
PROGRAMA DE PÓS-GRADUAÇÃO EM ECOLOGIA E BIODIVERSIDADE

**Padrões de Diversidade Genética e Filogeografia de *Tillandsia aeranthos*
(Lois.) L.B. Smith (Bromeliaceae)**

Felipe Aoki Gonçalves



Abril de 2018

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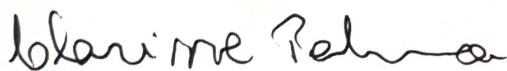
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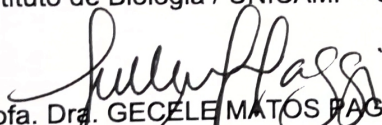
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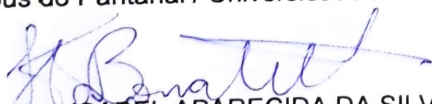
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Resumo

O continente sul-americano é o mais biodiverso da Terra, sendo palco da interação de complexos processos climáticos e geológicos que moldaram sua biota de forma muito heterogênea. Um crescente número de estudos de filogeografia de espécies Sul Americanas tem auxiliado no entendimento das respostas evolutivas envolvidas em tal diversificação. A família Bromeliaceae é caracterizada por extensa radiação adaptativa, apresenta heterogeneidade de estratégias reprodutivas e padrões distintos de fluxo gênico e estrutura genética. *Tillandsia aeranthos* (Lois.) L.B. Smith é uma bromeliácea epífita que habita matas ciliares por toda região dos Pampas. Sua ocorrência em densas populações ao longo de ambientes geograficamente distintos a torna um bom modelo para estudos sobre a influência de fatores geoclimáticos e ecológicos no padrão de distribuição da variabilidade genética e decorrentes processos de especiação ou manutenção da integridade da espécie. Esta dissertação foi dividida em dois manuscritos a fim de fornecer dados e análises úteis para a compreensão da evolução desta espécie neotropical.

No **Capítulo 1** foi realizada a amplificação heteróloga em *Tillandsia aeranthos* e *Tillandsia recurvata* de marcadores microssatélites nucleares previamente desenvolvidos para outras espécies de Bromeliaceae. Conjuntos de sete e seis marcadores apresentaram índices satisfatórios de polimorfismos em *T. aeranthos* e *T. recurvata*, respectivamente. A análise dos dados em duas populações de 20 indivíduos de cada espécie apresentou resultados compatíveis com sistemas reprodutivos distintos de cada espécie: fecundação cruzada predominante em *T. aeranthos* e auto-fecundação predominante em *T. recurvata*.

No **Capítulo 2** investigamos os padrões de variabilidade e estrutura genética e sistema reprodutivo de *Tillandsia aeranthos* ao longo da distribuição geográfica da espécie. Um total de 203 indivíduos de 13 localidades foi analisado a partir de sete marcadores microssatélites nucleares; 12 indivíduos tiveram 13 regiões universais plastidiais sequenciadas; e 74 indivíduos com 543 flores foram submetidos a experimentos de polinização manual. Os dados de microssatélites nucleares apontam altos níveis de diversidade genética em *T. aeranthos* ($H_E=0,806$; $H_O=0,745$) apesar de todas as regiões plastidiais sequenciadas terem sido monomórficas, sem diferenciação haplotípica. Foi observada também baixa diferenciação populacional ($F_{ST}=0,031$) sem correlação significativa entre as distâncias genéticas e geográficas das populações (isolamento-por-distância). Sinais moderados de eventos recentes de gargalo genético foram detectados em somente quatro das 13 populações, indicando que a maior parte das populações apresentou estabilidade demográfica durante o último máximo glacial. Os experimentos de manipulação polínica evidenciaram auto-incompatibilidade total em *T. aeranthos*. Em conclusão, os resultados demonstram altos níveis de diversidade genética e estabilidade demográfica na espécie, com fluxo gênico ocorrendo sem barreiras geográficas evidentes dentro da área de ocorrência de *Tillandsia aeranthos*.

Palavras chave: Filogeografia, microssatélites, genética de populações, Pampas, Bromeliaceae

Abstract

South America is the most biodiverse subcontinent of the planet, bearing interactions between complex geoclimatic processes that heterogeneously molded its biota. An increasing number of Phylogeographic studies in South American species have helped us to understand the evolutionary responses that gradually formed such great biodiversity. Bromeliaceae is a family of herbaceous plants characterized by extreme adaptive radiation, its species present a wide range of reproductive strategies and distinct patterns of gene flow and genetic structure. *Tillandsia aeranthos* (Lois.) L.B. Smith is an epiphyte that inhabits mainly riparian forests of the Pampas biome. It occurs in dense populations across distinct habitats and topographic profiles, which makes it a good model species in studies about the influence of geo-climatic and ecologic factors over patterns of genetic variability and structure, as well as subsequent evolutionary processes of speciation or species cohesion maintenance. This dissertation presents two manuscripts aiming to provide data and analysis that will allow a better comprehension of *T. aeranthos* evolutionary history.

In **Chapter 1**, we performed cross-amplifications of several nuclear microsatellite loci developed for other bromeliad species in *Tillandsia aeranthos* and *T. recurvata*. Sets of seven and six markers amplified satisfactorily and were polymorphic in *T. aeranthos* and *T. recurvata* respectively. The following analysis were carried in two populations of 20 individuals for each species and results were in accordance to opposite breeding systems of each species: predominant cross-pollination in *T. aeranthos* and predominant self-pollination in *T. recurvata*.

In **Chapter 2**, we investigated patterns of genetic diversity, phylogeographic structure and breeding system in *T. aeranthos* across most of its geographic distribution. Altogether, 203 individuals were analyzed from seven microsatellite markers; 12 individuals were analyzed from 13 chloroplast regions; and controlled pollination experiments were carried in 74 individuals bearing 543