occurrence of individuals with intermediate morphological features suggest that hybridization may play an important role in *Ortgiesia*. In Bromeliaceae, hand-pollination and molecular studies have demonstrated that hybridization is a feasible phenomenon (Wendt et al., 2001, 2002; Jabaily and Sytsma, 2010; Schulte et al., 2010; Palma-Silva et al., 2011; Zanella, 2013).The lack of detailed molecular studies in *Ortgiesia* hampered the evaluation of morphological characters and their taxonomic value, and so far it is still unclear to what extend hybridization and introgression may contribute to intermediate morphological attributes in *Ortgiesia*.

Previous phylogenetic studies in Bromeliaceae demonstrated a very low DNA sequence variation in standard plastid and nuclear markers (e.g., Barfuss et al., 2005; Horres et al., 2007; de Oliveira et al., 2007; Schulte et al., 2009; Maia et al., 2012; Versieux et al., 2012), which rendered it generally difficult to assess intra- and interspecific relationships. Amplified fragment length polymorphisms (AFLP) have been successfully used to elucidate interspecific relationship in closely related Bromeliaceae (Horres et al., 2007; Rex et al., 2007; Schulte et al., 2010; Jabaily and Systma, 2013). The AFLP technique provides numerous genetic markers that are distributed across the whole genome and usually exhibit moderate to high rates of variability. AFLPs require no prior

knowledge of the genome analyzed, and have been proven to be a time and cost efficient tool in assess interspecific relationships, also in complex plant groups (Meudt and Clarke, 2008; Gaudeul et al., 2012).

This study aims to gain insights into the diversification of bromelioids in the Atlantic rainforest using *Aechmea* subgenus *Ortgiesia* as model group. To this aim, we 1) reconstruct interspecific relationships in *Ortgiesia* based on AFLP data, 2) examine the evolution of key morphological characters and discuss their taxonomic value, 3) explore biogeographic patterns in *Ortgiesia*, and 4) discuss the role of hybridization for the evolution of the group.

2. Materials and Methods

2.1 Plant material

A total of 96 individuals belonging to 14 species of subgenus *Ortgiesia* plus one potential new species were sampled along the geographical distribution of the group in the Atlantic rainforest (Table 1). Fresh leaves of 1-9 individuals per species were sampled and dried on silica gel. Five species were included as outgroup based on previous molecular phylogenetic studies (Schulte et al., 2009; Silvestro et al., 2014): *Neoregelia laevis*, *Nidularium billbergioides*, *Nidularium innocentii*, *Wittrockia superba* from the core bromeliad clade, and *Bromelia antiacantha* as representative of an early lineage within the subfamily. Vouchers information is provided in Table 1.

Updated geographical distribution maps for *Ortgiesia* species were generated based on data obtained from field collections, Smith and Downs (1979), and the online database of major Brazilian herbaria *species*Link (http://www.splink.org.br) to explore biogeographical patterns.

2.2 AFLP procedures

Total genomic DNA was extracted with the CTAB method following Doyle and Doyle (1990). Amplified fragment length polymorphism protocols followed Vos et al. (1995) with some modifications as outlined by Schulte et al. (2010). In the digestion-ligation reaction 30 ng of DNA were digested with the restriction enzymes *Hind*III and *Mse*I (10U/µl; Fermentas) for 12 h at 37° C in a final volume of 30 µl, and the *Hind*III and

*Mse*I adapters were ligated to the DNA fragments using T4 DNA ligase (5U/µl; Fermentas). Pre-selective amplifications were carried out with primers with one selective base each (*Hind*III-A and *Mse*I-C, respectively) in a total volume of 10 µl containing 2 µl of the 1:10 diluted restriction-ligation product, 1 x *Taq* buffer (Kapa Biosystems), 0.5 µM of each primer, 2 mM of dNTP mix, and 0.25 U of *Taq* DNA polymerase (Kapa Biosystems). Selective amplifications were run with primers carrying three selective bases each (see below), and with fluorescently labeled *Hind*III primers (FAM, NED, or VIC; Applied Biosystems), with 2.5 µl 1:20 diluted pre-selective PCR product, 1 x *Taq* buffer (Kapa Biosystems), 0.5 µM of each primer, 2 mM of dNTP mix, and 0.25 U of *Taq* DNA polymerase (Kapa Biosystems). Primer screening was carried out with 54 primer combinations and eight representatives *Ortgiesia* samples. Based on the resulting banding patterns, eleven primer combinations were chosen for generation of the final AFLP profiles: (1) *H*-ACA and *M*-CAA, (2) *H*-AGC and *M*-CGA, (3) *H*-AAC and *M*-CTG, (4) *H*-ACA and *M*-CTA, (5) *H*-AGC and *M*-CTG, (6) *H*-AAC and *M*-CGA, (7) *H*-ACC and *M*-CAG, (8) *H*-AGC and *M*-CTA, (9) *H*-ACT and *M*-CAG, (10) *H*-ACA and *M*-CTC, and (11) *H*-ACA and *M*-CAT. To assess the accuracy and reproducibility of the AFLP markers, a subset of samples were run independently for each primer pair. Comparisons of the results from the two independent runs were satisfactory, yielding highly similar and reproducible banding patterns. Amplification products were electrophoresed at the Australian Genome Research Facility Ltd on an AB3730 DNA analyzer (Applied Biosystems) with an internal standard size (LIZ 500).

Genotypes were automatically scored in GeneMarker version 1.97 (SoftGenetics) using the AFLP analysis tool, carefully cross-checked, and manually edited where required. Fragments of a size range between 90 and 400 base pairs and with signal strengths above an intensity score of 500 were scored as a binary presence/absence matrix (1/0).

2.3 AFLP data analyses

In order to detect incompatible or ambiguous phylogenetic signal within the dataset a NeighborNet analysis (Bryant and Moulton, 2004) was conducted based on Nei and Li (1979) distances using SplitsTree4, version 4.13.1 (Huson and Bryant, 2006).

Interespecific relationships were reconstructing by neighbor-joining (NJ) analysis (Saitou and Nei, 2002), carried out based at Nei-Li's distance measure (Nei and Li, 1979) using PAUP* version 4.0b10 (Swofford, 2002). Statistical support values for nodes and clades were estimated by bootstrap analysis with 1000 replicates (Felsenstein, 1985).

2.4 Evaluation of major morphological transitions

To re-assess the taxonomic utility of morphological characters used in previous taxonomic treatments of *Aechmea* subgen. *Ortgiesia*, we encoded three key floral characters: petal color: (0) yellow, (1) blue, (2) pink, and (3) white; inflorescence type: (0) simple, and (1) compound; and inflorescence shape: (0) ellipsoid, (1) cylindric, and (2) pyramidal. The character state matrix was based on published data (Smith and Downs, 1979; Reitz, 1983; Wanderley and Martins, 2007), and on personal observations. Character state transitions were traced along the NJ tree under the maximum parsimony criterion using the software MESQUITE version 2.75 (Maddison and Maddison, 2008).

3. Results

3.1 NeighborNet and neighbor-joining analysis

AFLP profiles generated with eleven AFLP primer pairs for 96 *Ortgiesia* accessions yielded a data matrix comprising 942 fragments, and 1032 fragments including the outgroup. The number of scored fragments per primer pair ranged from 70 (*H*-ACC and *M*-CAG) to 123 (*H*-ACT and *M*-CAG), with an average of 93.8 fragments per primer combination.

The NeighborNet analysis of the AFLP dataset exhibited short internal nodes indicating an overall shallow genetic divergence within *Ortgiesia* (Fig. 1). Nevertheless, within the NeighorNet four subgroups were discernable: 1) a subgroup that unites all *A. recurvata* accessions, 2) a subgroup that comprises all studied *A. winkleri* samples, 3) a subgroup harboring seven out of nine *A. calyculata* accessions together with all *A. blumenavii* accessions, and termed the calyculata group in the following, and 4) a distinct subgroup formed by the outgroup.

In the NJ tree subgenus *Ortgiesia* formed a highly supported group with a bootstrap value (BV) of 98. Within *Ortgiesia*, the NJ tree exhibited short internal branches and split

into a dichotomy with two main branches: one branch comprised the calyculata group, including the two species *A. blumenavii* and *A. calyculata,* and the other one split into group I and II (Fig. 2).

The calyculata group split into a dichotomy, with one branch formed by four accessions of *A. calyculata*, and the other united two subgroups formed by *A. blumenavii* and a third comprising the remaining *A. calyculata* samples.

Group I was divided into two main branches, B and C. Branch B comprised three subgroups, one formed by *A. gracilis* accessions; a second moderately supported (BV 78) united *A. gamosepala* samples and a third and well supported subgroup (BV 89) included all the *A. recurvata* accessions. Branch C also harbored three subgroups, one formed by *A. coelestis* accessions, plus one *A. organensis* sample and one *A.* sp accession; the second included *A. organensis* accessions, and the third comprised all the remaining samples of *A.* sp.

Group II comprised 11 species and split into two branches, D and E. Branch D unified three subgroups, one formed by all accessions of *A. comata*, and the second and third subgroup comprised *A. kertesziae* accessions plus *A. kleinii*. Branch E split into a dichotomy, with one branch unifying all *A. winkleri* accessions, and the other comprising all accessions of *A. caudata* plus one accession each of *A. candida*, *A cylindrata, A. gamosepala, A. kertesziae, A. organensis*, as well as several representatives of *A. coelestis* and *A. gracilis*. The relationships within this subgroup remain largely uncertain.

3.2 Character evolution in *Ortgiesia*

The petal color of the majority of species in *Aechmea* subgen. *Ortgiesia* is yellow (eight species), followed by species with blue petals (six species). Tracing the character state transitions along the NJ tree inferred yellow petals as the ancestral character state for *Ortgiesia*. Yellow petals are found in all species of the calyculata group, and was inferred as the ancestral state for group II in the NJ tree, in which the majority of species possess yellow petals (Fig. 3a). Blue petals were reconstructed to have evolved twice and appear as the ancestral character state for group I in the NJ tree in which blue petals are the prevalent petal color, with the exception of one species, *A. recurvata*, with pink petals. Interestingly, blue petaled species are also found within branch E (group II) in the NJ tree, and this concerns accessions of species that are also found within group I. White and pink petals

were reconstructed as derived character states for *Ortgiesia,* and have each evolved only once (in *A. candida* and *A. recurvata*, respectively).

Most of the species in *Ortgiesia* possess simple inflorescences (12 species), and five species have compound inflorescences. For one species, *A. gracilis*, both character states have been reported (Smith and Downs, 1979; Wanderley and Martins, 2007). The reconstruction of character evolution along the NJ tree indicates that simple inflorescences are the ancestral character state in *Ortgiesia,* and that compound inflorescences evolved at least three times independently (Fig. 3b). Compound inflorescences were reconstructed as ancestral state for branch C in group I, and in *A. gracilis* (within branch B, group I), as well as for branch E in group II. Further, the character reconstruction implies that there have been several reversals to simple inflorescences, but this concerns samples of species, which were placed in different groups along the tree (e.g. *A. kertesziae* and *A. gamosepala*).

In *Ortgiesia*, the inflorescence shape of simple inflorescences can be ellipsoid or cylindric while compound inflorescences are always pyramidal. Character state reconstruction of the inflorescence shape implies high levels of homoplasy of this character within *Ortgiesia*, and the ancestral character state remained ambiguous (Fig. 3c).

4. Discussion

4.1 Hybridization within *Aechmea* subgen. *Ortgiesia*

The presented AFLP study revealed that some blue petaled accessions nested within group II, which was formed by species with yellow flowers. These blue flowered accessions concerned to species that were also found in group I, which united the majority of blue flowered taxa. *Aechmea caudata* was the yellow flowered species recovered as close related to the blue accessions from branch E in the NJ tree and they are found sympatrically in some areas (Fig. 4a). Thus, this pattern suggests the occurrence of hybridization between yellow and blue species of *Ortgiesia*.

Reproductive biology studies with *Ortgiesia* taxa revealed that some species present the same pollinator, i.e. the hummingbird *Thalurania glaucopsis* (observed in *A. caudata*, *A. comata*, and *A. organensis*), the bee *Bombus morio* (*A. caudata*, *A. comata*, *A. gamosepala*, and *A. winkleri*), and *B. brasiliensis* (*A. caudata* and *A. cylindrata*) (Araújo et al., 2004; Kaehler et al., 2005; Lenzi et al., 2006; Machado and Semir, 2006; Piacentini

and Varassin, 2007; Dorneles et al., 2011; Kamke et al., 2011; Büttow, 2012). These data reveal a lack of pollinator's specificity inside *Ortgiesia* not only between taxa that present the same petal color, but also among species with yellow (*A. caudata*, *A. comata*, *A. winkleri*) and blue (*A. cylindrata*, *A. gamosepala*, *A. organensis*) petals, indicating that interspecific gene flow is possible to occur. Isolation between species due to differences on flowering time is also almost absent in *Ortgiesia*, as most of the species are recognized as blossoming during autumn (March to June, Reitz, 1983; Wanderley and Martins, 2007). Thus, prezygotic isolation seems to be weak in *Ortgiesia* species.

The blue flowered taxa recovered in the NJ tree as close related to *A. caudata* may be from hybrid origin. In fact, in some populations it is difficult to distinguish *A. caudata* from the blue flowers taxa (*A. coelestis* and *A. organensis*) especially because the main differences among them are the petal and ovary color (Wanderley and Martins, 2007). Reitz (1965) described an *A. caudata* variety with light blue petals (*A. caudata* var. *eipperi*) later synonymized to *A. organensis* (Smith and Downs, 1979; Wanderley and Martins, 2007), which could have been a hybrid. Our results did not allow us to identify which of the blue petaled species may hybridize with *A. caudata*. Further population genetic studies and hand cross-pollination experiments involving *A. caudata* and blue petaled sympatric species should be carry out to investigate this hypothesis.

Artificial hybrids of bromeliads are easily produced and cultivated (e.g., Vervaeke, 2004; Zhang et al., 2012), which indicate weak pre and postzygotic isolation within Bromeliaceae. Wendt et al. (2008) studied 42 sympatric bromeliads belonging to nine genera and revealed that prezygotic isolation mechanisms were inefficient to avoid interspecific pollination. However, only one putative hybrid between two *Aechmea* species was observed. Field observations and hand-pollination experiments reported natural hybridization between two sympatric species of genus *Pitcairnia* (Wendt et al., 2001, 2002), which was further confirmed by a molecular study (Palma-Silva et al., 2011). The occurrence of natural hybridization was also detected between sympatric species from genera *Puya* and *Vriesea* (Jabaily and Sytsma, 2010; Schulte et al., 2010; Zanella, 2013), thus indicating that hybridization is an important factor involved in the speciation process of the family Bromeliaceae.

Identify isolation barriers in *Aechmea* subgen *Ortgiesia* will be of great importance to try to understand the diversification process inside this group. Geographical isolation

seems to be an important isolation factor inside *Ortgiesia* as putative hybrids were identified in contact zones. However, other factors should also be involved. Evolutionary changes in mating system, for example, can influence the degree of reproductive isolation between selfers and their outcrossing relatives (Sweigart and Willis, 2003). *Aechmea comata* and *A. kertesziae* are partially sympatric in the coast region of Santa Catarina state (Fig. 4b) and greatly differ in their mating system: *A. comata* is self-compatible (Lenzi et al., 2006), while *A. kertesziae* is an obligate outcrossing (Capra, 2012). In a study conducted with two sympatric bromeliads from genus *Pitcairnia* the authors concluded that the selfer species would be more protected against interspecific introgression than its close outcrossing relative (Palma-Silva, et al., 2011). So, the differences presented by *A. comata* and *A. kertesziae* in their mating system could have been acting as an isolation barrier, avoiding interspecific gene flow as the former was recovered as an exclusive lineage in the present study. Unfortunately data about mating system for other sympatric *Ortgiesia* species as *A. gracilis* and *A. organensis* for example, are not available. Further studies are needed to improve our knowledge on mating system as well as other characteristics of the reproductive biology of *Ortgiesia* species, which will be useful to understand the speciation process inside this group.

4.2 Interspecific relationships

Aechmea subgen. *Ortgiesia* was recovered as highly supported (BV 98) indicating being a natural group, confirming the results from previous studies (Schulte et al., 2005, 2009; Schulte and Zizka, 2008; Silvestro et al., 2014). The AFLP analysis revealed three main genetic groups of closely related species within *Ortgiesia*, namely calyculata group, group I and II.

Species of calyculata group present simple inflorescences, flowers with yellow petals and an inland geographical distribution. *Aechmea blumenavii* is restricted to the northern region of Santa Catarina state where it partially overlaps in geographical distribution with *A. calyculata*. *Aechmea calyculata* presents a more widespread range, which is also found further southwest, reaching Rio Grande do Sul state and Argentina (Fig. 5a). Besides this, the close relationship between *A. blumenavii* and *A. calyculata* was not suggested before (Reitz, 1983), especially because the differences in inflorescence shape: cylindric in the former and ellipsoid in *A. calyculata*. The results from NJ analysis revealed that some

accessions of *A. calyculata* were closer related to *A. blumenavii* than to the remaining samples of *A. calyculata* (Fig. 2 branch A). In face of weak prezygotic barriers, these close related species may hybridize and thus explain why these morphologically distinct taxa were recovered as non-exclusive lineages in the NJ tree. Studies addressing intra and interspecific gene flow involving a broader sampling, especially including *A. calyculata* populations from the sympatric areas, as well as chloroplast markers, are needed in order to elucidate the extend of hybridization between these species.

Group I united species with both simple and compound inflorescences, and blue and pink flowers. Taxa from branch B, *A. gracilis*, *A. gamosepala*, and *A. recurvata*, have in common the condition of propagate through rhizomes although their close relationship was not suggested by morphology (Smith and Downs, 1979; Reitz, 1983). *Aechmea recurvata* is a distinct species within *Ortgiesia* as it is the only with nested inflorescences and pink petals (Smith and Downs, 1979; Reitz, 1983). Its geographical distribution is the most widespread inside the subgenus, occurring from Paraná to Rio Grande do Sul state in Brazil, reaching Argentina, Paraguay, and Uruguay, co-occurring with *A. gracilis* and *A. gamosepala* (Fig. 5b), as well as with many of the *Ortgiesia* species.

Species from branch C of group I (*A. coelestis*, *A. organensis*, and *A.* sp) share blue petals and close areas of occurrence (Fig. 6). The taxonomy delimitation of these taxa plus *A. gracilis* has received researchers' attention for a long time. The characters traditionally used to delimitate them are very polymorphic and in many cases overlap and do not present clear boundaries (Wanderley and Martins, 2007; Abonanza, 2012). Still, there is no clear geographical isolation among these taxa, which are easily found in sympatry. Wanderley and Martins (2007) after long revision of herbarium specimens and some new field collections were able to separate these taxa in distinct species. However, Abonanza (2012) based on a morphological revision and on data obtained from nuclear microsatellite markers proposed the synonymization of *A. gracilis* and *A. organensis* in *A. coelestis* based on the lack of diagnosis characters and low genetic differentiation among species.

Our AFLP results revealed that *A. coelestis*, *A. gracilis*, *A. organensis*, and *A.* sp formed distinct genetic subgroups in the NJ tree, which may indicate that they are different species. The large number of fragments normally generated in AFLP studies can over-perform microsatellites in discriminating taxa and populations (Meudt and Clarke, 2008, and references therein), which may explain the discrepancies found among the present

study and that developed by Abonanza (2012). However, the difficulty in identifying morphological diagnosis characters, as revealed by previous studies (Wanderley and Martins, 2007, Abonanza, 2012), does not discard the occurrence of hybridization among them or that those taxa are cryptic species. To further shed light on the relationships inside this species complex a study with a broader sampling using multilocus sequence data (as those get by next-generation sequencing, McCormack et al., 2013) should be carry out.

Most of the species from group II present yellow flowers, with exception of branch E that harbored few blue petaled accessions. Branch D united species with simple inflorescences, restricted to Santa Catarina state, occurring at the coast (*A. comata* and *A. kertesziae*) and in high altitudes (*A. kleinii*, above 1000 m elevation, Fig. 4b). The close relationship of these species has been suggested by morphology (Smith and Downs, 1979; Reitz, 1983). However, *A. kleinii* was included in *Aechmea* subgenus *Pothuava* by Smith and Downs (1979) without any apparent reason. Wendt (1997) in a morphological revision of Brazilian *Pothuava* species removed *A. kleinii* from this subgenus and included it in *Ortgiesia*, position that is confirmed by the present study.

The NJ tree revealed that *A. winkleri* is close related to a subgroup formed by *A. caudata* and few blue petaled accessions. Despite the relationships inside the *A. caudata* subgroup is not resolved (see discussion about hybridization), *A. winkleri* and *A. caudata* can be considered close related species, which was already suggested by morphology, especially because they are the only yellow flowered *Ortgiesia* that present compound inflorescences (Smith and Downs, 1979).

The results obtained in the present study provided insights into the relationships within subgenus *Ortgiesia* for the first time. This allowed the identification of closely related species within the group and directions to further studies. The taxonomy difficulties highlighted here were already evident in previous studies within *Aechmea* (e.g., Faria et al., 2004, 2010; Schulte et al., 2009; Sass and Specht, 2010). The recent origin of *Aechmea* subgen. *Ortgiesia* allied to hybridization may have lead to these taxonomic difficulties.

4.3 Character evolution

Petal color is one of the main characters used to distinguish *Aechmea* subgen. *Ortgiesia* species (Smith and Downs, 1979; Reitz, 1983). Mapping this character onto the obtained NJ tree revealed that yellow, pink, and white petals evolved only once within

Ortgiesia, while blue petals was reconstructed to have evolved twice. However, the presence of blue petaled species inside group II could be a result of hybridization between yellow and blue flowered species, and so these blue accessions should better not be considered for character reconstruction. In doing so, blue colored petals could also be considered as had evolved only once within *Ortgiesia*. Petal color then seems to be a good taxonomic character to be used to distinguish species of subgenus *Ortgiesia*.

The reconstruction of inflorescence type and shape along the NJ tree revealed that these characters are homoplastic and have limited taxonomic value. Morphological characters often prove to be homoplastic within *Aechmea* (Faria et al., 2004). Inflorescence type within *Ortgiesia* shows considerable plasticity under field conditions with variation among individuals of a same population (M Goetze, personal observations). The difficulties in identifying morphological characters that do not overlap between species inside *Ortgiesia* could be a result of hybridization, which would have lead to many intermediate forms. These intermediate forms though may have been very important to conquest new environments, thus leading to a rapid diversification of *Ortgiesia* in Atlantic rainforest.

4.4 Biogeography

Serra do Mar in southeastern Brazil is hypothesized as the place of origin of the bromelioid clade (Schulte et al., 2005; Givnish et al., 2011). Serra do Mar is a range of mountains, parallel to the coastline distributed from 19° to 27°S (Almeida and Carneiro, 1998). Species from *Aechmea* subgen. *Ortgiesia* occur in this region, but most of them are restricted to the southern range of Serra do Mar, from southern São Paulo to northern Santa Catarina state (23° to 27°S). In the northern region of Santa Catarina state, five out of the seven yellow *Ortgiesia* occur, including species from calyculata group, which suggests this region as the center of origin for the subgenus. From this area we hypothesized three main migration routes based on the actual geographical distribution of the species: one towards the south via the coastal region reaching Rio Grande do Sul state and Uruguay; a second into the western inland regions, reaching Argentina and Paraguay; and the third towards north, getting into Espírito Santo state. The migration route towards south agrees with a floristic study which described that species from Atlantic rainforest migrated into Rio Grande do Sul and Uruguay from the north, via the coastline (Rambo, 1961). The decrease

in diversity towards west, where only two *Ortgiesia* species are found, was previously observed for other Atlantic rainforest taxa, and seems to be correlated to a decrease in humidity and rainfall (Oliveira-Filho and Fontes, 2000). This climatic difference would also explain the less species found towards northern Atlantic rainforest. Annual rainfall decreases from southern São Paulo to northern Rio de Janeiro, especially in the coastal region (Oliveira-Filho and Fontes, 2000), which may explain the occurrence of fewer *Ortgiesia* species in those areas.

Some *Ortgiesia* species are found as narrow endemics or in a patchy and disjoint pattern of distribution. This could be a result of forest contraction/ expansion, which occurred during the climatic changes of Pleistocene. Palynological studies describe a decrease in temperature and dryer conditions in south and southeastern Brazil during the Last Glacial Maximum and forest taxa may have being restricted to small areas of refugia in deep valleys or to more humid areas along rivers. In the beginning of Holocene there was a significant increase in temperature and humidity, which allowed forest expansion (Behling and Negrelle, 2001; Behling et al., 2004; Behling and Pillar, 2007). These shifts in vegetation range may have fragmented species distribution leading to geographical isolation and subsequent secondary contact. Geographical isolation could have promoted population differentiation leading to adaptations to new ecological conditions, whereas secondary contact may have allowed hybridization, promoting the emergence of new adaptive traits. For *Ortgiesia* this scenario would explain why some species present small geographical range, as *A. comata* and *A. kleinii*; the disjoint distribution of *A. winkleri*; and the occurrence of some accession of putative hybrid origin (branch E).

5. Conclusions

The results obtained in the presented study revealed that *Aechmea* subgen. *Ortgiesia* is a natural group. Interspecific relationships however were not well resolved and merit further investigation. For *Ortgiesia*, hybridization may have played an important role during the diversification process in Atlantic rainforest, which will explain the great diversity in morphology and the difficulties in identifying diagnosis characters for the species. However, to understand to what extension hybridization may have had impact in the evolution of *Ortgiesia*, further studies should use chloroplast markers, to assess the

occurrence of ancients events of hybridization inside the group. The mapping of key morphological characters allowed us to confirm petal color as a valuable taxonomic character while inflorescence type and shape were found as homoplastic. The current pattern of geographical distribution of *Ortgiesia* species suggests the northern region of Santa Catarina state as their diversity center, with further expansion towards south, west and northern Atlantic rainforest areas.

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Table 1. List of samples included in this study.

Species	Sample name	Location/ population code	Voucher
A. blumenavii	611, 612, 616, 617, 632, 633, 640, 642	Blumenau - SC/ BL	FURB 13803
A. calyculata	325, 327	Derrubadas - RS/ DE	HAS 66416
	530	Concórdia - SC/ CO	FURB 34426
	516	Barracão - RS/ BA	ICN 119812
	581	Putinga - RS/ PU	HVAT 46
	429, 431	Maquiné - RS/ MA	HAS 36048
	480, 510	São Francisco de Paula - RS/ SF	ICN 165253
A. candida	711	Guaratuba - PR/ GU	Cultivated
A. caudata	8	Guarujá - SP/ GA	SP 385012
	56	Ilhota - SC/ IL	FURB 14688
	59	Serra do Rio do Rastro - SC/ SR	HAS 66425
	170	Araquari - SC/ AR	FURB 28062
	239, 266	Florianópolis - SC/ FL	FURB 22585
A. coelestis	09, 15, 17, 20	Ubatuba - SP/ UB	SP 397027
	291, 292, 295	Santa Teresa - ES/ ST	T. S. Coser 321 (R)
	305, 306	Santa Maria do Jetibá - ES/ SM	T. S. Coser 341 (R)
A. comata	196, 199, 203, 205, 648, 649, 650, 655	Florianópolis - SC/ FL	ICN 165256
A. cylindrata	363	Serra da Graciosa - PR/ SG	MBM 180463
A. gamosepala	154, 156	Serra Dona Francisca - SC/ SD	ICN 191154
	421, 422	Matinhos - PR/ MT	ICN 165259
A. gracilis	02, 03, 07	Pariquera-Açu - SP/ PA	B. F. Abonanza 13 (SP)
	283, 284	Iporanga - SP/ IP	B. F. Abonanza 23 (SP)
	386, 392, 396, 497	Serra da Graciosa - PR/ SG	ICN 191151
A. kertesziae	102, 588, 600	Itajaí - SC/ IT	ICN 191153
	659, 664	Bombinhas - SC/ BO	CESJ 62360
	219, 734	Florianópolis - SC/ FL	UPBC 35253
	40, 44	Laguna - SC/ LA	ICN 167498
A. kleinii	278, 279, 280	Antônio Carlos - SC/ AC	ICN 167501

Table 1. Cont.

A. organensis	731	Teresópolis - RJ/ TE	B. F. Abonanza 17 (SP)
	388, 397, 398, 406, 736	Serra da Graciosa - PR/ SG	ICN 191150
A. recurvata	29, 30, 31, 32	Viamão - RS/ VI	ICN 115402
	119, 120, 121	Itajaí - SC/ IT	FURB 14370
	737	Monte Castelo - PR/ MC	ICN 191149
A. sp	312, 313, 314, 315, 316	Tapiraí - SP/ TA	B. F. Abonanza 3 (SP)
A. winkleri	130, 133, 140, 739	Corupá - SC/ CR	ICN 191152
	176, 178, 181, 735	Santa Cruz do Sul - RS/ SC	ICN 189267
Bromelia antiacantha	725	Viamão - RS	ICN 61643
Neoregelia laevis	705	Serra da Graciosa - PR	ICN 190907
Nidularium billbergioides	710	Garuva - SC	UPCB 22872
N. innocentii	706	Serra Dona Francisca - SC	FURB 20521
Wittrockia superba	726	Jacinto Machado - SC	FURB 25282

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

Figure Legends

Fig. 1. NeighborNet diagram of 96 *Ortgiesia* accessions plus outgroup based on Nei and Li (1979) distances of 1032 AFLP characters derived from eleven primer pairs. The scale bar indicates genetic distances.

Fig. 2. Neighbor-joining tree of 96 *Ortgiesia* accessions plus outgroup based on Nei and Li (1979) distances of 1032 AFLP characters obtained with eleven primer pair combinations. Bootstrap values are shown above branches. The similarity scale is indicated by a horizontal bar.

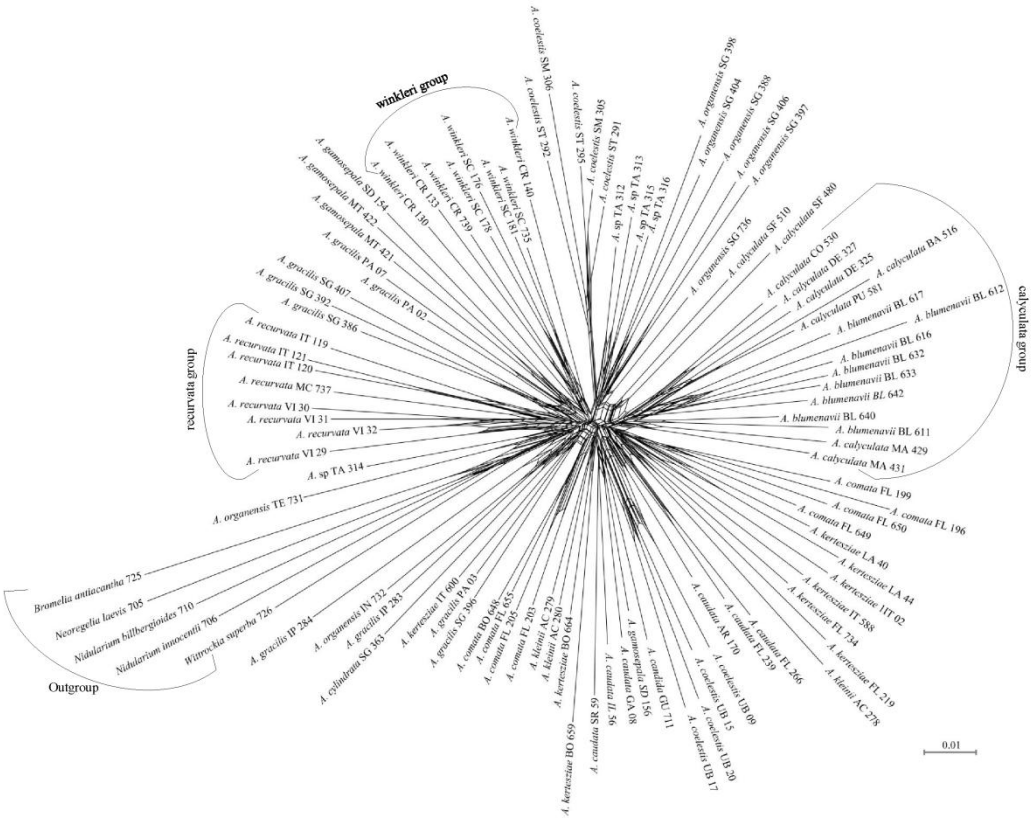
Fig. 3. Most parsimonious reconstruction of the evolution of (A) petal color, inflorescence type (B), and inflorescence shape (C) in *Ortgiesia*, based on the neighbor-joining tree obtained from the analysis of 1032 AFLP characters.

Fig. 4. Maps showing the geographical distribution of *Ortgiesia* species accordingly to herbarium records. Each branch is enlarged from Fig. 2 and the placement of these branches in the NJ tree is indicated with a gray box on the reduced full tree. The symbol of each species/ individual name in the NJ tree or in the key matches the symbol on that species on the maps. Populations/ individuals sampled are indicated and codes correspond to those in Table 1. (A) Branch E. (B) Branch D. Brazilian Federal states: SP, São Paulo; PR, Paraná; SC, Santa Catarina; RS, Rio Grande do Sul.

Fig. 5. Maps showing the geographical distribution of *Ortgiesia* species accordingly to herbarium records. Each branch is enlarged from Fig. 2 and the placement of these branches in the NJ tree is indicated with a gray box on the reduced full tree. The symbol of each species/ individual name in the NJ tree or in the key matches the symbol on that species on the maps. Populations/ individuals sampled are indicated and codes correspond to those in Table 1. (A) Branch A. (B) Branch B. Brazilian Federal states: SP, São Paulo; PR, Paraná; SC, Santa Catarina; RS, Rio Grande do Sul.

Fig. 6. Map showing the geographical distribution of species of branch C, accordingly to herbarium records. The branch is enlarged from Fig. 2 and the placement of it in the NJ tree is indicated with a gray box on the reduced full tree. The symbol of each species/ individual name in the NJ tree or in the key matches the symbol on that species on the map. Populations/ individuals sampled are indicated and codes correspond to those in Table 1. Brazilian Federal states: ES, Espírito Santo; RJ, Rio de Janeirio; SP, São Paulo; PR, Paraná.

Fig. 1


winkleri group
recurvata group
calyculata group
Outgroup
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A. coelestis ST 295
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A. coelestis ST 291
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A. sp TA 313
A. sp TA 315
A. sp TA 316
A. organensis SG 398
A. organensis SG 404
A. organensis SG 388
A. organensis SG 406
A. organensis SG 397
A. winkleri SC 176
A. winkleri CR 739
A. winkleri SC 178
A. winkleri SC 181
A. winkleri CR 140
A. winkleri SC 755
A. gamosepala SD 154
A. winkleri CR 133
A. winkleri CR 130
A. gamosepala MT 422
A. gamosepala MT 421
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A. gracilis PA 02
A. gracilis SG 407
A. gracilis SG 392
A. gracilis SG 386
A. recurvata IT 119
A. recurvata IT 121
A. recurvata IT 120
A. recurvata MC 737
A. recurvata VI 30
A. recurvata VI 31
A. recurvata VI 32
A. recurvata VI 29
A. sp TA 314
A. organensis TE 731
Bromelia antiacantha 725
Neoregelia laevis 705
Nidularium billbergioides 710
Nidularium innocentii 706
Wittrockia superba 726
A. gracilis IP 284
A. organensis IN 732
A. gracilis IP 283
A. cylindrata SG 363
A. kertesziae IT 600
A. gracilis PA 03
A. gracilis SG 390
A. comata BO 648
A. comata FL 655
A. comata FL 205
A. comata FL 203
A. kleinii AC 279
A. kleinii AC 280
A. kertesziae BO 664
A. kertesziae BO 659
A. caudata SR 59
A. comata IL 36
A. comata GO 98
A. gamosepala SD 156
A. caudata GU 711
A. coelestis UB 17
A. coelestis UB 20
A. coelestis UB 15
A. coelestis UB 09
A. caudata AR 170
A. caudata FL 239
A. caudata FL 266
A. kertesziae FL 219
A. kleinii AC 278
A. kertesziae FL 734
A. kertesziae IT 588
A. kertesziae IT 02
A. kertesziae LA 44
A. kertesziae LA 40
A. comata FL 649
A. comata FL 650
A. comata FL 196
A. comata FL 199
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A. calyculata MA 429
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A. blumenavii BL 640
A. blumenavii BL 642
A. blumenavii BL 633
A. blumenavii BL 632
A. blumenavii BL 616
A. blumenavii BL 612
A. blumenavii BL 617
A. calyculata PU 581
A. calyculata DE 325
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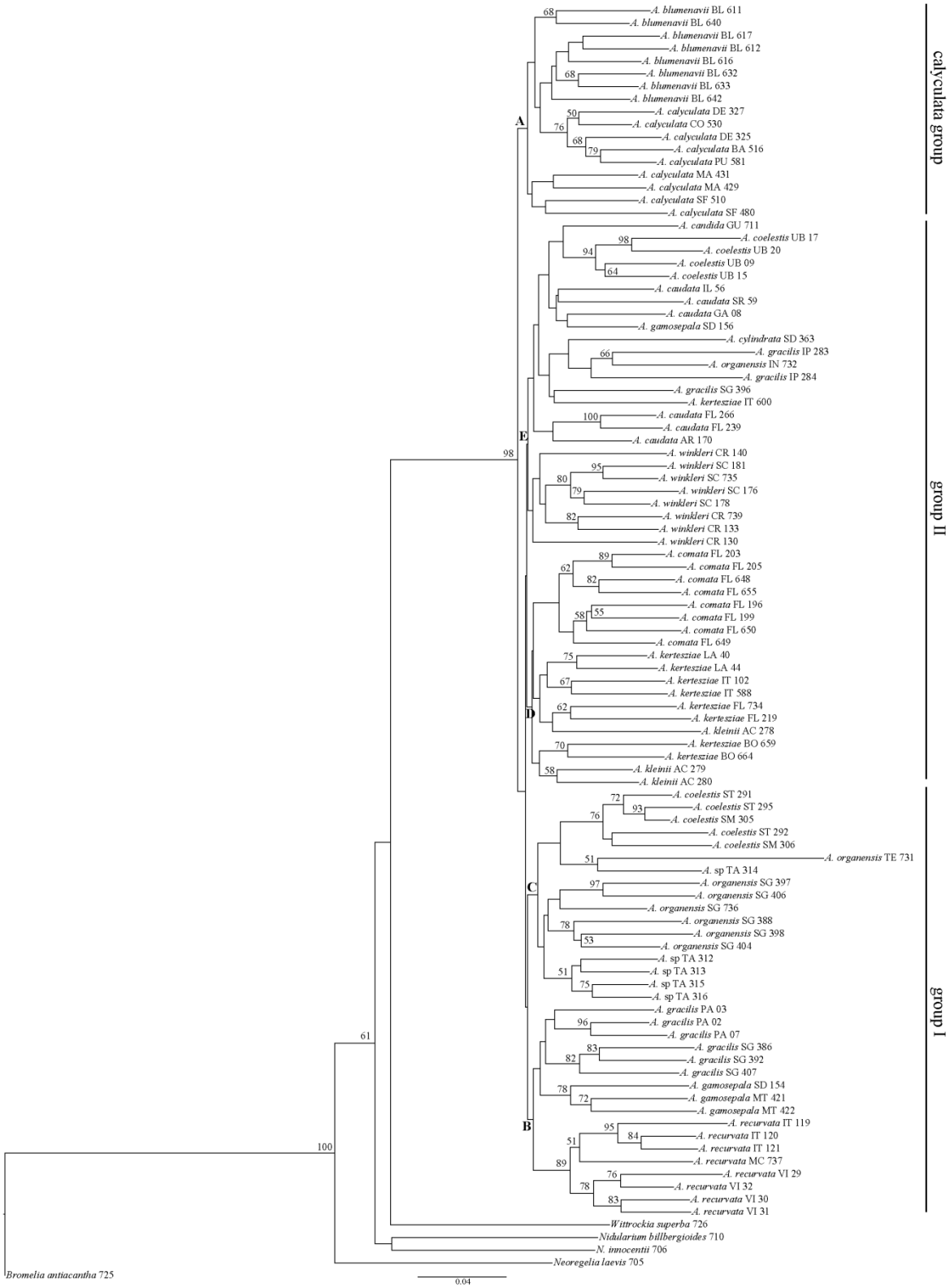
Fig. 2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A. caudata GA 08
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A. cylindrata SD 363
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A. caudata FL 239
A. caudata AR 170
A. winkleri CR 140
A. winkleri SC 181
A. winkleri SC 735
A. winkleri SC 176
A. winkleri SC 178
A. winkleri CR 739
A. winkleri CR 133
A. winkleri CR 130
A. comata FL 203
A. comata FL 205
A. comata FL 648
A. comata FL 655
A. comata FL 196
A. comata FL 199
A. comata FL 650
A. comata FL 649
A. kertesziae LA 40
A. kertesziae LA 44
A. kertesziae IT 102
A. kertesziae IT 588
A. kertesziae FL 734
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A. kleinii AC 278
A. kertesziae BO 659
A. kertesziae BO 664
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A. kleinii AC 280
A. coelestis ST 291
A. coelestis ST 295
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A. coelestis ST 292
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A. sp TA 314
A. organensis SG 397
A. organensis SG 406
A. organensis SG 736
A. organensis SG 388
A. organensis SG 398
A. organensis SG 404
A. sp TA 312
A. sp TA 313
A. sp TA 315
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A. gracilis PA 03
A. gracilis PA 02
A. gracilis PA 07
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A. gamosepala SD 154
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A. recurvata IT 119
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A. recurvata MC 737
A. recurvata VI 29
A. recurvata VI 32
A. recurvata VI 30
A. recurvata VI 31
Wittrockia superba 726
Nidularium billbergioides 710
N. innocentii 706
Neoregelia laevis 705
Bromelia antiacantha 725
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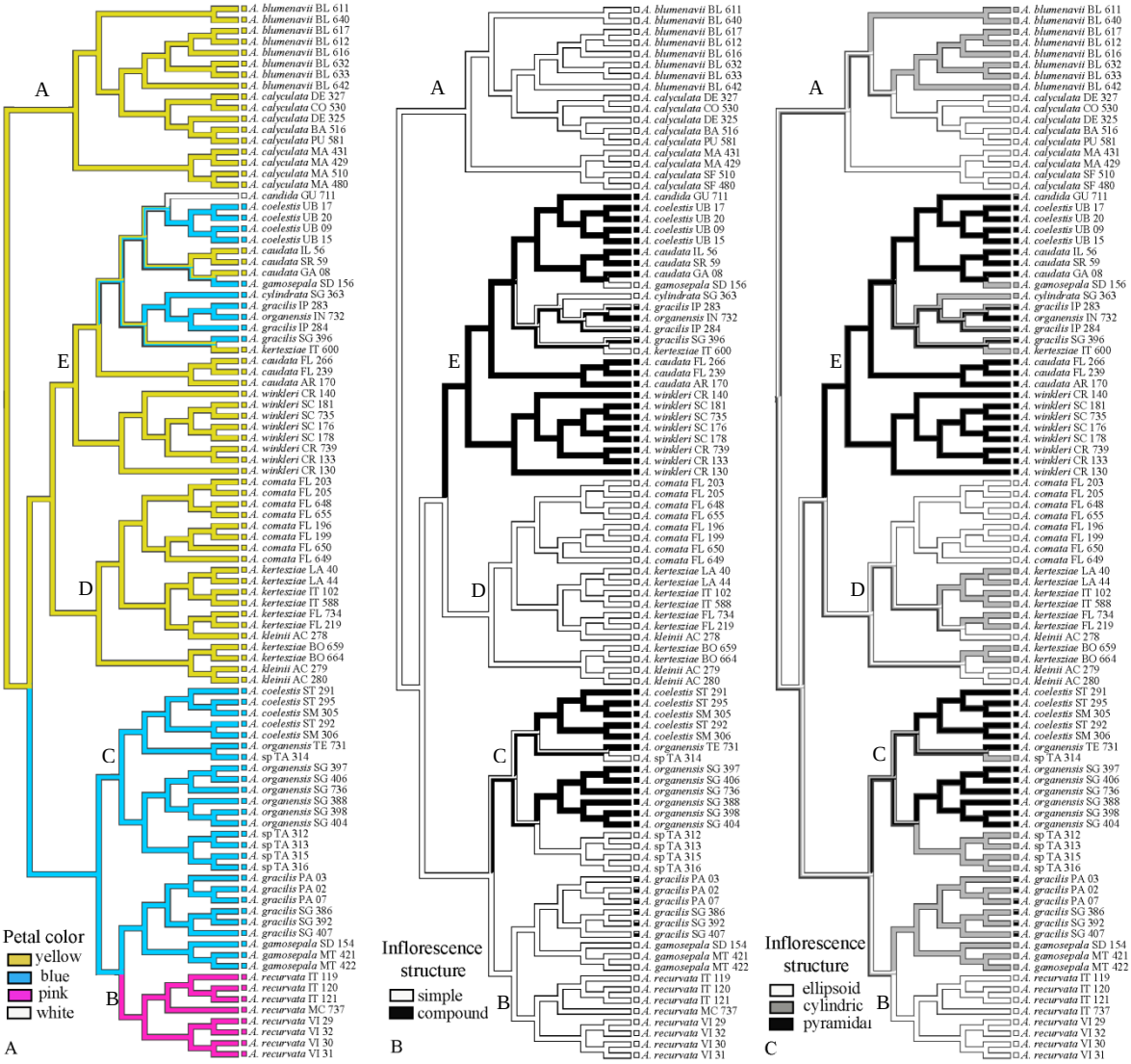
A
B
C
D
E
calyculata group
group II
group I

Fig. 3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A. calyculata MA 480
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A. coelestis UB 20
A. coelestis UB 09
A. coelestis UB 15
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A. caudata SR 59
A. caudata GA 08
A. gamosepala SD 156
A. cylindrata SG 363
A. gracilis IP 283
A. organensis IN 732
A. gracilis IP 284
A. gracilis SG 396
A. kertesziae IT 600
A. caudata FL 266
A. caudata FL 239
A. caudata AR 170
A. winkleri CR 140
A. winkleri SC 181
A. winkleri SC 735
A. winkleri SC 176
A. winkleri SC 178
A. winkleri CR 739
A. winkleri CR 133
A. winkleri CR 130
A. comata FL 203
A. comata FL 205
A. comata FL 648
A. comata FL 655
A. comata FL 196
A. comata FL 199
A. comata FL 650
A. comata FL 649
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A. kertesziae LA 44
A. kertesziae IT 102
A. kertesziae IT 588
A. kertesziae FL 734
A. kertesziae FL 219
A. kleinii AC 278
A. kertesziae BO 659
A. kertesziae BO 664
A. kleinii AC 279
A. kleinii AC 280
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A. coelestis ST 295
A. coelestis SM 305
A. coelestis ST 292
A. coelestis SM 306
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A. sp TA 314
A. organensis SG 397
A. organensis SG 406
A. organensis SG 736
A. organensis SG 388
A. organensis SG 398
A. organensis SG 404
A. sp TA 312
A. sp TA 313
A. sp TA 315
A. sp TA 316
A. gracilis PA 03
A. gracilis PA 02
A. gracilis PA 07
A. gracilis SG 386
A. gracilis SG 392
A. gracilis SG 407
A. gamosepala SD 154
A. gamosepala MT 421
A. gamosepala MT 422
A. recurvata IT 119
A. recurvata IT 120
A. recurvata IT 121
A. recurvata MC 737
A. recurvata VI 29
A. recurvata VI 32
A. recurvata VI 30
A. recurvata VI 31
A
B
C
D
E
Petal color
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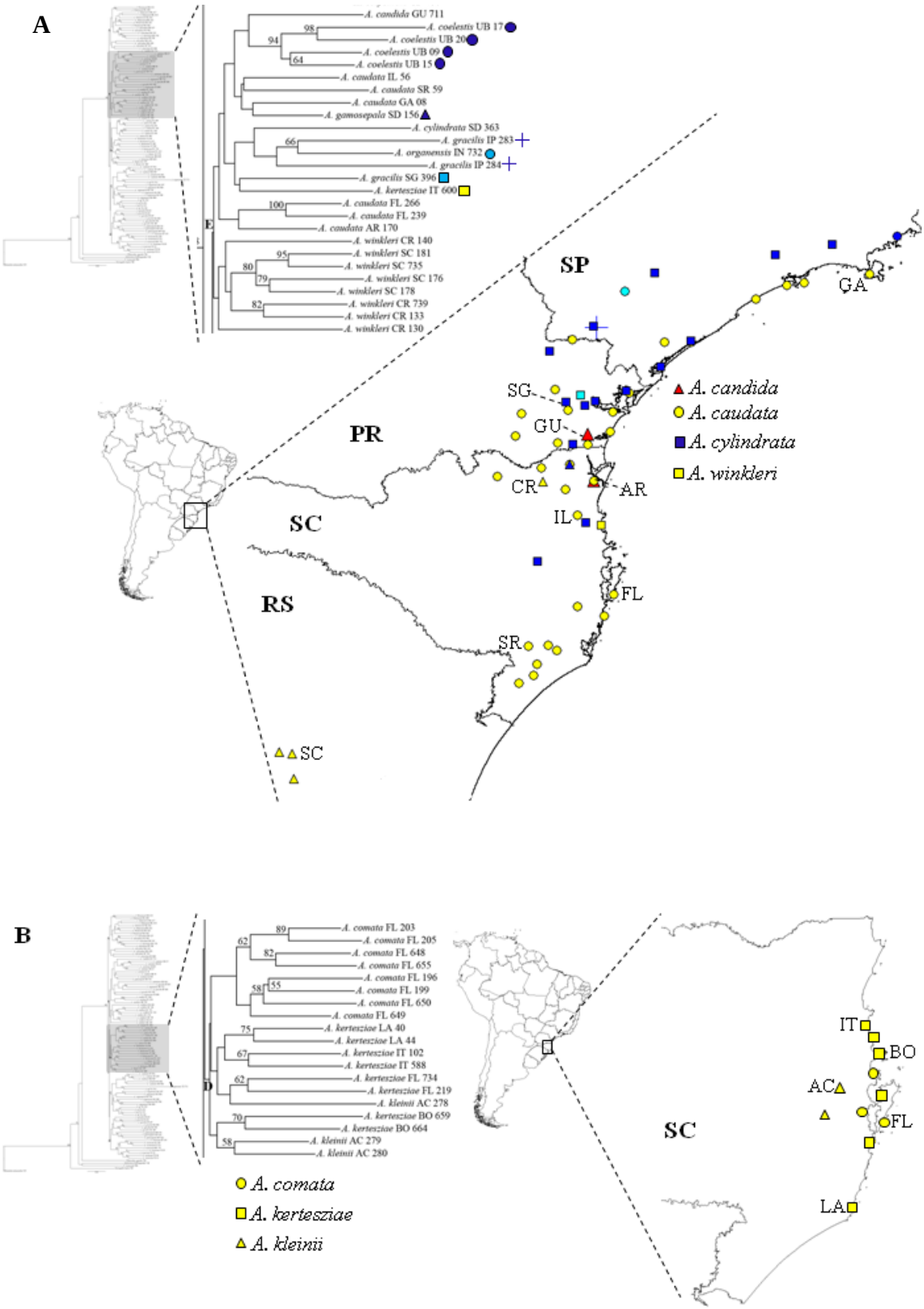
blue
pink
white
Inflorescence structure
simple
compound
Inflorescence structure
ellipsoid
cylindric
pyramidal

Fig.4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A. caudata SR 59
A. caudata GA 08
A. gamosepala SD 156
A. cylindrata SD 363
A. gracilis IP 283
A. organensis IN 732
A. gracilis IP 284
A. gracilis SG 396
A. kertesziae IT 600
A. caudata FL 266
A. caudata FL 239
A. caudata AR 170
A. winkleri CR 140
A. winkleri SC 181
A. winkleri SC 735
A. winkleri SC 176
A. winkleri SC 178
A. winkleri CR 739
A. winkleri CR 133
A. winkleri CR 130
SP
GA
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PR
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IL
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RS
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SR
SC
A. candida
A. caudata
A. cylindrata
A. winkleri
B
A. comata FL 203
A. comata FL 205
A. comata FL 648
A. comata FL 655
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A. comata FL 650
A. comata FL 649
A. kertesziae LA 40
A. kertesziae LA 44
A. kertesziae IT 102
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A. kertesziae FL 734
A. kertesziae FL 219
A. kleinii AC 278
A. kertesziae BO 659
A. kertesziae BO 664
A. kleinii AC 279
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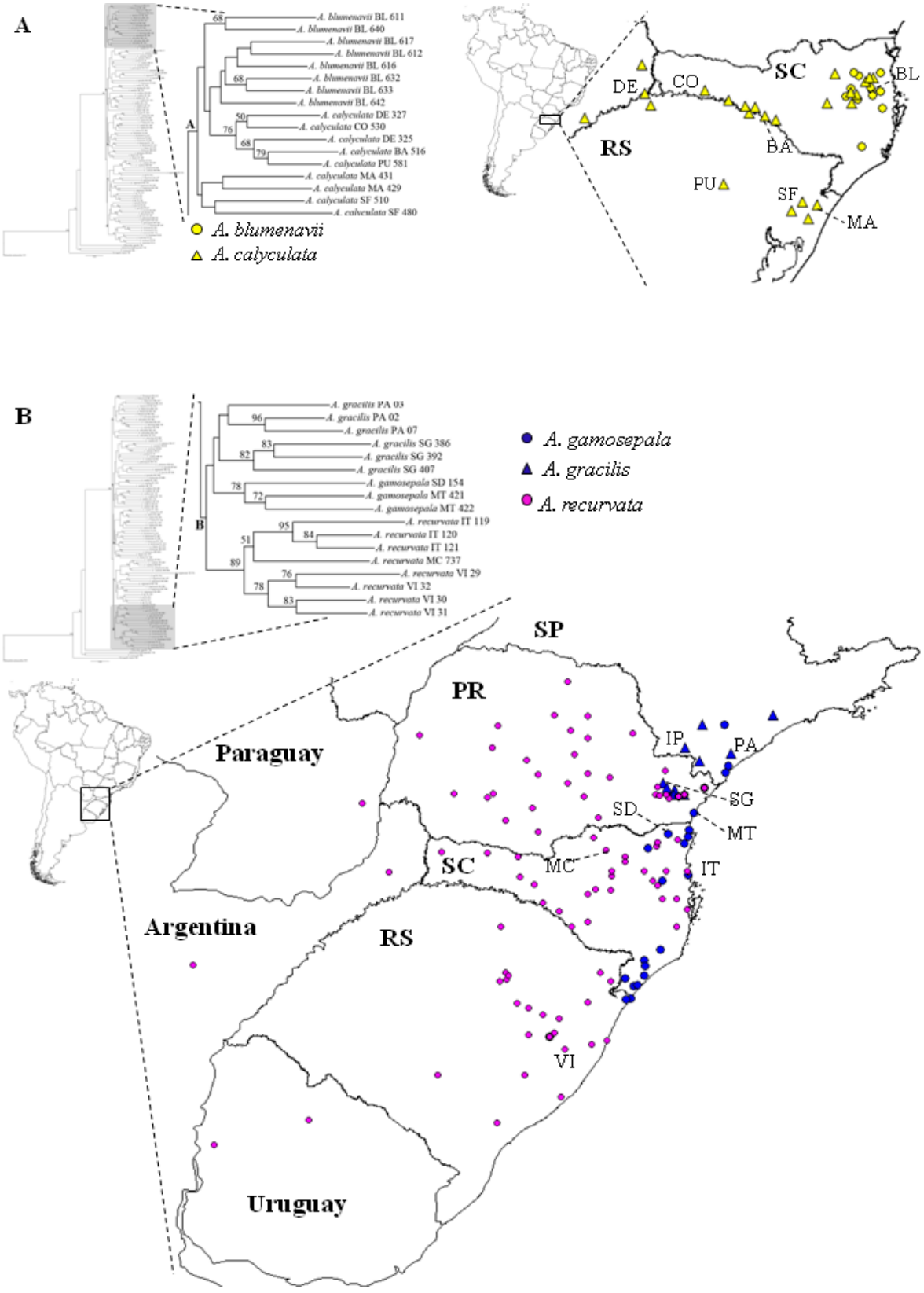
IT
BO
AC
FL
SC
LA
A. comata
A. kertesziae
A. kleinii

Fig. 5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A. blumenavii BL 633
A. blumenavii BL 642
A. calyculata DE 327
A. calyculata CO 530
A. calyculata DE 325
A. calyculata BA 516
A. calyculata PU 581
A. calyculata MA 431
A. calyculata MA 429
A. calyculata SF 510
A. calyculata SF 480
A. blumenavii
A. calyculata
DE
CO
SC
BL
RS
BA
PU
SF
MA
B
A. gracilis PA 03
A. gracilis PA 02
A. gracilis PA 07
A. gracilis SG 386
A. gracilis SG 392
A. gracilis SG 407
A. gamosepala SD 154
A. gamosepala MT 421
A. gamosepala MT 422
A. recurvata IT 119
A. recurvata IT 120
A. recurvata IT 121
A. recurvata MC 737
A. recurvata VI 29
A. recurvata VI 32
A. recurvata VI 30
A. recurvata VI 31
A. gamosepala
A. gracilis
A. recurvata
SP
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PA
SG
SD
MT
MC
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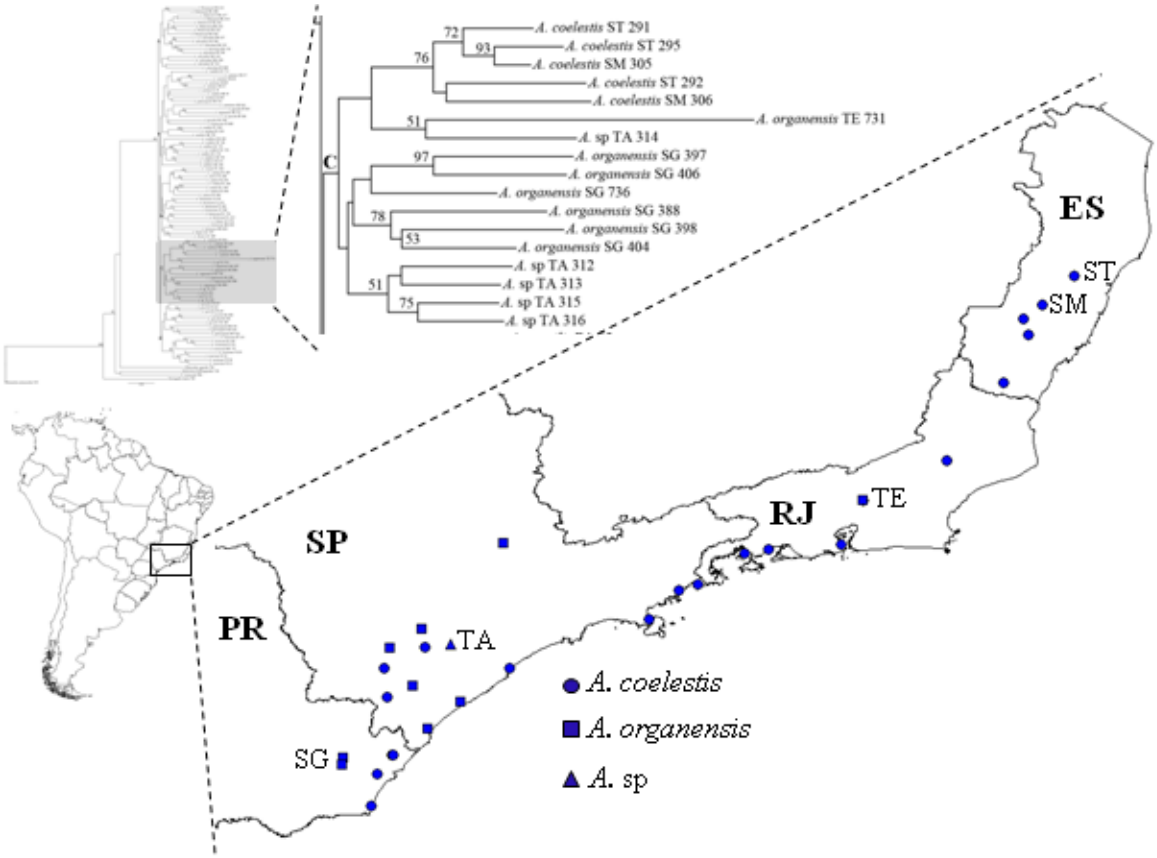
SC
Argentina
RS
VI
Uruguay

Fig. 6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A. organensis SG 736
A. organensis SG 388
A. organensis SG 398
A. organensis SG 404
A. sp TA 312
A. sp TA 313
A. sp TA 315
A. sp TA 316
C
72
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97
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51
75
ES
ST
SM
RJ
TE
SP
PR
TA
SG
A. coelestis
A. organensis
A. sp

Capítulo III

Phylogeography and population differentiation of seven *Aechmea* species (Bromeliaceae) from Atlantic rainforest

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Phylogeography and population differentiation of seven *Aechmea* species (Bromeliaceae) from Atlantic rainforest

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Short running title: Phylogeography and population differentiation of seven *Aechmea* species

In the present study we investigated the genetic differentiation and diversity of seven *Aechmea* species, endemic to Atlantic rainforest, to better understand their relationship and diversification. Samples from all seven species were collected throughout most of their geographical range and sequenced for two chloroplast and one nuclear region, respectively. Thirty-two plastid haplotypes were recovered, which were partially structured accordingly to the species to which the individuals belong. For the nuclear region, 54 haplotypes were found, eleven of them shared between species. Our results suggest that the speciation process inside this group is still ongoing. High levels of genetic diversity were observed in populations located at the northern region of Santa Catarina state, south Atlantic rainforest, which may be considered of conservation and evolutionary value.

ADDITIONAL KEYWORDS: ancient hybridization - bromeliads - conservation - cpDNA - incomplete lineage sorting - speciation.

INTRODUCTION

The speciation process has been fascinating evolutionary biologists for a long time (Sobel *et al*., 2009). Geographical isolation and adaptations to new ecological conditions are among the main factors invoked to explain the appearance of new species (Lorenz-Lemke *et al*., 2010; Pavan *et al*., 2011; Hope *et al*., 2012; Fregonezi *et al*., 2013; Gehring *et al*., 2013; Wachowiak *et al*., 2013; Christie *et al*., 2014; Turchetto *et al*., 2014). For plants diversification, hybridization seems also to play a role since 50-70% of the species present hybrid origin (Ellstrand *et al*. 1996; Rieseberg, 1997; Soltis and Soltis, 2009). Also of great importance, especially in recent groups or in those where the speciation process is still ongoing, is the sharing of ancestral polymorphism (Martinsen *et al*., 2001). All these processes may lead to novel genetic combinations, thus raising divergent populations and distinct genetic lineages (Seehausen, 2004). So studies focusing in the distribution of genetic diversity are of great importance to understand intra and interspecific genetic differentiation (Hewitt, 2001).

Phylogeography provides valuable insights into diversification as it deals with the spatial arrangements of genetic lineages, and try to understand the processes that underline the distribution of genetic variation, especially within and among closely related species (Avise, 2009; Knowles, 2009). Phylogeographical approaches can be used to answer a broad kind of questions as for example, species delimitation, identification of historical hybridization events, current introgression, and geographic determinants of isolation (reviewed in Hickerson *et al*., 2010). However, plant phylogeographical studies remain scarce when compared to animals (Beheregaray, 2008), especially in highly biodiverse areas as South America, where only 17% of such studies focused on plants (Turchetto-Zolet *et al*., 2013).

Subgenus *Ortgiesia* (*Aechmea*, Bromeliaceae) encompasses ca. 17 species found in Argentina, Paraguay, Uruguay, and south and southeastern Brazil (Smith & Downs, 1979). The delimitation of taxa is difficult because of its high morphological variability in inflorescence branching pattern, and possible hybridization between some species (Reitz, 1983; Wanderley & Martins, 2007; Goetze, unpublished data). A study conducted with 15 species of *Ortgiesia*, based on AFLP molecular markers, revealed shallow genetic divergence within the subgenus (Goetze, unpublished data). Nevertheless three main genetic groups were recovered, which could in broad scale be characterized by petal color:

two formed by species with yellow flowers (despite few blue petaled species where nested inside one of the groups, which was likely an outcome of hybridization events); the third by blue and pink flowered species (Goetze, unpublished data).

The seven yellow flowered species are distributed mainly in south Brazil, with one species reaching the southeastern region, and other extending into Argentina in the southwest (Reitz, 1983; Smith & Downs, 1979). Almost all species overlap in geographical distribution (Fig. 1), they blossom mainly during the autumn (March to June; Reitz, 1983), and some species share pollinators (Lenzi *et al*., 2006; Dorneles *et al*., 2011; Kamke *et al*., 2011; Büttow, 2012). From the two yellow flowered groups recovered in the study based on AFLP, one was formed by *A. blumenavii* and *A. calyculata* while the other united the five remaining species: *A. caudata*; *A. comata*; *A. kertesziae*, *A. kleinii*, and *A. winkleri* (Goetze, unpublished data). However, the relationship of these species inside each group was not well resolved and hybridization was pointed out as an important mechanism during the diversification process of this group.

To get new insights into the relationship and diversification of yellow flowered *Ortgiesia* species, chloroplast markers and a nuclear gene were used in the present study. As chloroplast genome is probable maternally inherited in bromeliads as in most angiosperms (Ennos, 1994) it is useful for tracking colonization histories and identify ancient hybridization events. In addition we used the nuclear gene phytocrome C (*phyC*), which has been used in other bromeliads studies (Jabaily & Systma, 2010; Krapp *et al*., 2014; Louzada *et al*., 2014; Silvestro *et al*., 2014) and provide data about gene flow by pollen. By using chloroplast and nuclear markers, and samples from most of the range of all seven yellow flowered *Ortgiesia* species we specifically aim to: 1) examine if morphologically identified species are genetically differentiated; 2) identify or confirm hybridization in areas of distributional overlap; and 3) quantify genetic diversity and its distribution among populations to identify areas of value for conservation.

MATERIAL AND METHODS

SPECIES DESCRIPTION

The yellow flowered *Ortgiesia* species occur in south and southeastern Brazilian Atlantic rainforest with one species also found in Argentina (Smith & Downs, 1979; Reitz, 1983;

Fig. 1). *Aechmea caudata* is the most widespread species within this group, occurring as epiphyte, terrestrial or saxicolous in mountain and coastal areas. *Aechmea blumenavii* is restricted to the northern area of Santa Catarina state, occurring above 500 m mostly as epiphyte in the forest, while *A. kertesziae* is found in the coastal region of the same state, growing on rocks or as terrestrial. *Aechmea comata* and *A. kleinii* are narrow restricted species, with the former occurring in an island at Santa Catarina state, and the later above 1000 m high in the continent. *Aechmea winkleri* presents a disjoint geographical range, with only two regions known for its occurrence, distant around 480 km from each other. *Aechmea calyculata* is the only species that reach the west region of south Brazil, entering Argentina. Recently it was also found occurring in the central-north region of Santa Catarina state. This species is found mostly as an epiphyte in the forest (Smith & Downs, 1979; Reitz, 1983, Fig. 1).

SAMPLE COLLECTION, DNA EXTRACTION, AMPLIFICATION AND SEQUENCING

A total of 204 individuals were sampled at 26 populations (Table 1; Fig. 1) spanning most of the range of all seven yellow flowered species of subgenus *Ortgiesia*. Young leaves were collected for genetic analysis and dried in silica gel. The total genomic DNA was extracted following protocol described by Doyle & Doyle (1990).

Nine chloroplast (cp) DNA regions (*psbA-trnH*, *trnTa-trnLb*, *trnLFc-trnLFf*, *rpL16-rpL16*, *trnD-trnT*, *petG-trnP*, *trnL* intron, *rpl32-trnL*, and *rpS16-trnK*), were initially screened using representative samples from each species and population. Only two regions were polymorphic, *rpl32-trnL* and *rps16-trnK*, which were amplified and sequenced for all individuals, using primers described by Shaw *et al*. (2007). The nuclear (nr) gene *phyC* was amplified for 179 individuals using primers described by Louzada *et al*. (2014). All PCR reactions were carried out in a total volume of 20 µl containing 10 ng DNA template, 1x Go*Taq* buffer, 2.5 mM $MgCl_2$, 0.25 mM dNTP mix, 5 ρmol forward and reverse primers or 2.5 ρmol (*phyC*) and 0.5 U of Go*Taq* DNA polymerase (Promega, Madison, WI, USA) and run using the following parameters: denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 54°C (*rpl32-trnL*) or 58°C (*rpS16-trnK*) or 64°C (*phyC*) for 1min, and 72°C for 1 min, and a final extension for 10 min at 72°C. PCR amplifications were performed in a Veriti 96-Well Thermal Cycler (Applied Biosystems). PCR products were sequenced from both ends using BigDye Kit (Applied Biosystems) by

Macrogen Inc. (Seoul, Korea). All sequences have been deposited in GenBank with accessions numbers XX for *rpl32-trnL*, XX for *rps16-trnK*, and XX for *phyC*.

DATA ANALYSIS

Sequences were analyzed and edited manually to obtain the consensus using the software MUSCLE (Edgar, 2004) implemented in MEGA version 5.1 (Tamura *et al*., 2011). Length variations in mononucleotide repeats were excluded due to ambiguous alignment. Haplotypes in sequences of *phyC* with heterozygous nucleotide positions were resolved by using a Bayesian algorithm implemented in PHASE version 2.1 (Stephens *et al*., 2001; Stephens & Donnelly, 2003). The analysis was run with the default values for 10 000 iterations. All the analyses were performed with two data sets: chloroplast data set - two concatenated cpDNA regions (*rpl32-trnL* and *rpS16-trnK*); and nrDNA (*phyC* sequences).

GENETIC DIVERSITY AND POPULATION STRUCTURE

Haplotype (h) and nucleotide (π) diversities (Nei, 1987) were estimated for each species using the software ARLEQUIN version 3.11 (Excoffier *et al*., 2005). Haplotypes were identified using DnaSP 5.10.01 (Librado & Rozas, 2009) and the evolutionary relationships among them were estimated with NETWORK 4.6.1.2 (available at http://www.fluxus-engineering.com) using the median-joining (MJ) method (Bandelt *et al*., 1999). For nrDNA the MJ network was post processed with a maximum parsimony algorithm (Polzin & Daeschmand, 2003) to remove unnecessary links and median vectors. An Analyses of Molecular Variance (AMOVA) was conducted to assess the genetic differentiation among species and populations using Kimura 2-parameters (Kimura, 1980) as genetic distance. This analysis was performed in ARLEQUIN under 10 000 permutations. *Aechmea kleinii* was not included because only one population was sampled.

BAPS version 5.3 (Corander *et al*., 2008) was employed to analyze the population genetic structure by clustering sampled individuals into groups. We carried out a genetic mixture analysis to determine the most probable number of groups (K) given the data (Corander & Tang, 2007; Corander *et al*., 2008). Under its default settings, BAPS includes K as a parameter to be estimated, and the best partition of the data into K clusters is identified as the one with the highest marginal log likelihood. The clustering with linked loci analysis was chosen to account for the linkage present between sites within aligned

sequences. Ten iterations of *K* (from 1 to 8) were conducted to determine the optimal number of genetically homogeneous groups. The admixture analysis was then applied to estimate individual admixture proportions with regards to the most likely number of *K* clusters identified (Corander & Marttinen, 2006; Corander *et al*., 2008). Admixture inference was based on 100 iterations using different allele frequencies.

RESULTS

GENETIC VARIATION

The *rpl32-trnL* and *rps16-trnK* intergenic spacers of cpDNA concatenated totalized 1696 base pairs (bp). A total of 42 polymorphic sites (19 transitions, 14 transversions, and 11 indels) were observed. Thirty-two haplotypes were found in the 204 individuals analyzed for the seven species (Supporting Information Table S1). For the nrDNA all the individuals had their genotypes resolved after PHASE analysis, totaling 179 individuals and 358 allele copies, with 918 bp in length. Thirty-five polymorphic sites (33 transitions and 2 transversions) were observed, recovering 54 haplotypes (Supporting Information Table S2). For both genomes, basic genetic diversity parameters are presented in Table 2. *Aechmea kertesziae*, *A. comata*, and *A. caudata* showed the higher values for both haplotype and nucleotide diversity for cpDNA. *Aechmea comata* besides occurring restricted to an island presented similar values of diversity as the other two widespread species. For nrDNA *A. caudata* and *A. kertesziae* showed the higher values for haplotype and nucleotide diversity (Table 2). Considering all the populations sampled, the highest number of cpDNA haplotypes were found in KL (five haplotypes) and AL and WC (with four haplotypes each), whereas AA and KI with 13 and 11 haplotypes, respectively, were the most diverse populations for nrDNA (Table 1).

HAPLOTYPES RELATIONSHIP

The relationship of the 32 cpDNA haplotypes can be visualized in Figure 2. The network was partially structured accordingly to the species to which the individuals belong. Exclusive haplotypes were found to *A. blumenavii* (H1 to H3), *A. calyculata* (H4 and H5), and to *A. kleinii* (H32). H1 and H4 were connected to the central haplotype by a median vector and a single mutational step, respectively, while H32 is a more divergent lineage.

Three haplotypes were shared among the some species: H8, H12, and H17. The central haplotype H8 was shared by individuals of *A. caudata*, *A. comata*, *A. kertesziae*, and *A. winkleri* from geographically distant populations (Table 1). H12 was shared by *A. caudata*, *A. kertesziae*, and *A. winkleri* while H17 was found in *A. comata* and individuals of *A. kertesziae* from KL population (Fig. 2; Table 1). Haplotype 20, identified as belonging to *A. comata*, was found connecting *A. caudata* haplotypes from inland and coastal populations, which could indicate an ancient hybridization event with chloroplast capture (Fig. 2; Table 1).

The network analysis concerning nrDNA showed less structure that those of cpDNA, with none species presenting exclusive lineages, haplotypes separated by one or a few mutational steps and an extensive sharing of haplotypes (11 in total; Fig. 3). The central haplotype Hn3 was shared by *A. blumenavii*, *A. calyculata*, *A. caudata*, and *A. kertesziae*. The second most frequent haplotype, Hn35, was shared by *A. comata* and *A. kertesziae*, the later species almost exclusively represented by individuals from KL population (Table 1), similar to the pattern observed for cpDNA (Fig. 2). Hn4 was shared by all species except *A. comata* and *A. winkleri* whereas many other peripheral haplotypes were found in more than one species (Fig. 3).

POPULATION DIFFERENTIATION

The Bayesian Analysis of Population Structure (BAPS) pointed to the most probable number of six genetic clusters for cpDNA (Fig. 4). *Aechmea blumenavii*, *A. calyculata*, and *A. kleinii* were strongly associated to only one genetic cluster (I, II, and VI, respectively), similar to the pattern recovered in the network. Further insights into population structure were obtained plotting the six genetic clusters on the map (Fig. 4B), which showed that most of the coastal *A. caudata* populations (AG, AP, AM, AA, and AF) were strongly associated to cluster III. Also similar to the pattern already recovered in the network analysis was the occurrence of cluster V only in *A. comata* and individuals of *A. kertesziae* from KL population. Haplotype 20 (*A. comata*), as indicated in the cpDNA network, belongs to cluster III, which characterizes *A. caudata* coastal populations (Fig. 4).

BAPS using nrDNA revealed $K = 5$ genetic groups (Fig. 5). Once again a great share of polymorphism was recovered. However, *A. comata* is strongly associated to cluster V while populations of *A. calyculata* from the central-west region of occurrence in Rio

Grande do Sul state (CD, CC, CB, and CP) were composed by genetic cluster II (Fig. 5). Another pattern found was the shared of cluster IV only between *A. caudata* and *A. winkleri* populations (Fig. 5).

AMOVA analysis revealed less pronounced population structure for nrDNA than for cpDNA (Table 3). For cpDNA 51.01% of the genetic variation could be attributed to diversity among populations within species and 35.94% was explained by differences among species. Concerning nrDNA, genetic diversity was partitioned due to variation within population (45.76%) and among population within species (30.11%), with the remaining 24.14% attributed to differences among species (Table 3).

DISCUSSION

The comparison of plastid and nuclear DNA provided new insights into the relationship and diversification of yellow flowered *Ortgiesia*, and permitted us to identify that together with hybridization, the shared of ancestral polymorphism (incomplete lineage sorting) also played a role during the speciation process within this group. Hybridization is a very common event when populations invade new environments or when allopatric taxa contact, especially in recent adaptive groups (Seehausen, 2004). Still, recent divergent lineages may share polymorphism for a certain time (incomplete lineage sorting) while some parts of the genome are already differentiated (Wu, 2001; Nosil *et al*., 2009). So, it is a challenge task to distinguish between hybridization and shared of ancestral polymorphism and in some cases, both mechanisms may be involved in the diversification (e.g. *Dyckia* – Krapp *et al*., 2014; *Petunia* - Segatto *et al*., 2014).

ANCIENT RETICULATION EVENT

At least one ancient event of hybridization could be identified within the yellow flowered *Ortgiesia* group, involving *A. comata* and *A. caudata*. Currently, these species overlap in geographical distribution in Santa Catarina island (Fig. 1) where the putative hybrid was collected, and they share pollinators (hummingbirds and bumble bees; Lenzi *et al*., 2006; Dorneles *et al*., 2011; Kamke *et al*., 2011). This individual was morphologically identified as *A. comata* but presented plastid haplotype (Fig. 2) and genetic cluster composition (BAPS analysis; Fig. 4) of *A. caudata*. As the chloroplast is probable inherited maternally as in most angiosperms, this implies pollination of *A. caudata* flowers by *A. comata* pollen,

followed by recurrent backcrosses of the resulting hybrid with *A. comata*. However, as only one individual with this pattern was sampled and we do not have nuclear data for it, more studies are needed to confirm and clarified this event.

HAPLOTYPE SHARING AND SPECIES LIMITS

The persistence of ancestral polymorphism is evident not only because most of the haplotypes shared in the networks based on both cp and nrDNA is among geographically distant populations (Fig. 2-3; Table 1), but also by the recent origin of subgenus *Ortgiesia*. It is hypothesized that the diversification process between extant lineages from this subgenus initiated as recent as the late Pliocene (2.5 Ma; Silvestro *et al*., 2014). *Ortgiesia* underwent a rapid diversification process as revealed by a previous study (Goetze, unpublished data), and bromeliads are a well known example of recent adaptive radiation, occurring in a great variety of environments, presenting morphologically and edaphically diversity (Benzing, 2000; Givnish *et al*., 2014). Thus the yellow flowered *Ortgiesia* species may have not had much time to accumulate genetic differences suggesting an ongoing process of diversification.

Despite the great variation observed in inflorescence branching pattern, the main character used to identify *Ortgiesia* species, they present other morphological and ecological conditions which are useful to distinguish them as sepal color, ovary shape, flower length, and habitat specificity (Smith & Downs, 1979; Reitz, 1983). The only exception is *A. comata* and *A. kertesziae* that does not show clear boundaries, overlap in geographical distribution, and individuals with intermediate morphology are found in the field (M. Goetze, personal observations). BAPS and network analyses revealed that *A. comata* preferentially share genetic variation with *A. kertesziae*, especially with individuals from KL population (Fig. 2-5). However, no signs of hybridization between *A. comata* and *A. kertesziae* were recovered in the previous study based on AFLP (Goetze, unpublished data). As these species are close related, it is very difficult to distinguish between hybridization and incomplete lineage sorting. Further investigation involving *A. comata* and *A. kertesziae* is needed to depict their relationship and evaluate if hybridization or incomplete lineage sorting, or both, is responsible for the sharing of haplotypes.

Aechmea kleinii was recovered as a divergent lineage in the analysis with cpDNA while two haplotypes were found with nrDNA, one exclusive and the second shared with

four other species. In the AFLP study it was found in an unresolved relationship with *A. kertesziae* (Goetze, unpublished data). *Aechmea kleinii* nowadays present habitat isolation when compared with the remaining yellow flowered species, only occurring in high altitudes. It is also the only species within *Ortgiesia* described as having nocturnal anthesis (Reitz, 1983). Ancient hybridizations events were not detected with cpDNA in the presented study and the shared of nuclear haplotypes seems to be due incomplete lineage sorting.

Aechmea blumenavii and *A. calyculata* were recovered as close related species by the AFLP study although in an unresolved relationship, which make the authors suggest the occurrence of hybridization among them (Goetze, unpublished data). In the present study these species were recovered as distinct lineages in the network analysis based on cpDNA (Fig. 2) and they shared only one nuclear haplotype (Hn4; Fig. 3). During field collection for this study, no sympatric population or individual with intermediate morphology was found. However, *A. calyculata* was recently collected (2011, 2012; *species*link, available at http://www.splink.org.br) in the region though to only occur *A. blumenavii*. The use of rapidly evolving chloroplast and nuclear microsatellite markers seems to be a good tool to be used allied to a broader sampling to clarify this pattern.

Aechmea winkleri was the most recent described species within this group (Reitz, 1975) and as *A. caudata* presents compound inflorescences. Their close relationship is clearly revealed by nuclear data, as *A. winkleri* preferentially shared haplotypes with *A. caudata* (Fig. 3) and the genetic cluster IV from BAPS analysis is only found in these two taxa (Fig. 5).

Although more markers would be needed to improve the resolution of the level of genetic differentiation between the yellow flowered *Ortgiesia* species, our data suggest ongoing speciation and limited present-day gene flow between taxa (or restricted to narrow geographical overlapping areas). This pattern is best explained by incomplete lineage sorting with some localized hybridization events. Geographical and habitat isolation seem to be important barriers to interspecific gene flow in face of sharing of pollinators and blossom periods.

INTRASPECIFIC GENETIC STRUCTURE

The outstanding pattern recovered in this study by all the analyses done of more pronounced genetic structure of cpDNA when compared to nrDNA is in line with other bromeliads species (*Alcantarea* – Barbará *et al*., 2008; *Vriesea* – Palma-Silva *et al*., 2009; Paggi *et al*., 2010; and *Pitcairnia* – Palma-Silva *et al*., 2011) and may be the result of the restricted dispersion of seeds which carry the maternally inherited chloroplast genome. Nuclear markers should be use in further studies addressing species cohesion as they show higher intraspecific gene flow (Petit & Excoffier, 2009).

Genetic substructure was detected for *A. caudata* with cpDNA. Populations from the coast were associated to cluster III in BAPS analysis while inland to clusters I and IV (Fig. 4B), which suggests independent demographic and/or evolutionary histories for these populations.

For *A. calyculata* populations' substructure was revealed by nrDNA, between the central-west and east region of the sampled distribution (Fig. 5B). This pattern may indicate recent origin of the western populations of *A. calyculata*, founded by individuals from the east region. Similar pattern was recovered in a phylogeographical study conducted with a mouse species from the Atlantic rainforest, which suggested a southwest expansion of the inland populations that are probable younger than populations having persisted in historically more stable areas in the coast region of the biome (Valdez & d'Elía, 2013).

DISTRIBUTION OF GENETIC DIVERSITY

The genetic diversity observed in the seven yellow flowered *Ortgiesia* seems not to be correlated to the geographical range occupied by the species. One example is the similar diversity found between *A. comata*, *A. caudata*, and *A. kertesziae* besides the former species is endemic to an island (Table 2; Fig. 1). However, the great genetic variation observed in *A. comata* could be an indicative of hybrids inside the populations. Indeed one putative hybrid was identified in the present study.

The levels of genetic variation inside each species varied a lot when comparing plastid and nuclear DNA (Table 2). For example, *A. calyculata* presented a nuclear haplotype diversity of 0.4120 while at cpDNA only 0.0465, one of the lowest indices within the group (Table 2). This incongruence between uni and biparently inherited

markers may be attributed to differences in the effective population size, with the former being more strongly affected by demographic changes and genetic drift (Ennos, 1994; Petit & Excoffier, 2009).

Our analyses provide some insights about the distribution of genetic diversity in the yellow flowered *Ortgiesia*, revealing that populations located in the north region of Santa Catarina state harbor the greatest amount of haplotypes (Table 1). Populations WC and AL for cpDNA and AA and KI for nuclear genome presented high diversity and should be considered as potential areas of conservation value. This area is inside the region considered the center of diversity for the entire subgenus *Ortgiesia* (Goetze, unpublished data) which thus also suggest this region as putative refugia during the Pleistocene climatic oscillations in south Brazil and merits further investigation.

As yellow petaled *Ortgiesia* species are restricted to one of the most threatened ecoregions of the world, the Atlantic rainforest (Myers *et al*., 2000), they can also be considered in danger. Populations of yellow flowered *Ortgiesia* are normally small, occurring in urban or agricultural areas. Still, many populations sampled by other researchers in the 50 and 60's decades were not found anymore, which suggest that a great amount of individuals were locally extinct, pattern that could be observed by the occurrence of some median vector in the networks. Currently only *A. kleinii* and *A. winkleri* have been included in the Brazilian Official List of Threatened Flora Species (MMA, 2008) and we suggest that all the yellow flowered *Ortgiesia* species should be part of that list, especially because of their limited geographical range.

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Table 1. Details of the seven yellow flowered *Ortgiesia* species used in the study, geographical coordinates, elevation, sample sizes, and haplotypes found.

Species	Population	Code	Latitude S	Latitude W	Elevation (m)	Sample size		Haplotypes	
						cpDNA	nrDNA	cpDNA	nrDNA
A. blumenavii	Blumenau/ SC	BP	27°03'	49°05'	285	9	8	H1-2	Hn1-5
	Blumenau/ SC	BS	27°00'	49°06'	308	9	7	H2-3	Hn1, Hn3-6
A. calyculata	Derrubadas/ RS	CD	27°08'	53°52'	141	8	8	H4	Hn3
	Concórdia/ SC	CC	27°17'	52°07'	396	8	8	H4-5	Hn3
	Barracão/ RS	CB	27°37'	51°31'	758	2	3	H4	Hn3
	Putinga/ RS	CP	29°04	52°13	207	8	8	H4	Hn3
	São Francisco de Paula/ RS	CS	29°27'	50°33'	580	8	6	H4	Hn3-4, Hn7-9
	Maquiné/ RS	CM	29°30'	50°14'	104	9	7	H4	Hn3, Hn10-13
A. caudata	Guarujá/ SP	AG	23°59'	46°11'	31	7	6	H6	Hn14
	Praia Grande/ SP	AP	24°01'	46°03	30	4	4	H6	Hn3, Hn6, Hn14
	Piraquara/ PR	AI	25°31	49°02	1021	5	4	H7-8	Hn4, Hn15-16
	Matinhos/ PR	AM	25°47'	48°31'	8	5	5	H6, H7, H9	Hn3-4, Hn17-19
	Araquari/ SC	AA	26°22'	48°43	8	11	9	H6, H10-11	Hn4, Hn15-18, Hn20-27
	Ilhota/ SC	AL	26°48'	48°55'	569	7	7	H12-15	Hn3-4, Hn17, Hn28-29
	Florianópolis/ SC	AF	27°45'	48°29'	84	7	8	H6	Hn16, Hn25, Hn30
	Serra Rio do Rastro/ SC	AS	28°23'	49°31'	804	8	8	H12, H15, H16	Hn16, Hn19, Hn27, Hn31-33
A. comata	Florianópolis/ SC	OF	27°31'	48°30'	95	8	7	H17-19	Hn34-35
	Florianópolis/ SC	OS	27°45'	48°30	12	9	7	H8, H20-21	Hn35-37

Table 1. Cont.

A. kertesziae	Itajaí/ SC	KI	26°55'	48°38'	19	15	15	H12, H22	Hn3-4, Hn26-27, Hn38-44
	Camboriu/ SC	KC	27°00'	48°34'	5	5	4	H8, H12	Hn3, Hn45
	Bombinhas/ SC	KB	27°08	48°29'	19	8	6	H8, H12, H23	Hn3-4, Hn8, Hn11, Hn46-48
	Florianópolis/ SC	KF	27°31'	48°30'	139	8	6	H8, H24-25	Hn3-4, Hn49
	Laguna/ SC	KL	28°30'	48°45'	11	16	11	H17, H26-29	Hn3, Hn35, Hn50
A. kleinii	Antônio Carlos/ SC	LA	27°27'	48°52'	637	3	2	H32	Hn4, Hn54
A. winkleri	Corupá/ SC	WC	26°23'	49°20'	425	8	7	H8, H12, H30-31	Hn17, Hn19, Hn26, Hn51, Hn52
	Santa Cruz do Sul/ RS	WS	29°41'	52°26'	125	9	8	H12	Hn15, Hn53
Total						204	179	32	54

Brazilian Federal States: SP – São Paulo; PR – Paraná; SC – Santa Catarina; RS – Rio Grande do Sul.

Table 2. Sample sizes, haplotypes observed and genetic diversity for seven yellow flowered *Ortgiesia* species based on cp and nrDNA.

Species	N	NH	Haplotypes	*h* (SD)	π (SD)
			cpDNA		
Aechmea blumenavii	18	3	H1, H2, H3	0.5686 (0.0964)	0.000000 (0.000000)
Aechmea calyculata	43	2	H4, H5	0.0465 (0.0439)	0.000028 (0.000077)
Aechmea caudata	54	11	H6 to H16	0.8190 (0.0431)	0.001508 (0.000913)
Aechmea comata	17	6	H8, H17 to H21	0.8529 (0.0473)	0.001891 (0.001150)
Aechmea kertesziae	52	11	H8, H12, H17, H22 to H29	0.8703 (0.0282)	0.002172 (0.001239)
Aechmea kleinii	3	1	H32	0.0000	0.000000
Aechmea winkleri	17	4	H8, H12, H30, H31	0.4191 (0.1413)	0.000427 (0.000375)
			nrDNA		
Aechmea blumenavii	15	6	Hn1 to Hn6	0.7885 (0.0524)	0.001718 (0.001171)
Aechmea calyculata	40	9	Hn3, Hn4, Hn7 to Hn13	0.4120 (0.0681)	0.000688 (0.000604)
Aechmea caudata	51	23	Hn3, Hn4, Hn6, Hn14 to Hn33	0.9196 (0.0127)	0.002820 (0.001687)
Aechmea comata	14	4	Hn34 to Hn37	0.3730 (0.1065)	0.000432 (0.000462)
Aechmea kertesziae	42	20	Hn3, Hn4, Hn8, Hn11, Hn26, Hn27, Hn35, Hn38 to Hn50	0.8445 (0.0257)	0.002356 (0.001463)
Aechmea kleinii	2	2	Hn4, Hn54	0.6667 (0.2041)	0.002179 (0.001828)
Aechmea winkleri	15	7	Hn15, Hn17, Hn19, Hn26, Hn51 to Hn53	0.7885 (0.0541)	0.002722 (0.001680)

N, sample size; NH, number of haplotypes; *h*, haplotype diversity; π, nucleotide diversity; SD, standard deviation.

Table 3. AMOVA results among the species for the different markers.

Source of variation	Percentage of variation	*F* statistics	*P*-value
	cpDNA		
Among species	35.94	$F_{CT} = 0.359$	< 0.01
Among populations within species	51.01	$F_{ST} = 0.870$	< 0.001
Within populations	13.05	$F_{SC} = 0.796$	< 0.001
	nrDNA		
Among species	24.14	$F_{CT} = 0.241$	< 0.001
Among populations within species	30.11	$F_{ST} = 0.542$	< 0.001
Within populations	45.76	$F_{SC} = 0.397$	< 0.001

Table S1. Variable sites of aligned sequences of 32 cpDNA haplotypes for seven yellow flowered *Ortgiesia* species. Dots (.) indicate the same character as for haplotype H1. (N) Haplotype frequency in number of individuals.

	Nucleotide variable positions																																									
	rpl32-trnL																										*rps16-trnK*															
Haplotype	0067	0090	0148	0217	0244	0260	0265	0278	0300	0331	0350	0356	0395	0423	0432	0452	0462	0463	0515	0526	0532	0554	0573	0687	0753	0822	0933	1077	1131	1168	1176	1208	1369	1379	1431	1461	1483	1484	1497	1533	1546	1618
H1 (11)	G	-	A	A	T	C	-	C	A	T	C	C	G	A	C	-	G	-	C	T	G	-	T	A	T	A	A	-	G	G	T	C	C	C	T	C	-	-	T	A	C	A
H2 (2)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	T	G	.	.	.	.
H3 (5)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	G	.	.	.	.
H4 (42)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	G	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H5 (1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	G	A	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H6 (21)	.	.	.	G	.	.	.	.	.	.	.	.	T	.	.	.	.	A	.	G	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.
H7 (2)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	C	.	.	.
H8 (18)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H9 (3)	.	.	.	G	.	.	.	.	.	.	.	.	-	.	.	.	.	A	.	G	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.
H10 (5)	.	.	.	G	.	.	.	.	.	.	.	.	T	.	.	.	T	A	.	G	.	.	.	G	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	T	.
H11 (4)	.	.	.	G	.	.	.	.	.	.	.	.	T	.	.	.	T	A	.	G	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	T	.
H12 (32)	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H13 (2)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.
H14 (2)	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	G	.	.
H15 (6)	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.
H16 (1)	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H17 (4)	.	.	C	.	.	.	.	.	.	.	.	.	.	C	.	G	.	A	.	G	.	T	.	.	.	.	G	.	.	.	A	.	.	.	C	.	.	.	.	.	.	.
H18 (3)	.	.	C	.	.	.	.	.	.	.	.	.	.	C	.	G	.	A	.	G	.	T	.	.	.	.	G	.	.	.	A	-	.	.	C	.	.	.	.	.	.	.
H19 (3)	.	.	C	.	.	.	.	.	.	.	.	.	.	C	.	G	.	A	.	G	.	T	.	.	.	.	G	.	.	.	A	.	A	.	C	.	.	.	.	.	.	.
H20 (1)	.	.	.	G	.	.	.	.	.	.	.	.	T	.	.	.	.	A	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.
H21 (3)	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	A	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Table S1. Cont.

H22 (3)	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	G	.	.	.	.	.	C	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.
H23 (3)	A	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	A	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H24 (3)	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	A	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H25 (4)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	G	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H26 (3)	.	.	C	.	.	T	.	.	.	.	.	.	.	C	.	G	.	A	.	G	.	T	.	C	.	.	G	.	.	.	A	.	.	.	C	.	.	.	.	.	.	-
H27 (5)	.	.	C	.	.	T	.	.	.	.	.	.	.	C	.	G	.	A	.	G	.	T	.	.	.	.	G	.	.	.	A	.	.	.	C	.	.	.	.	.	.	-
H28 (5)	.	.	C	.	.	.	.	.	.	.	.	.	.	C	.	G	.	A	.	G	.	T	.	C	.	.	G	.	.	.	A	.	.	.	C	.	.	.	.	.	.	.
H29 (1)	.	.	C	.	.	T	.	.	.	.	.	.	.	C	.	G	.	A	.	G	.	T	.	.	.	.	G	.	.	.	A	.	.	.	C	.	.	.	.	.	.	.
H30 (2)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.
H31 (1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	T	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H32 (3)	.	.	.	.	.	.	.	T	.	G	T	T	.	.	T	G	.	A	.	G	.	.	A	.	.	.	.	.	T	A	A	.	.	.	C	.	.	.	.	.	.	.

-, indel.

Table S2. Variable sites of aligned sequences of 54 nrDNA haplotypes for seven yellow flowered *Ortgiesia* species. Dots (.) indicate the same character as for haplotype Hn1. (N) Haplotype frequency in number of allele copies.

	Nucleotide variable positions																																		
	0	0	0	1	1	1	1	1	1	1	1	2	2	3	3	3	3	4	4	4	4	4	5	6	6	6	6	6	7	7	7	7	8	8	8
	2	4	8	0	1	4	5	6	7	7	8	4	6	4	6	8	8	4	4	6	6	7	6	4	5	6	6	7	2	2	8	8	0	3	5
Haplotype	2	3	8	9	3	4	3	5	1	4	0	4	3	4	5	6	8	0	6	1	4	3	9	1	6	5	8	1	3	4	0	8	6	9	3
Hn1 (12)	G	G	T	A	C	T	T	G	G	G	T	A	G	C	T	C	G	C	T	T	C	A	C	T	T	C	C	G	T	C	T	C	G	G	C
Hn2 (3)	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hn3 (99)	C	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hn4 (28)	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hn5 (5)	C	.	C	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hn6 (6)	C	.	C	.	.	.	C	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hn7 (3)	C	A	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hn8 (2)	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hn9 (2)	C	A	C	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hn10 (3)	C	.	C	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hn11 (9)	C	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.
Hn12 (1)	C	.	C	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.
Hn13 (1)	C	.	C	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.
Hn14 (15)	C	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.
Hn15 (9)	.	.	C	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T
Hn16 (19)	.	A	C	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hn17 (9)	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T
Hn18 (2)	.	A	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hn19 (9)	C	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T
Hn20 (1)	C	.	C	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	T
Hn21 (1)	C	.	C	.	.	.	C	A	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	T
Hn22 (2)	.	A	C	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	T
Hn23 (2)	C	.	C	.	.	.	C	A	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T
Hn24 (1)	.	A	C	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T
Hn25 (3)	.	A	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T
Hn26 (6)	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.

Table S2. Cont.

Hn27 (6)	.	.	C	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hn28 (2)	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hn29 (2)	.	.	C	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hn30 (2)	.	A	C	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T
Hn31 (1)	.	A	C	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.
Hn32 (1)	C	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	T
Hn33 (2)	C	.	C	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T
Hn34 (4)	C	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	G	.	.	.	.	.	.	.	.	.	T	.	.	.
Hn35 (38)	C	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.
Hn36 (1)	C	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	G	.	.	.	.	.	.	.	T	.	.	.	.	.
Hn37 (1)	C	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	T	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.
Hn38 (5)	C	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.
Hn39 (2)	.	.	C	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.
Hn40 (1)	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.
Hn41 (1)	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	T	.	.	.
Hn42 (1)	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	T	.	.	.
Hn43 (2)	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.
Hn44 (1)	C	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.
Hn45 (1)	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hn46 (2)	.	.	C	.	.	.	.	.	A	.	.	.	.	.	C	.	A	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.
Hn47 (1)	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C	.	A	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.
Hn48 (1)	C	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hn49 (2)	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.
Hn50 (3)	C	.	C	.	T	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	G	.	C	.	.	.	.	.	.	.	.	.	.	.
Hn51 (6)	C	.	C	.	.	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T
Hn52 (2)	C	.	C	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T
Hn53 (12)	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.
Hn54 (2)	C	.	C	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

FIGURE LEGENDS

Figure 1. Map showing the populations sampled of yellow flowered *Ortgiesia* species and their described geographical distribution accordingly to herbarium records: purple, *A. blumenavii*; yellow, *A. calyculata*; green, *A. caudata*; orange, *A. comata*; blue, *A. kertesziae*; gray, *A. kleinii*; and red, *A. winkleri*. Population codes correspond to those in Table 1.

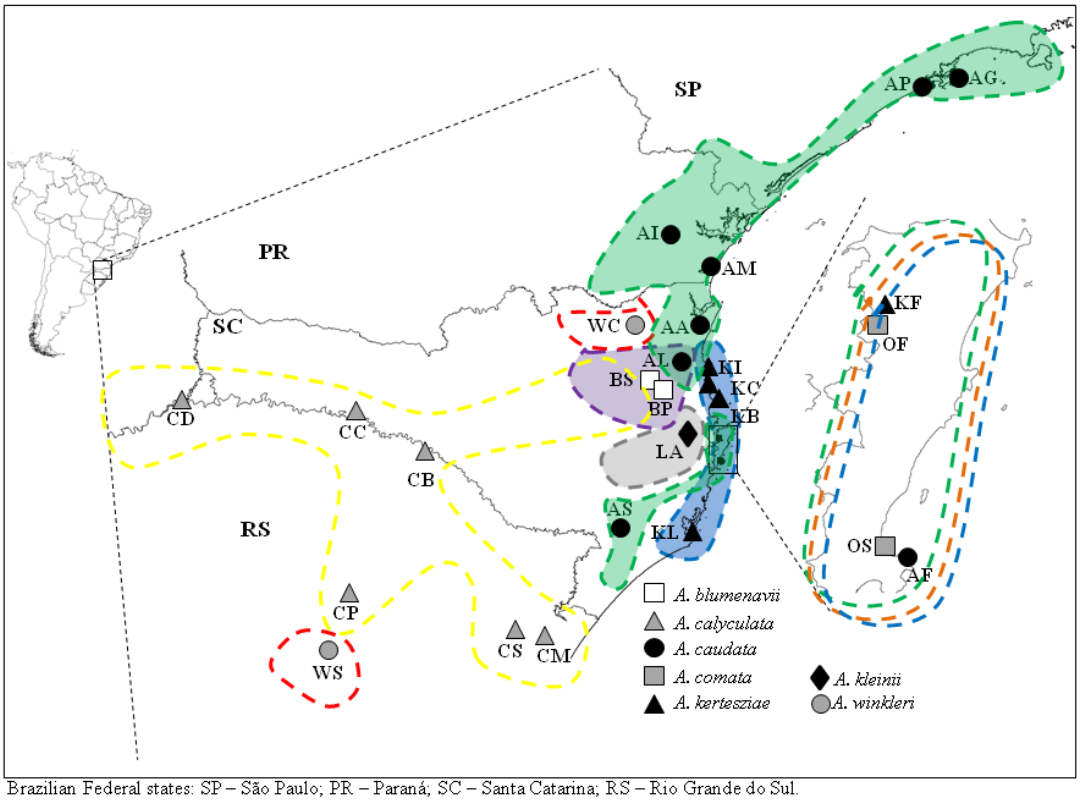
Figure 2. Median-joining network showing the genealogical relationship recovered for 32 cpDNA haplotypes; each circle represents one haplotype, its diameter is proportional to its total frequency; more than one mutational step required to explain transitions among haplotypes are indicated by numbers along the network. Each color represents one of the seven species as indicated by the key on the left.

Figure 3. Median-joining network showing the genealogical relationship recovered for 54 nrDNA haplotypes; each circle represents one haplotype, its diameter is proportional to its total frequency; more than one mutational step required to explain transitions among haplotypes are indicated by numbers along the network. Each color represents one of the seven species as indicated by the key on the left.

Figure 4. Population genetic structure based on cpDNA. a) Bayesian admixture proportions inferred with BAPS for individuals of seven yellow flowered *Ortgiesia* species for a $K = 6$ groups model. Species are separated by dashes lines and symbols correspond to the key on the right. b) Distribution of the clusters recovered in BAPS analysis. Population codes correspond to those in Table 1.

Figure 5. Population genetic structure based on nrDNA. a) Bayesian admixture proportions inferred with BAPS for individuals of seven yellow flowered *Ortgiesia* species for a $K = 5$ groups model. Species are separated by dashes lines and symbols correspond to the key on the right. b) Distribution of the clusters recovered in BAPS analysis. Population codes correspond to those in Table 1.

Fig. 1



Brazilian Federal states: SP – São Paulo; PR – Paraná; SC – Santa Catarina; RS – Rio Grande do Sul.

Fig.2

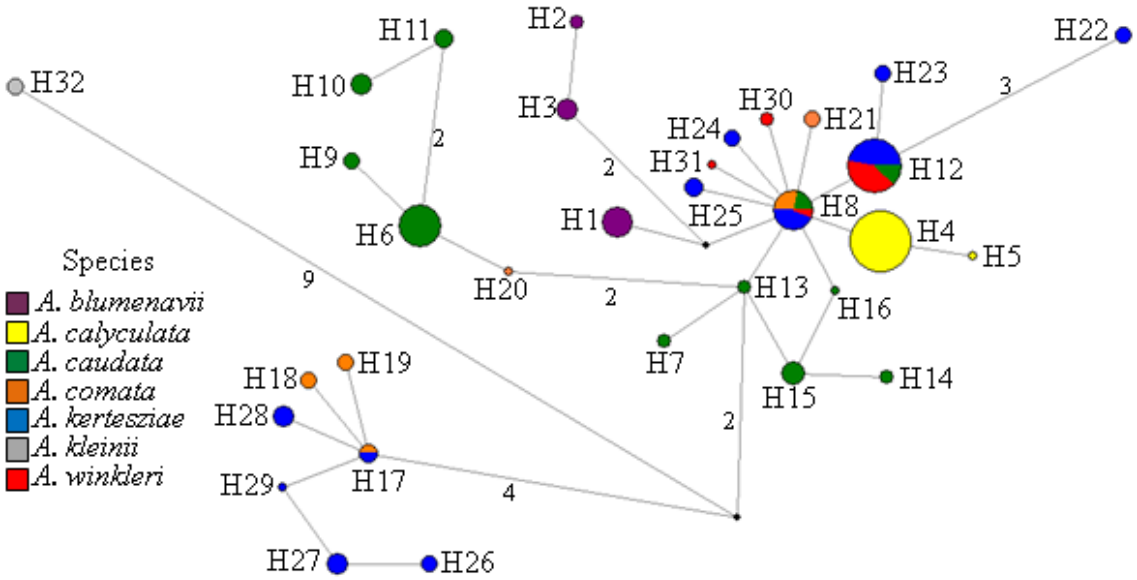


Fig. 3

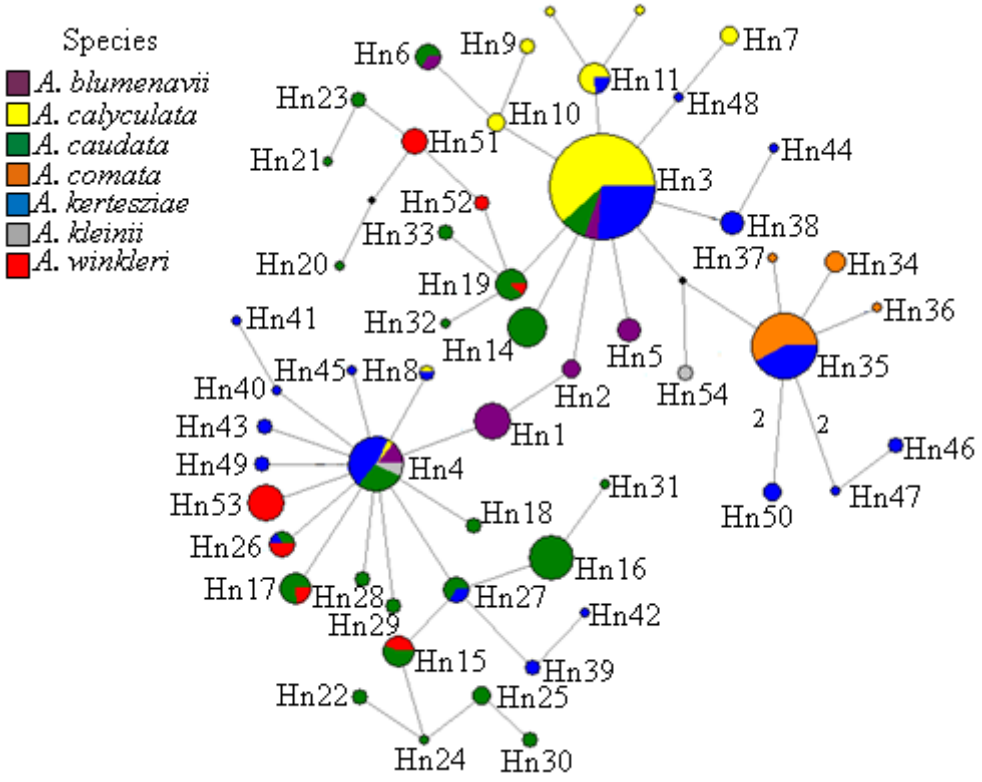
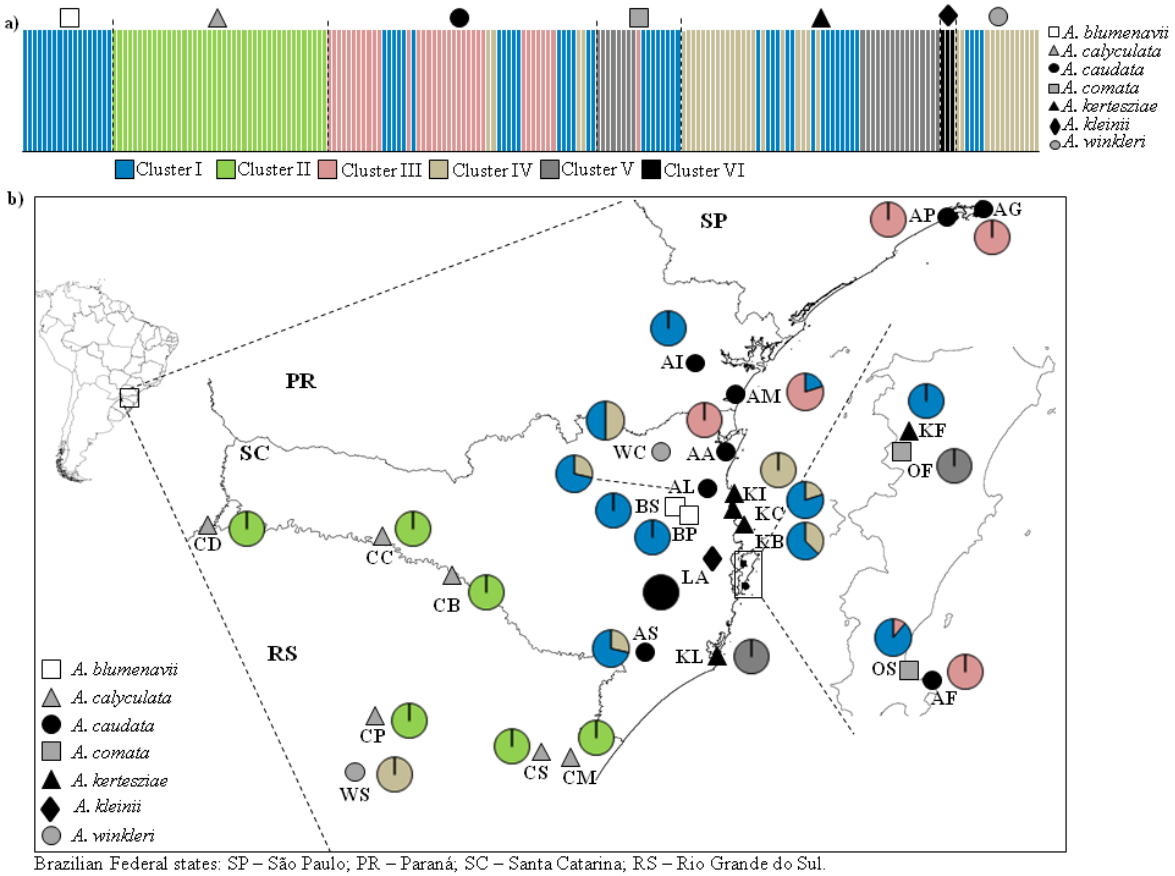
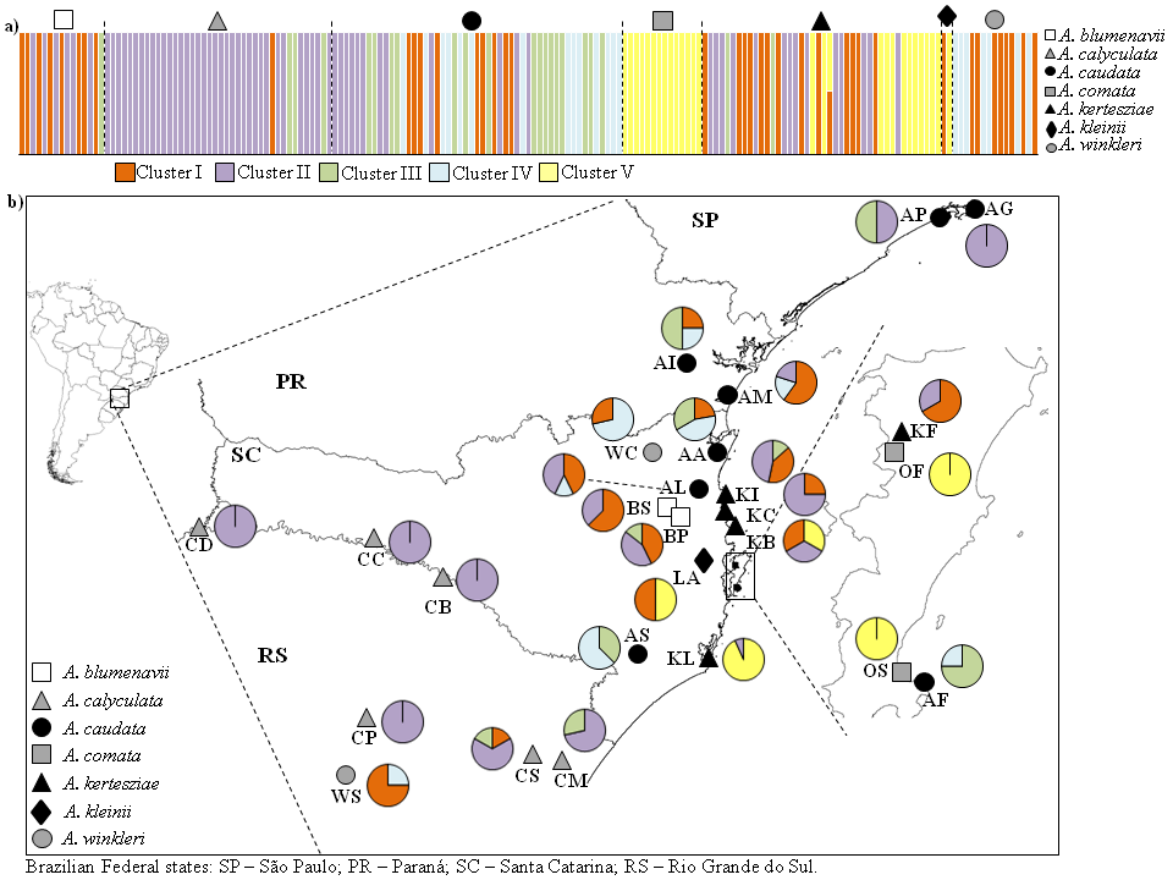

Species
A. blumenavii
A. calyculata
A. caudata
A. comata
A. kertesziae
A. kleinii
A. winkleri
Hn6
Hn9
Hn7
Hn11
Hn23
Hn10
Hn48
Hn21
Hn51
Hn44
Hn3
Hn52
Hn38
Hn33
Hn37
Hn34
Hn20
Hn19
Hn36
Hn41
Hn32
Hn14
Hn5
Hn35
Hn45
Hn8
Hn2
Hn54
Hn40
Hn43
Hn1
2
2
Hn46
Hn49
Hn4
Hn31
Hn53
Hn18
Hn50
Hn47
Hn26
Hn16
Hn17
Hn28
Hn27
Hn42
Hn29
Hn15
Hn39
Hn22
Hn25
Hn24
Hn30

Fig. 4



Brazilian Federal states: SP – São Paulo; PR – Paraná; SC – Santa Catarina; RS – Rio Grande do Sul.

Fig. 5



Brazilian Federal states: SP – São Paulo; PR – Paraná; SC – Santa Catarina; RS – Rio Grande do Sul.

Capítulo IV

Development of microsatellite markers for genetic diversity analysis of *Aechmea caudata* (Bromeliaceae) and cross-species amplification in other bromeliads

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Development of microsatellite markers for genetic diversity analysis of *Aechmea caudata* (Bromeliaceae) and cross-species amplification in other bromeliads

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1. Introduction

Aechmea comprises ~250 species being considered the largest genus in Bromelioideae subfamily (Luther, 2008). *Aechmea caudata* Lindm. is a lithophytic species endemic to the coastal region of southeastern Brazil (Smith and Downs, 1979) and few small natural populations are currently found, since it is threatened by habitat loss and fragmentation (Goetze, 2010). This species is diploid ($2n = 50$ chromosomes; Ceita et al., 2008) and belongs to the epiphyte clade of bromelioids, which is hypothesized to have originated nearly 5.5 million years ago and to have undergone a rapid diversification, mainly in the Brazilian Atlantic rainforest (Givnish et al., 2011). Thus, *A. caudata* may provide a valuable model system for evaluating mechanisms underlying species evolution in this biome.

Patterns of diversity and gene flow in *A. caudata* and other plant species from the Brazilian Atlantic rainforest are poorly known. This knowledge is crucial for understanding the distribution of genetic variation that may influence the evolutionary potential of the species and help define recommendations for *in situ* and *ex situ* conservation efforts.

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Microsatellite markers are distributed along the genome; they have codominant inheritance and have been widely used by population geneticists due to their high degree of polymorphisms even in small populations. Moreover, they have been used as valuable tools in studies of genetic diversity (Palma-Silva et al., 2009; Boisselier-Dubayle et al., 2010; Zanella et al., 2011), species delineation (Barbará et al., 2007a, 2009), phylogeography (Palma-Silva et al., 2009), and variation in mating systems (Paggi, 2009; Buttow, 2012), as well as the detection of interspecific hybridization and introgression (Palma-Silva et al., 2011) in bromeliads. Besides some microsatellite loci have already been published in Bromeliaceae (e.g. Palma-Silva et al., 2007; Paggi et al., 2008; Wörhmann and Weising, 2011; Wörhmann et al., 2012a; Zanella et al., 2012a), none of them focused on *Aechmea* genus. Here we report the development of 10 microsatellite markers for *A. caudata* aiming to evaluate the genetic diversity of this species and the usefulness of these markers in other related bromeliads.

2. Materials and methods

2.1. Sample collection and DNA extraction

Fresh leaves were collected from 37 individuals from the two biggest natural populations of *A. caudata* currently found in Brazilian Atlantic rainforest (Table 1), and stored in Petri dishes with silica gel. Total genomic DNA was extracted according to the protocol described by Doyle and Doyle (1990).

2.2. Construction of a microsatellite-enriched library and primer design

Markers isolation involved the construction of a genomic enriched library of *A. caudata* as described by Billote et al. (1999). Genomic DNA was digested with the *Rsa*I restriction enzyme (Invitrogen). The library was enriched for $(CT)_n$ and $(GT)_n$ repeats using biotinylated probes, and the target fragments were captured using streptavidin-coated magnetic beads as described by Kijas et al. (1994).These microsatellite-enriched DNA fragments were ligated into pGEM-T Easy Vector (Promega) and used to transform XL1-Blue *Escherichia coli* competent cells. The recombinant clones obtained were sequenced using the BigDye terminator Cycle Sequencing Kit (v.3.1) on an ABI 3730 DNA Analyzer Sequencer (Applied Biosystems).

For clones containing SSR motifs, forward and reverse sequences were aligned using SeqMan software (DNASTAR) and primers were designed with Primer3 software (http://frodo.wi.mit.edu/primer3/). Forward primers were synthesized with a 19-bp M13 tail (5′-CACGACGTTGTAAAACGAC-3′) at the 5′ end to allow labeling with a tailed fluorescent dye M13 primer during genotyping procedures, following the method of Schuelke (2000).

2.3. Amplification conditions and genotyping

Polymerase chain reaction (PCR) amplifications were performed in a 10 μl reaction volume containing 10 ng DNA template, 1 × PCR Buffer, 2 mM $MgCl_2$, 100 μM dNTPs mix, 1 pmol forward primer, 4 pmol reverse primer, 0.4 pmol universal M13 primer labeled with different fluorochromes (FAM, VIC, PET, or NED), and 0.5 U Go*Taq* polymerase (Promega). PCRs were performed in a Veriti 96-Well Thermal Cycler (Applied Biosystems) using a touchdown cycling program as described by Palma-Silva et al. (2007). Loci were genotyped on an ABI 3730 DNA Analyzer Sequencer and sized against a LIZ molecular size standard using GeneMarker software (SoftGenetics).

2.4. Data analysis

To estimate microsatellite variation, the number of alleles (A), and both observed (H_O) and expected heterozygosities (H_E), and the polymorphism information content (PIC), were estimated for each loci using CERVUS version 3.0.3 (Kalinowski et al., 2007). GENEPOP on the Web (Raymond and Rousset, 1995) was used to test departures from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) between all pairs of loci.

To measure genetic diversity of *A. caudata*, allelic richness (R_S), expected (H_O) and observed heterozygosities (H_E) were estimated for each population using FSTAT (Goudet, 1995) and MSA (Dieringer and Schlötterer, 2003), such as the within population inbreeding coefficient (F_{IS}) using GENEPOP on the web. Departures from HWE for each population were identified using exact tests in GENEPOP on the web. The relative amount of genetic variation within and among populations was determined by performing analyses of molecular variance (AMOVA) using Arlequin 3.11 (Excoffier et al., 2005).

Table 1
Location of the populations sampled and genetic diversity in *Aechmea caudata*.

Population	Latitude S	Longitude W	N	R_S	H_O	H_E	F_{IS}
Araquari	26°22′92″	48°43′51″	12	3.237	0.516	0.676	0.225*
Florianópolis	27°45′46″	48°29′24″	25	3.007	0.448	0.638	0.300*

Number of individuals sampled (N), allelic richness (R_S), observed heterozygosity (H_O), expected heterozygosity (H_E), and within inbreeding coefficient (F_{IS}).
*Significant departure from Hardy–Weinberg equilibrium ($P < 0.001$).

2.5. Cross-species amplification in other bromeliads

All polymorphic markers were tested for cross-amplification in 21 species belonging to three Bromeliaceae subfamilies: Bromelioideae, Tillandsioideae, and Pitcairnioideae. The PCR conditions were the same as described above. The amplification products were visualized on 2% agarose LE (USB Corp.) gels stained with GelRed (Biotium) under UV light. The loci were considered successfully amplified when at least one band of the expected size was visualized. A 50-bp DNA ladder (Ludwig Biotec) was used as molecular size marker.

3. Results

3.1. Identification of microsatellites and primers design

From the 96 colonies obtained and sequenced only 21 showed microsatellites repeat motifs. Seven sequences did not meet the primer designing criteria. A set of 14 loci were synthesized and checked for all of the samples, and 10 amplified with a polymorphic pattern (Table 2).

3.2. Microsatellite polymorphism in A. caudata

A total of 85 alleles were detected at the 10 microsatellite loci in the 37 samples of *A. caudata*. The number of alleles ranged from 4 to 14 with an average of 8.5 alleles per locus. The observed and expected heterozygosities ranged from 0.118 to 0.667 and from 0.271 to 0.893, respectively. The polymorphism informative content ranged from 0.259 to 0.867 with an average of 0.674 (Table 2). Except Ac78 (PIC = 0.259), all markers were highly informative (PIC > 0.5) and useful in genetic diversities studies. Three loci showed a significant departure from HWE in the overall sample (Table 2) and LD was detected only between pairs Ac11 and Ac25 ($P < 0.001$).

3.3. Genetic diversity analysis

The results of the analysis of genetic diversity for *A. caudata* populations are shown in Table 1. The highest values of allelic richness, observed and expected heterozygosities were observed in population Araquari. The two populations presented high positive within inbreeding coefficient (F_{IS}) values, and significantly deviated from HWE, indicating a deficit of heterozygotes (Table 1). The AMOVA showed that most genetic variation (81%) was attributed to variation within, rather than among, populations ($F_{ST} = 0.190$, $P < 0.001$).

3.4. Microsatellite cross-amplification

Cross-species amplification tests revealed that five out of the 10 loci developed amplified in more than 50% of the species (Table 3). Locus Ac25 amplified in 19 species, followed by locus Ac11 (17), Ac78 (16), Ac01 and Ac55 (12 species each). These

Table 2
Characteristics of 10 polymorphic microsatellite markers developed in *Aechmea caudata*. All forward primers were M13-tailed at the 5′ end.

Locus	Primer sequences (5′-3′)	Repeat motif	Size range (bp)	GenBank accession number	A	H_O	H_E	PIC
Ac01	F: CCTGACAACAAAAGGAGTGG R: TACGACGATTCCAAAAGAGG	$(AC)_{13}$	263–275	JX399856	4	0.667	0.712	0.648
Ac11	F: TACTGCCCTCCATTTCCAC R: CGCGAATGTGTATGATCTTG	$(CA)_{10}$	198–208	JX399857	6	0.639	0.714	0.668
Ac12	F: TTGCCCTTTTTCACTCACTC R: TAACCAGGAGAGGATCACCA	$(AC)_6NN(CA)_{13}$ $(CT)_7$	236–248	JX399858	14	0.412	0.866*	0.838
Ac25	F: ATACTGCCCTCCATTTCCAC R: GCTGATCTCAAACTACGAGCA	$(CA)_9$	170–180	JX399859	7	0.486	0.630	0.583
Ac35	F: GCAACGTTTCGATGTCAAC R: CAACAACAACCCCCACAC	$(TC)_8TT(TC)_6NN(CT)_5NN(TG)_{14}$	174–192	JX399860	8	0.667	0.614	0.572
Ac40	F: GCAGCACCAGAGACAGCA R: GTGGGAGAGTGTGGAGAGGT	$(AG)_{18}$	188–202	JX399861	13	0.657	0.872	0.845
Ac55	F: GTAGCTGAGGTTTCCAGATCC R: CTTGTATGGGCCTTTTTGG	$(CT)_{27}$	147–165	JX399862	12	0.656	0.893	0.867
Ac64	F: CCGTGGTTTTGTTTGTCTCT R: GGGGTCAGGAAAGGAGAATA	$(AG)_{29}$	214–228	JX399863	8	0.192	0.784*	0.738
Ac78	F: GACTTGTTCTGAAACGCAAAA R: TTGCCCTCTAAGAGAGACTGG	$(CA)_3CG(CA)_4A$ $(AC)_8$	173–195	JX399864	6	0.176	0.271	0.259
Ac88	F: CAGTTGCGCCCTAAGTGTA R: CAGCAGCCCAGATACAGATT	$(GA)_8AA(GA)_{17}$	241–259	JX399865	7	0.118	0.783*	0.725
Mean					8.5	0.467	0.714	0.674

Number of alleles per locus (A), observed heterozygosity (H_O), expected heterozygosity (H_E), and polymorphic information content (PIC).

Table 3
Cross-amplification of 10 microsatellite loci isolated from *Aechmea caudata* across Bromelioideae, Pitcairnioideae and Tillandsioideae subfamilies of Bromeliaceae.

Species	Ac01	Ac11	Ac12	Ac25	Ac35	Ac40	Ac55	Ac64	Ac78	Ac88
Bromelioideae										
Acanthostachys strobilacea (Schult. f.) Klotzsch.	+	+	–	+	–	–	–	–	+	–
Aechmea coelestis (K. Kock) E Morren	+	+	–	+	–	+	+	–	+	–
Aechmea comata Baker	–	+	–	+	+	–	+	–	+	–
Aechmea gamosepala Wittm.	+	+	+	–	–	–	+	–	+	–
Aechmea kertesziae Reitz	–	+	+	+	+	–	–	–	+	–
Aechmea recurvata (Klotzsch) L.B. Sm.	+	+	–	+	–	–	+	+	+	–
Aechmea winkleri Reitz	+	+	+	+	–	–	+	–	+	–
Bilbergia amoena (Lodd.) Lindley	+	–	–	+	–	+	–	–	+	–
Bromelia antiacantha Bertoloni	+	+	–	+	–	–	–	–	+	–
Edmundoa lindenii (Regel) Leme	–	+	–	+	–	–	+	–	+	+
Hohenbergia augusta (Vell.) E. Morren	+	+	–	+	–	+	+	–	+	–
Neoregelia guttata Leme	+	–	+	+	–	+	+	–	+	–
Nidularium procerum Lindm.	–	+	+	+	–	+	+	–	+	–
Quesnelia quesneliana (Brongn.) L.B. Sm.	+	+	–	+	–	+	+	–	+	–
Pitcairnioideae										
Dyckia distachya Hassl.	–	+	–	+	–	–	+	–	–	+
Dyckia leptostachya Baker	–	+	–	+	+	–	+	–	+	–
Tillandsioideae										
Alcantarea extensa (L.B. Sm.) J.R. Grant.	–	+	–	+	–	–	–	–	–	+
Vriesea carinata Wawra	+	–	–	–	+	–	–	–	–	–
Vriesea gigantea Gaudich.	–	+	–	+	–	+	–	–	–	–
Vriesea incurvata Gaudich.	+	–	–	+	–	+	–	–	+	–
Vriesea reitzii Leme & Costa, Andrea	–	+	–	+	+	–	–	–	–	+
Total = 21	12	17	5	19	5	8	12	1	16	4

'+' indicates successful amplification; '–' indicates unsuccessful amplification.

loci amplified even in some species belonging to other subfamilies than Bromelioideae, suggesting their potential usefulness in studies of population genetics involving other bromeliad species. A typical electropherogram from cross-amplification test is showed in Supplementary data.

4. Discussion

The 10 microsatellite loci isolated from *A. caudata* were highly polymorphic. Although three loci deviated from HWE the polymorphic information content were high in nine of them. Since few individuals are currently observed in the remaining populations of the species, the deviation from HWE could be explained by small effective population size leading to inbreeding and or genetic drift. Moreover, heterozygote deficiency in a population can occur as a result of inbreeding, selection against heterozygotes, the Wahlund effect, or the presence of null alleles (Sun and Salomon, 2003). This set of microsatellite markers are the first described for *Aechmea* and will be useful not only in this genus but even in species from other Bromeliaceae subfamilies, increasing the number of loci available.

High rates of markers transferability were also observed in other recent studies with microsatellites (Palma-Silva et al., 2007; Paggi et al., 2008; Wörhmann and Weising, 2011; Wörhmann et al., 2012a, 2012b; Zanella et al., 2012a). As shown by these studies, the markers were more effectively transferred to species belonging to the same subfamily. Microsatellites transferability in bromeliads may be considered an exception among plants, where transfer rates are approximately 10% between genera, and this might be due to low sequence divergence, which is common in recent adaptive radiations such as Bromeliaceae (Barbará et al., 2007b). Despite it become faster and easier to develop new microsatellite markers using 454 pyrosequencing technology, the transferability of SSR is a useful tool mainly in comparisons between closely related taxa for addressing mechanisms involved in population divergence and speciation (Noor and Feder, 2006), species cohesion and reproductive isolation (Barbará et al., 2007a; Palma-Silva et al., 2011).

Besides *A. caudata* populations are small the levels of genetic diversity were similar to values observed in other bromeliads (Zanella et al., 2012b). A moderate level of population differentiation were detected ($F_{ST} = 0.190$, $P < 0.001$) as measured by AMOVA analysis. This is consistent with the expectations, since seed dispersion may be though birds as for other *Aechmea* species (Fischer and Araujo, 1995; Lenzi et al., 2006). The microsatellite markers developed were useful to the study of genetic diversity in *A. caudata*. Given that the species presents small populations sizes, and that the populations sampled in the present study are the biggest observed, we recommended that *A. caudata* should be included in the Official List of Endangered Brazilian Flora Species. Both populations, Araquari and Florianópolis, presented high genetic diversity, and so, these areas are of great importance for the species survival in long-term. The moderate genetic structure observed can became a great threat to *A. caudata*, increasing inbreeding and genetic drift, leading to a decrease in genetic variation, affecting the species evolutionary potential. So, effective conservation strategies are needed to avoid *A. caudata* extinction.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bse.2012.12.022.

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Capítulo V

Genetic structure in *Aechmea calyculata* (Bromeliaceae) explained by different forests formations in southern Atlantic Forest

Manuscrito a ser submetido a American Journal of Botany

Goetze et al Genetic structure in *A. calyculata*

GENETIC STRUCTURE IN *AECHMEA CALYCULATA* (BROMELIACEAE) EXPLAINED BY DIFFERENT FORESTS FORMATIONS IN SOUTHERN ATLANTIC FOREST [1]

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Premise of the study: Studies addreessing genetic structure and diversity in plants of Atlantic rainforest may provide insights into the processes underlying diversification in this region. Here we use a combination of molecular data to assess genetic variation and structure within and among populations of *Aechmea calyculata*, to detect intraspecific differentiation and the pattern of genetic diversity distribution.
Methods:Twelve nuclear microsattelites markers, two cpDNA regions, and one nuclear gene were used to genotype and sequence 144 individuals from six populations of *A. calyculata*. For all sets of markers, we estimated genetic diversity and population differentiation. Bayesian structure analysis of nuclear markers and plastid haplotype network were used to infer population structure.
Key results: High genetic diversity was found within and between populations of *A. calyculata* with nuclear markers. Strong intraspecific genetic structure was detected, with limited gene flow among *A. calyculata* populations from the east and western south Atlantic rainforest.
Conclusions: *Araucaria* forest may be acting as a barrier to gene flow among *A. calyculata* populations from the east (located at ombrophilous forest) and west (semi-desciduous).

Key words: *Aechmea*; Atlantic Forest; Bromeliad; cpDNA; Genetic Diversity; Microsattelites; Phylogeography.

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The Atlantic Forest (AF) is one of the most diverse biomes on Earth, harbouring one of the higher degrees of species richness and endemism (Ribeiro et al., 2009). It is considered a biodiversity hotspot and priority area for conservation mainly due the high rates of deforestation, which threats its flora and fauna, and endemism (Myers et al., 2000). This biome presents a large geographical extend, *ca* 98 800 km^2, occuring from northeastern to southern Brazil in the coast and also in inland regions reaching Argentina and Paraguay limits in the west (Morellato and Haddad, 2000; Oliveira-Filho and Fontes, 2000). It is floristically diverse, presenting many vegetation types (Rizzini, 1979). In its subtropical portion, Atlantic mixed forest (*Araucaria* forest) separates the ombrophilous forest, which occurs in the coast, from the semi-deciduous forest that occurs in the inland-west regions (Oliveira-Filho and Fontes, 2000).

Several phylogeographical studies have been conducted trying to understand the mechanisms responsible for the huge biodiversity encountered in AF but the processes that shaped the currently patterns found are complex (see review in Martins, 2011). The Pleistocene refuges theory, neotectonic events, riverine barriers, and the latitudinal gradient are the main hypotheses that have been proposed to explain the contemporary patterns of species distribution and diversity, and population differentiation in AF (Dantas

et al., 2011). However, according to the results obtained in the studies published to date, the biogeographical history of this biome seems to be complex, with not only one single factor triggering the diversification but probably a combination of them (Turchetto-Zolet et al., 2013).

In a general way, phylogeographycal studies of animal and plant species that occur in AF point out to the formation of two major groups. A northern group, composed by populations occurring in northeastern and southeastern regions that presents high genetic diversity and a southern group. Large areas of climatic stability and refugia have been predictited as occuring in the northeastern region of AF during the Last Glacial Maximum with forest expansion towards the south in Holocene (Carnaval and Moritz, 2008; Carnaval et al., 2009; Palma-Silva et al., 2009; d'Horta et al., 2011; Porto et al., 2013). Nevertheless, other studies in the southern portion of AF found forested areas with divergent lineages and high genetic diversity, refuting the idea of recent colonization from northern areas (Grazziotin et al., 2006; Cabanne et al., 2007; Fitzpatrick et al., 2009; Thomé et al., 2010; Pinheiro et al. 2011; Amaro et al., 2012; Valdez and D'Elía, 2013). Indeed a study conducted with a bird species, *Basileuterus leucoblepharus*, reported demographyc stability of the populations along its entire geographical distribution, even in the southern region of AF (Batalha-Filho et al., 2012).

The genus *Aechmea* Ruiz & Pav. is one of the most diverse genera within the subfamily Bromelioideae (Bromeliaceae), presenting 240 species with 70% of them occurring in Brazil (Smith and Downs, 1979; Luther, 2008). *Aechmea* species occur from Mexico to Uruguay and AF is the diversity center of the genus (Smith, 1934; Benzing, 2000; Givnish et al., 2011). *Aechmea calyculata* (Morren) Baker occurs in the southern region of AF from the coast (ombrophilous forest) region until medium altitudes (above 500 m) reaching northeast Argentina (semi-deciduous forest) (Smith and Downs, 1979; Reitz, 1983). This species grows as an epiphyte in the rainforest (Reitz, 1983), is diploid with 2n = 50 chromosomes (Palma-Silva et al., 2004), presents berry fruits (Fagundes and Mariath, 2010) and seeds are probable dispersed by birds as occur for other *Aechmea* species (Fischer and Araujo, 1995; Lenzi et al., 2006). *Aechmea calyculata* belongs to subgenus *Ortgiesia* which is part of the core bromelioid clade (Schulte et al., 2009). The core bromelioid clade diverged from the basal lineages of subfamily Bromelioideae in the late Miocene, 7.08 Ma, being the diversification within *Ortgiesia* estimated to have started

around 2.5 Ma (Silvestro et al., 2014). By studying populations of *A. calyculata* that occurs in two different forest formations, we aimed to test if there is any pattern corresponding to ombrophilous and deciduous forests and understand how a species restricted to the southern portion of AF experienced the Pleistocene climatic changes. We used nuclear and chloroplast markers to address the following questions: 1) how is the genetic diversity distributed along the geographical range of *A. calyculata*? Is it possible to identify regions that can be considered stable during the Pleistocene climatic oscillations? 2) Is there an east-west genetic structure among *A. calyculata* populations? If yes, can this pattern be associated to different forests formations in south AF?

MATERIALS AND METHODS

***Sampling design and DNA extraction*—**Six populations of *A. calyculata* were sampled in most of its geographical distribution, totalizing 144 individuals (Fig. 1 and Table 1). The distance between populations ranged from 30 to 437 km. Up to 33 individuals per population were randomly sampled in each site (Table 1), at least 10 m apart to avoid sampling clones. Young leaves were collected in a way to not damage the plants, cut into small pieces and stored in silica gel for drying. Total genomic DNA was extracted following the protocol described by Doyle and Doyle (1990).

***Microsatellite markers and genotyping assays*—**Twelve nuclear microsatellite markers (SSR) were used in this study (Appendix S1). For each SSR, the forward primers were synthesized with a 19-bp M13 tail (5′-CACGACGTTGTAAAACGAC-3′) at the 5′ end to allow labeling with a tailed fluorescent dye M13 primer during genotyping procedures, following the method of Schuelke (2000). All polymerase chain reaction (PCR) amplifications were performed in a Veriti 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA) following the protocol described by Goetze et al. (2013). The microsatellite alleles were resolved on a ABI 3100 DNA Analyzer Sequencer (Applied Biosystems) and sized against the GS500 LIZ molecular size standard (Applied Biosystems) using GeneMarker Demo version 1.97 (SoftGenetics, State College, PA, USA).

***Sequence data: amplification and sequencing*—**Nine cpDNA regions (*psbA-trnH*, *trnTa-trnLb*, *trnLFc-trnLFf*, *rpL16-rpL16*, *trnD-trnT*, *petG-trnP*, *trnL* intron, *rpl32-trnL* and *rpS16-trnK*) were initially screened using representative samples from each population. Only two regions were polymorphic, *rpl32-trnL* and *rps16-trnK*, which were amplified and sequenced using primers described by Shaw et al. (2007). In addition one nuclear gene, phytocrome C (*phyC*), was amplified using primers described by Louzada et al. (2014). All PCR reactions were carried out in a total volume of 20 µl containing 10 ng DNA template, 1x Go*Taq* buffer, 2.5 mM $MgCl_2$, 0.25 mM dNTP mix, 5 ρmol forward and reverse primers or 2.5 ρmol (*phyC*) and 0.5 U of Go*Taq* DNA polymerase (Promega, Madison, WI, USA) and run using the following parameters: denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 54°C (*rpl32-trnL*) or 58° C (*rpS16-trnK*) or 64° C (*phyC*) for 1min, and 72°C for 1 min, and a final extension for 10 min at 72°C. PCR amplifications were performed in a Veriti 96-Well Thermal Cycler (Applied Biosystems). PCR products were sequenced from both ends using BigDye Kit (Applied Biosystems) by Macrogen Inc. (Seoul, Korea). All sequences have been deposited in GenBank with accessions numbers XX for *rpl32-trnL*, XX for *rps16-trnK*, and XX for *phyC*.

***Microsatellite data analysis*—**The genetic diversity of *A. calyculata* populations were characterized by the number of alleles (A), number of private alleles (AP), allelic richness (R_S), observed (H_O) and expected (H_E) heterozygosities and inbreeding coefficient (F_{IS}; Weir and Cockerham, 1984), calculated using the programs FSTAT 2.9.3.2 (Goudet, 1995) and MSA 4.00 (Dieringer and Schlötterer, 2003). Departures from the Hardy–Weinberg equilibrium (HWE) for each population were identified using exact tests in GENEPOP 4.0 (Raymond and Rousset, 1995). Population BA was not included in SSR analysis due to its small sample size (three individuals).

The genetic differentiation of the populations was assessed by the estimates of F_{ST} (Weir and Cockerham, 1984), and the standardized genetic differentiation measure G'_{ST} (Hedrick, 2005) calculated in the software FSTAT. Pairwise comparisons of F_{ST} between populations were estimated using the program ARLEQUIN 3.11 (Excoffier et al., 2005). Partitioning of genetic diversity within and among populations was examined by analysis of molecular variance (AMOVA; Excoffier et al., 1992) implemented in the software ARLEQUIN. The hypothesis that populations are differentiated because of isolation-by-

distance (Wright, 1965) was tested by calculating the correlation between geographic and genetic distance matrices (F_{ST}) with a standardized Mantel test using GENEPOP. The significance was assessed through a randomization test using 10 000 Monte Carlo simulations.

Population genetic structure was investigated using a Bayesian clustering method implemented in STRUCTURE 2.3.4 (Pritchard et al., 2000). A set of models was chosen in which individuals had admixed ancestries and correlated allele frequencies with the information of sampling localities considered in the assignment tests. The number of *K* was set from a minimum of one to a maximum of seven, and the simulations were run for each *K*-value with a burn-in of 100 000 and run length of 500 000. To define the most probable number of genetic clusters (*K*) present in the data, we used the method proposed by Evanno et al. (2005), which is based on an *ad hoc* measure ΔK that evaluates the second-order rate of change of the likelihood function with respect to *K*.

Each population was tested for recent population sizes reductions (e.g., genetic bottlenecks) using the *M*-statistic method described by Garza and Williamson (2001), implemented in the software ARLEQUIN. Significance was assessed by comparison between the mean value *M* across all loci and the value $M = 0.680$, the threshold value below which a population can reasonably be assumed to have undergone a reduction in population size (Garza and Williamson, 2001).

***Sequence data analysis*—**Sequences were analyzed and edited manually to obtain the consensus using the software MUSCLE (Edgar, 2004) implemented in MEGA version 5.1 (Tamura et al., 2011). As most of the variation was observed in mononucleotide microsatellites they were maintained in the alignment in the plastidial regions. Haplotypes in sequences of *phyC* with heterozygous nucleotide positions were resolved by using PHASE version 2.1 (Stephens et al., 2001; Stephens and Donnelly, 2003). All the analyses were performed with two data sets: plastidial data set - two concatenated cpDNA regions (*rpl32-trnL* and *rpS16-trnK*); and nuclear data set - nrDNA (*phyC*) sequences. Haplotype (*h*) and nucleotide (π) diversities (Nei, 1987) were estimated for each population using the software ARLEQUIN. The relationships between haplotypes were estimated with the NETWORK 4.6.1.1 program (available at http://www.fluxus-engineering.com) using the Median-joining method (Bandelt et al., 1999).

An analysis of molecular variance (AMOVA) was performed to examine the partition of genetic diversity between and among populations in ARLEQUIN. The genetic differentiation of the populations was assessed by estimating G_{ST}, using the program PERMUT/ CpSSR 2.0 (Pons and Petit, 1996). The population genetic structure was estimated by a Bayesian clustering approach implemented in the software BAPS 5.3 (Corander et al., 2008). The admixture analysis based on mixture clustering of individuals (Corander and Marttinen, 2006) was conducted with the following settings: minimal size of clusters, three individuals; 100 iterations to estimate the admixture coefficients for the individuals; 200 simulated reference individuals from each population; and 100 iterations to estimate the admixture coefficients for the reference individuals. The optimal *K* cluster population partition was characterized by the highest marginal log-likelihood. We performed 10 algorithm repetitions for each *K* between 1 and 8.

RESULTS

***Genetic diversity*—**High levels of genetic variation were found in *A. calyculata* populations genotyped with the 12 nuclear microsatellite markers (Table 2). The number of alleles ranged from 42 to 80, and the allelic richness ranged from 3.25 to 4.23. The observed and expected heterozygosities per population ranged from 0.389 to 0.638 and from 0.504 to 0.623, respectively. Two from 14 private alleles were found in almost all populations. The inbreeding coefficients were low and ranged from – 0.039 to 0.181 and all populations departed significantly from the HWE. Across all sites and loci the *M*-statistic ranged from 0.601 to 0.699, identifying bottlenecks in TU, PU, and SF populations (Table 2).

For the two plastidial regions concatenated we obtained in total an alignment of 1700 bp long with five variable sites. For *phyC* region (nrDNA) the alignment was 916 bp in length with six variable sites. Table 2 shows the basic genetic diversity results of these two data sets. A total of five haplotypes were found for the cpDNA, varying from one to two per population (Table 2 and Fig. 1A). On the other hand, more variation was observed with nrDNA, which displayed nine haplotypes, varying from one to five per population. Four out of six populations showed one fixed nuclear haplotype (Hn1; Table 2 and Fig. 1C).

***Genetic differentiation revealed by nuclear microsatellite markers*—**High levels of genetic differentiation across populations were found considering F_{ST} (0.231), and G'_{ST} (0.236). The pairwise F_{ST} values also revealed high genetic structure, mainly between the populations from the east part of the distribution (MA and SF) and the west (TU, CO, and PU), ranging from 0.068 to 0.359 (Table 3). No correlation among genetic and geographic distances was detected in the Mantel test ($r^2 = 0.036$, $P = 0.622$), indicating the absence of isolation by distance between the collecting sites. Bayesian analysis performed in STRUCTURE identified $K = 2$ genetic groups as show in Appendix S2.The assignment proportion (Q) of each individual into each group is shown in Fig. 2. Populations from the west distribution (TU, CO, and PU) are strongly associated to cluster I and those from the east side of distribution (SF and MA) to cluster II, with few individuals that can be identified as migrants between the two genetic groups. AMOVA results indicate that the majority of the genetic variation resides within populations (76.76%, $P < 0.0001$), and 23.24% is found among populations.

***Genetic differentiation revealed by sequence data*—**The plastidial markers analysis found a well-resolved network, with the most frequent haplotype (H1) shared by individuals of populations from the west group (TU, CO, BA, and PU). Haplotypes 2 and 3 were exclusive of populations CO and PU, respectively, while H4 and H5 only occur in SF and MA, respectively (Figs. 1A and B). For the nrDNA, Hn1 was the most frequent haplotype shared by individuals from all the populations. Exclusive haplotypes were found in SF and MA populations (Figs. 1C and D). Genetic differentiation was high, with $G_{ST} = 0.866$ for plastidial and $G_{ST} = 0.486$ for nuclear regions. According to AMOVA results, a great proportion of the genetic variation resides among the populations (92.59%, $P < 0.001$) for the plastidial data. On the other hand, as already indicated by nuclear SSR markers, for the nuclear DNA region, the genetic variation resides within (63.95%, $P < 0.001$) rather than among populations. According to BAPS analysis the cpDNA and nrDNA are optimally partitioned into three and four genetically structured groups, respectively (Fig. 3). Based on the cpDNA data set, the Bayesian analysis identified one genetic group occurring in the populations from the west side of the geographical distribution of *A. calyculata* (TU, CO, BA, and PU) while distinct genetic clusters were found for SF and MA populations (Fig. 3A). For nrDNA four genetic groups were found

which are distributed along the populations from the east side, while populations from the west group (TU, CO, BA and PU), are composed by only one genetic cluster (Fig. 3B).

DISCUSSION

***Levels of genetic diversity and stable areas in South Atlantic Forest*—**Studies including phylogeography of plants from AF are relatively scarce in the literature, especially those contemplating the southern region of this biome. Most of the phylogeographical studies with plant species that reach the south AF reported a decrease in genetic diversity in this portion of the biome when compared to the southeastern and northern regions (Lorenz-Lemke et al., 2005; Palma-Silva et al., 2009; Turchetto-Zolet et al., 2012; Zanella, 2013). The results obtained here, using nuclear microsatellites markers, showed high levels of genetic diversity within populations of *A. calyculata* (Table 2). Similar results were reported to other *Aechmea* species that are both restricted to southern AF, or present a more widespread range (Goetze, 2010; Abondanza, 2012; Capra, 2012; Goetze et al., 2013). Different from the pattern reported for bromeliads of genus *Vriesea*, which showed reduced genetic diversity in southern populations of AF (Palma-Silva et al., 2009; Zanella, 2013), *Aechmea* seems to not follow this pattern. *Aechmea* subgenus *Ortgiesia* seems to have its center of species diversity in southern Brazil (Goetze, unpublished data), which may explain the difference when compared with bromeliads from genus *Vriesea*, which present one of its diversity centers in southeastern AF (Costa et al., 2009).

Three out of five populations sampled here showed signals of reduction in population size (TU, PU, and SF), which seems to not have affected the levels of genetic variation that were similar among all populations, showing that even after suffering a bottleneck, the populations probably were large enough to maintain the genetic diversity. The only exception was the number of private alleles, which was reduced in the populations that suffered bottleneck (Table 2). Individuals from MA, located in the eastern region of geographical distribution, showed a great number of private alleles (14) when compared with all the other populations, which may indicate long-term persistence of individuals in this region.

Concerning the sequence data results, low genetic variation was observed with cpDNA in all the populations (Table 2, Figs. 1, 3A). The lack of within population variation in plastidial genome indicates low levels of gene flow by seeds and/or founder effect. Also, as plastidial genome represents half the effective population size when compared with nuclear genome, historical demographical changes may have higher effect on genetic diversity loss in plastidial than in nuclear genome due to the effect of genetic drift (Ennos, 1994). In bromeliads the low sequence variation in cpDNA has been previously detected by other studies (e.g., Barfuss et al., 2005; Horres et al., 2007; Givnish et al., 2004, 2007; Schulte et al., 2009). The chloroplast sequence divergence in Bromeliaceae seems to be three times lower than the observed for other families (Maia et al., 2012), as a result of the short divergence time.

In the present study, populations from the east region of *A. calyculata* distribution (SF and MA) are the most diverse when nrDNA is considered, but no clear pattern comes out with cpDNA and nuclear microsatellites. Palynological studies describe deep valleys in southern AF as possible refugia during the Pleistocene (Behling et al., 2004; Behling and Pillar, 2007; Leonhardt and Lorscheitter, 2010). Some paleomodeling studies though hypothesized severe forest contraction in southern AF (Carnaval and Moritz, 2008; Carnaval et al., 2009; Porto et al., 2013) whereas Thomé et al. (2010) recovered a refugia in the central-north region of Rio Grande do Sul state, in the south. Valdez and D' Elía (2013), in a phylogeographical study of *Akodon montensis*, a mouse species widespread in AF, invoked a refugia at the southern coastal end of southern AF (Rio Grande do Sul state), but without a precise location. Thus, discussing the initial question of whether is possible to identify regions that can be considered stable during the Pleistocene climatic changes, our results did not allow to identify a refugia using data of *A. calyculata*, especially due to low genetic variation observed with cpDNA. The east region (represented by SF and MA populations) could be considered as an initial evidence of stable region, because of the great diversity found in nrDNA and the high number of private alleles recovered with microsatellites. However, to propose a more complete scenario, the inclusion of other cpDNA genes should be carried out in future studies.

East-west pattern and population's genetic structure—The presence of a strong population genetic structure was found between the populations from the west (TU, CO,

BA, and PU) and the east (SF and MA) as revealed by the STRUCTURE Bayesian analysis (Fig. 2), the F_{ST} pairwise comparisons (Table 3) and BAPS analysis using both cpDNA and nrDNA (Fig. 3). In a study conducted with AFLP molecular markers (Goetze, unpublished data), two groups of *A. calyculata* were also recovered, corresponding to populations in the east and western regions. The eastern and western regions of southern AF correspond to distinct forest formations: ombrophilous forest in the coast (east) and semi-deciduous forest in the western side. Nowadays these two forest formations are partially separated by *Araucaria* mixed forest, which could being acting as a barrier to gene flow among eastern and western population of *A. calyculata*.

Another factor that should be considered to explain the genetic structure revealed by this study is the occurrence of hybridization. Populations of *A. calyculata* from the western region of Rio Grande do Sul state are closer related to *A. blumenavii* than to the remaining *A. calyculata* populations from the east, as revealed by the AFLP study (Goetze, unpublished data). Although populations from the western region were morphologically identified as *A. calyculata*, they could be from hybrid origin, and thus some isolation mechanism between populations from the east and west may be acting as a barrier to gene flow. This hypothesis should be investigated by using chloroplast and nuclear markers, involving populations from the two species, especially from sympatric areas.

***Conclusions*—**Our results revealed high levels of genetic diversity using nuclear markers for *A. calyculata* populations in southern AF. More studies focusing on species restricted to the southern portion of AF need to be carried out to confirm the east region of south Brazil as stable during the Pleistocene climatic oscillations. The use of plastidial and nuclear markers showed the occurrence of two genetic groups highly structured suggesting limited gene flow among east and western populations of *A. calyculata*. The three different forest formations in southern AF seem to be the responsible for this pattern, with *Araucaria* forest acting as a putative barrier to gene flow between the east (ombrophilous) and west (semi-deciduous) populations of *A. calyculata*.

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TABLE 1. Populations of *Aechmea calyculata* sampled in southern Atlantic Forest with their ID, geographical coordinates, elevation, forest type, and sample size for nuclear and plastidial markers.

Population	ID	Latitude S	Latitude W	Elevation (m)	Type of forest	Sample size		
						SSR	cpDNA	nrDNA
Derrubadas - RS	TU	27°08'	53°52'	141	Semi-deciduous	33	8	8
Concórdia - SC	CO	27°17'	52°07'	396	Semi-deciduous	33	8	8
Barracão - RS	BA	27°37'	51°31'	358	Semi-deciduous	-	2	3
Putinga - RS	PU	29°04	52°13	207	Semi-deciduous	12	8	8
São Francisco de Paula - RS	SF	29°27'	50°33'	580	Ombrophilous	33	8	6
Maquiné - RS	MA	29°30'	50°14'	104	Ombrophilous	33	9	7
Total						144	43	40

Notes: RS = Rio Grande do Sul state; SC = Santa Catarina state.

TABLE 2. Characterization of genetic variability in six populations of *Aechmea calyculata* in southern Atlantic Forest.

	SSR							cpDNA			nrDNA		
Population	A	PA	R_S	H_O	H_E	F_{IS}[a]	M[b]	NH	π	h	NH	π	h
TU	66	5	3.88	0.638	0.623	-0.039	0.673[c]	1	0	0	1	0	0
CO	61	3	3.73	0.439	0.533	0.134	0.689	2	0.000147	0.2500	1	0	0
BA	-	-	-	-	-	-	-	1	0	0	1	0	0
PU	42	0	3.25	0.389	0.504	0.181	0.601[c]	2	0.000147	0.2500	1	0	0
SF	63	2	3.84	0.497	0.602	0.152	0.635[c]	1	0	0	5	0.001766	0.7879
MA	80	14	4.23	0.562	0.620	0.095	0.699	1	0	0	5	0.001173	0.7253

Notes: A, number of alleles; PA, number of private alleles; R_S, allelic richness; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient; NH, number of haplotypes; π, nucleotide diversity; h, haplotype diversity. For populations abbreviations see Table 1.
[a] All inbreeding coefficient (F_{IS}) departed significantly from Hardy-Weinberg equilibrium (HWE) at the $P < 0.001$ level. [b] Population is considered to have undergone a bottleneck if its M-value falls below a threshold of 0.680, following the procedure described by Garza and Williamson (2001). [c] Populations in which bottlenecks were detected according to Garza and Williamson (2001).

TABLE 3. Pairwise F_{ST} among populations of *Aechmea calyculata* based on 12 microsatellite loci.

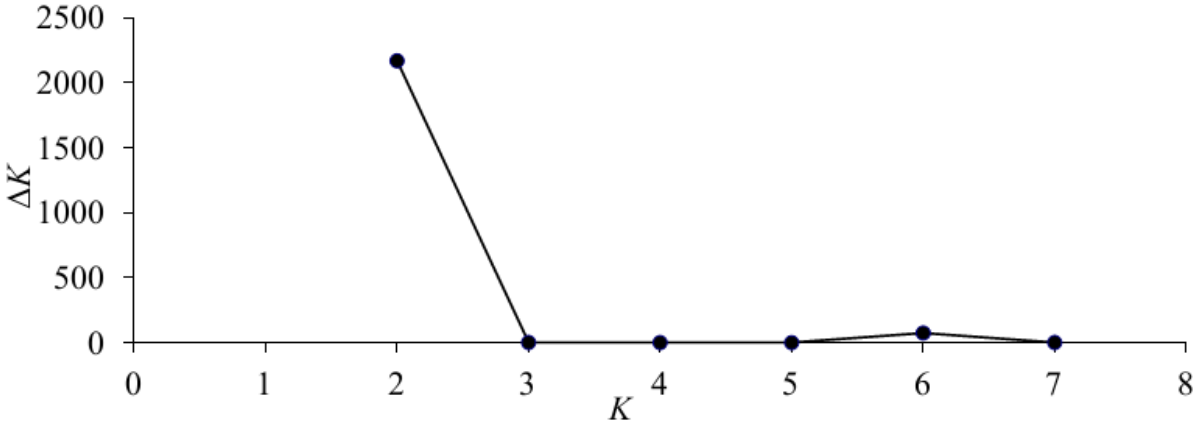
Population	TU	CO	PU	SF
CO	0.068			
PU	0.101	0.068		
SF	0.328	0.359	0.351	
MA	0.253	0.198	0.222	0.233

Notes: For populations abbreviations see Table 1. All values were significant at $P < 0.0001$.

Appendix S1. Nuclear microsatellite markers used in the study of genetic diversity and structure of populations of *Aechmea calyculata*.

Loci	Species	Study
Ac01	*Aechmea caudata*	Goetze et al., 2013
Ao6, Ao13	*A. coelestis* complex	Abondanza, 2012
Aw03	*A. winkleri*	Goetze et al., 2010
Acom_12.12, Acom_67.2, Acom_71.3, Acom_78.4, Acom_82.8	*Ananas comosus*	Wörhmann and Weising, 2011
PaZ01	*Pitcairnia albiflos*	Paggi et al., 2008
e6b	*Tillandsia fasciculata*	Boneh et al., 2003
VgC01	*Vriesea gigantea*	Palma-Silva et al., 2007

Appendix S2. Magnitude of Δ*K* from STRUCTURE analysis of *K* (mean ± SD over 10 replicates), calculated by following the Δ*K* method proposed by Evanno et al. (2005), for *Aechmea calyculata* microsatellite data. The modal values of these distributions indicate the true *K* or the uppermost level of structure is two "genetic clusters".



FIGURES LEGENDS

Fig. 1. Map showing the populations sampled of *Aechmea calyculata* in southern Atlantic Forest and the genealogical relationship of the haplotypes recovered. A) Pie charts reflect the frequency of occurrence of each cpDNA and (C) nrDNA haplotype in each population. Haplotype colors correspond to those shown in panel B and D, respectively. B) Median-joining network for cpDNA and (D) nrDNA. Circle sizes are proportional to the frequency of the haplotypes. More than one mutational step between haplotyes is shown by crossed lines.

Fig. 2. Bayesian admixture proportions (*Q*) of individual plants of *Aechmea calyculata* for a $K = 2$ population model. For population abbreviations see Table 1.

Fig. 3. Distribution of clusters observed with BAPS analysis in six populations of *Aechmea calyculata* for (A) cpDNA and (B) nrDNA. For populations abbreviations see Table 1.

Fig. 1

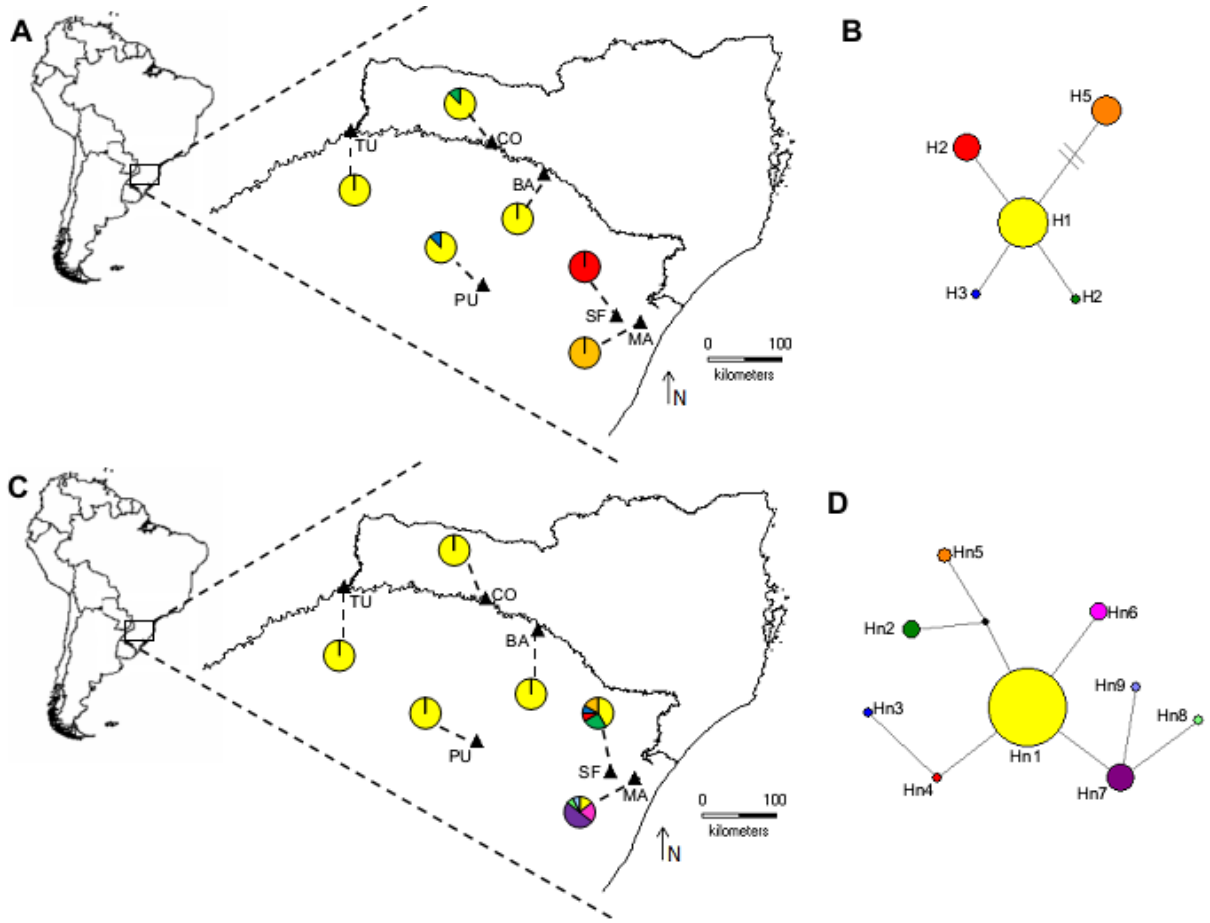

A
TU
CO
BA
PU
SF
MA
0
100
kilometers
N
B
H5
H2
H1
H3
H2
C
TU
CO
BA
PU
SF
MA
0
100
kilometers
N
D
Hn5
Hn6
Hn2
Hn9
Hn3
Hn8
Hn1
Hn4
Hn7

Fig. 2

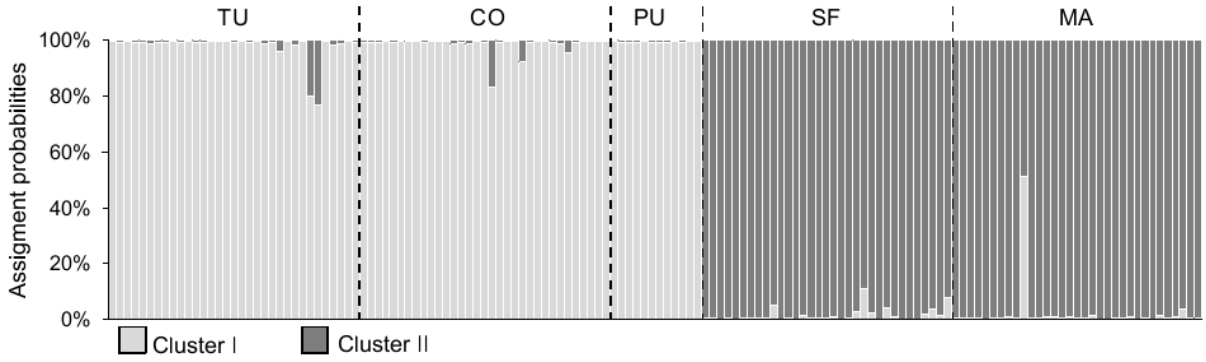
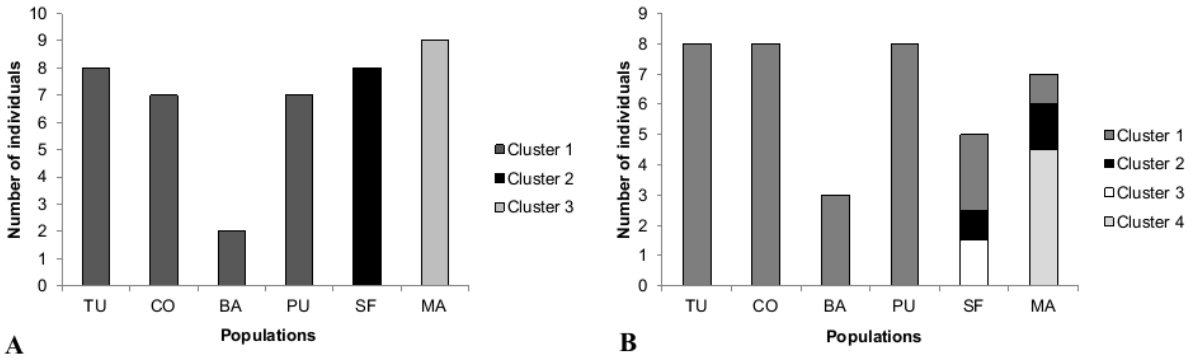

TU
CO
PU
SF
MA
100%
80%
60%
40%
20%
0%
Assigment probabilities
Cluster I
Cluster II

Fig. 3


Number of individuals
Populations
TU
CO
BA
PU
SF
MA
Cluster 1
Cluster 2
Cluster 3
A
Number of individuals
Populations
TU
CO
BA
PU
SF
MA
Cluster 1
Cluster 2
Cluster 3
Cluster 4
B

Capítulo VI
Considerações finais

Considerações finais

Os estudos com espécies do subgênero *Ortgiesia* no Núcleo de Genética e Conservação de Plantas tiveram início em 2008 com a espécie *A. winkleri*, para a qual foram investigados aspectos de diversidade e estruturação genética (Goetze, 2010), biologia reprodutiva, fertilidade e fluxo de pólen (Büttow, 2012). A partir desses dois estudos o grupo tomou conhecimento da problemática taxonômica que ocorre nesse subgênero, com vários *taxa* não apresentando limites claros de separação. Aliado a isso, várias espécies do grupo apresentam distribuição geográfica restrita ao sul da Mata Atlântica, o que chamou a atenção, pois a maioria dos estudos de filogeografia com espécies desse bioma inclui preferencialmente *taxa* do sudeste e nordeste do Brasil.

A presente tese foi dividida em quatro artigos que discutiram aspectos importantes do processo de especiação do subgênero *Ortgiesia* e dos padrões de diversidade genética desse grupo. Os resultados do presente estudo trouxeram dados importantes sobre os possíveis fatores envolvidos na diversificação de espécies do grupo e sobre a distribuição da diversidade genética na porção sul da Mata Atlântica.

O capítulo II da presente tese teve como objetivo principal investigar o relacionamento interespecífico no subgênero *Ortgiesia*. Para tanto, foram utilizados marcadores moleculares AFLP, uma vez que dados obtidos com o sequenciamento de regiões plastidiais e nucleares tradicionalmente utilizadas em estudos filogenéticos apresentaram baixa variação genética (Goetze, dados não publicados). Onze combinações de primers AFLP foram utilizadas, a partir das quais foram obtidos 942 fragmentos polimórficos para o grupo interno. As análises de neighbor-joining revelaram três principais grupos de espécies, que de uma maneira geral puderam ser caracterizados pela cor das pétalas. O grupo calyculata reuniu indivíduos de *A. blumenavii* e *A. calyculata*. Essas duas espécies apresentam flores com pétalas amarelas e inflorescência simples e parcialmente coocorrem na região norte do estado de Santa Catarina. O segundo grupo recuperado pelas análises de neighbor-joining reuniu indivíduos de seis espécies, cinco delas de pétalas azuis (*A. coelestis*, *A gamosepala*, *A. gracilis*, *A. organensis* e *A.* sp,) e uma com flores cor de rosa (*A. recurvata*). O último grupo reuniu as demais espécies do subgênero que apresentam flores com pétalas amarelas (*A. caudata*, *A. comata*, *A. kertesziae*, *A. kleinii* e *A. winkleri*) e alguns indivíduos de *A. coelestis*, *A. cylindrata*, *A.*

gamosepala, *A. gracilis* e *A. organensis* (pétalas azuis) e *A. candida* (pétalas brancas). De uma maneira geral o relacionamento interespecífico ficou moderadamente resolvido, com exceção do terceiro grupo. A presença de alguns indivíduos de espécies com pétalas azuis dentro do terceiro grupo, majoritariamente formado por *taxa* com pétalas amarelas, sugere que possa ocorrer hibridação entre essas espécies. Essa hipótese é suportada pelo fato de não haver especificidade de polinizador para esse grupo de espécies (Araújo *et al*., 2004; Kaehler *et al*., 2005; Machado e Semir, 2006; Piacentini e Varassin, 2007; Kamke *et al*., 2011) e a época de floração ser a mesma (Smith e Downs, 1979; Reitz, 1983; Wanderley e Martins, 2007). Ainda, essas espécies são encontradas em simpatria a partir da região norte de Santa Catarina, observando-se alguns indivíduos com morfologia intermediária (Smith e Downs, 1979; Wanderley e Martins, 2007). Entretanto, a hipótese de hibridação precisa ser confirmada, com a utilização de marcadores plastidiais e estudos de biologia da polinização. Os resultados encontrados também permitiram afirmar que o isolamento geográfico e de hábitat parecem ser importantes barreiras ao fluxo gênico interespecífico em *Ortgiesia*. A dificuldade em encontrar linhagens únicas observadas no presente trabalho corroboram resultados de estudos prévios, baseados tanto em morfologia (Faria *et al*., 2004; Wanderley e Martins, 2007), como em marcadores moleculares (Schulte *et al*., 2005, 2009; Horres *et al*., 2007; Schulte e Zizka, 2008; Sass e Specht, 2010; Silvestro *et al*., 2014). Essas dificuldades taxonômicas podem ser atribuídas a recente origem do subgênero *Ortgiesia* (2,5 milhões de anos atrás; Silvestro *et al*., 2014), aliado a um rápido processo de diversificação, conforme evidenciado na presente tese (Capítulo II).

A partir da árvore de neighbor-joining gerada com os dados de AFLP, a evolução dos principais caracteres morfológicos utilizados para a distinção de espécies dentro do subgênero *Ortgiesia* foi investigada e a utilidade taxonômica deles discutida. Cor de pétalas (amarela, azul, rosa ou branca), tipo (simples ou composta) e forma de inflorescência (elipsoide, cilíndrica ou piramidal) foram avaliadas. As análises revelaram que cor de pétala amarela e inflorescência simples são os caracteres de estado ancestral dentro do subgênero, enquanto altos níveis de homoplasia foram observados para forma de inflorescência. Cor de pétala foi considerada um bom caractere taxonômico, enquanto tipo e forma de inflorescência devem ser utilizados com cautela para a separação das espécies dentro de *Ortgiesia*.

A análise da distribuição geográfica atual das espécies do subgênero *Ortgiesia* revelou que cinco dos sete *taxa* do grupo com pétalas amarelas ocorrem na região norte de Santa Catarina, o que fez a área ser considerada o centro de origem para o subgênero. A partir dessa região três rotas migratórias principais foram propostas, uma em direção ao sul, atingindo o Rio Grande do Sul e o Uruguai (por exemplo, *A. recurvata*). Outra para o norte, alcançando a região central do Espírito Santo (*A. coelestis*), e a terceira para a região oeste do país, atingindo também a região noroeste da Argentina e Paraguai (*A. calyculata* e *A. recurvata*). Ainda, a ocorrência de espécies de *Ortgiesia* com distribuição geográfica disjunta e restrita indica que as oscilações climáticas do Pleistoceno podem também ter influenciado o processo de especiação do grupo. As populações teriam ficado isoladas durante esse período, o que pode ter levado a adaptações específicas para cada habitat, culminando com diferenças morfológicas e ecológicas que caracterizam cada uma das espécies atualmente.

No capítulo III a diversidade e estruturação genética das sete espécies de pétalas amarelas de *Ortgiesia*, *A. blumenavii*, *A. calyculata*, *A. caudata*, *A. comata*, *A. kleinii*, *A. kertesziae* e *A. winkleri* foi investigada, utilizando marcadores plastidiais (cpDNA) e um gene nuclear (nrDNA). Foram encontrados 32 haplótipos de cpDNA para os sete *taxa*, dos quais três foram compartilhados entre mais de uma espécie. Para o nrDNA, 54 haplótipos foram recuperados e 11 compartilhados. O compartilhamento de haplótipos ocorreu preferencialmente entre populações geograficamente distantes, indicando a ocorrência de retenção de polimorfismo ancestral nesse grupo. Além disso, os resultados encontrados também indicaram que as espécies desse grupo ainda não acumularam diferenças genéticas suficientes, resultado da recente diversificação do grupo, sugerindo que o processo de especiação ainda está em andamento. Ainda, entre duas espécies, *A. caudata* e *A. comata*, foi identificada a ocorrência de hibridação, com um indivíduo (Haplótipo 20 – Fig. 2, Fig. 3; Capítulo III) apresentando DNA de cloroplasto de *A. caudata* e morfologia de *A. comata*, caracterizando um evento antigo de hibridação com captura de cloroplasto. Essas duas espécies compartilham polinizadores, apresentam sobreposição do período de floração (Lenzi *et al*., 2006; Kamke *et al*., 2011), e são encontradas ocorrendo em simpatria na ilha de Santa Catarina (Fig. 1; Capítulo III). Os resultados encontrados nesse capítulo salientaram mais uma vez a importância do isolamento geográfico e de habitat no processo de diversificação em *Ortgiesia*.

Os maiores níveis de diversidade genética no grupo formado pelas espécies de pétalas amarelas de *Ortgiesia* foram encontrados na região norte de Santa Catarina, tanto no genoma plastidial como no nuclear. As populações de Ilhota e Corupá, apresentaram quatro haplótipos de cpDNA cada uma, enquanto que em Araquari e Itajaí, 13 e 11 haplótipos, respectivamente, foram observados (Fig. 1 e Tablea 1; Capítulo III). Portanto, a região norte de Santa Catarina é de valor imprescindível tanto para a conservação como evolutivamente, merecendo ser investigada como possível área de refúgio durante o Pleistoceno no sul da Mata Atlântica.

Dez locos de microssatélites nucleares (SSR) foram isolados e caracterizados para o gênero *Aechmea* no capítulo IV. Esses marcadores apresentaram alto polimorfismo (PIC > 0,5 em nove locos, Tabela 2; Capítulo IV) e foram utilizados para avaliar a diversidade genética de *A. caudata*. Os maiores índices de diversidade genética foram encontrados na população de Araquari (região norte de Santa Catarina), corroborando os resultados encontrados no Capítulo III. Os dez locos foram testados também quanto à amplificação heteróloga em 21 espécies pertencentes a três subfamílias de Bromeliaceae. Cinco primers (Ac01, Ac11, Ac25, Ac55 e Ac78) amplificaram em mais de 50% das espécies testadas. Esse foi o primeiro grupo de marcadores microssatélites nucleares desenvolvidos para o gênero *Aechmea* e serão de fundamental importância para estudos de diversidade genética e estruturação, entre outros, e também poderão ser úteis para as demais espécies do gênero e da subfamília Bromelioideae. Esse conjunto de marcadores foi empregados nos estudos com *A. calyculata* (Capítulo V), *A. kertesziae* (Capra, 2012) e com *Dyckia distachya* Hassler (Janke, 2014), pertencente a Pitcairnioideae, mostrando a utilidade desses locos também em espécies de outras subfamílias.

No capítulo V a estruturação e diversidade genética de *A. calyculata* foi investigada. Essa é a única espécie do grupo de pétalas amarelas de *Ortgiesia* que ocorre tanto na floresta ombrófila densa como na semidescídua (Reitz, 1983). Populações do leste da área amostrada para esse estudo estão localizadas na floresta ombrófila, enquanto as do centro-oeste ocorrem na semidescídua. Doze SSR, duas regiões de cpDNA e um gene nuclear foram utilizados nesse estudo. Os resultados encontrados revelaram altos níveis de divesidade genética para *A. calyculata*, similares aos já observados para outras espécies do subgênero (Goetze, 2010; Abonanza, 2012; Capra, 2012; Goetze *et al*., 2013). Entretanto, baixa variação genética foi observada para o genoma plastidial (um a dois haplótipos por

população, Fig.1; Capítulo V). Uma forte estruturação genética foi observada entre as populações do leste e centro-oeste com os marcadores microssatélites e com o gene nuclear *phyC*. A Mata de Araucária, que parcialmente separa a floresta ombrófila densa da semidescídua no sul do Brasil, foi considerada uma barreira ao fluxo gênico entre as populações de *A. calyculata*.

Por fim, o estudo desenvolvido na presente tese revelou o quão complexa é a evolução do subgênero *Ortgiesia.* Esse trabalho pode ser considerado como ponto de partida para muitos outros, pelo fato de ter identificado espécies proximamente relacionadas, e grupos que devem ser investigados de maneira conjunta, como por exemplo, *A. blumenavii* e *A calyculata*, e *A. comata* e *A. kertesziae*. Estudos futuros devem fazer uso de várias ferramentas, combinando dados genéticos, morfológicos, climáticos, de biologia reprodutiva, entre outros, para elucidar e obter mais informações sobre os processos responsáveis pela especiação nesse grupo.

Capítulo VII

Referências bibliográficas dos capítulos I e VI

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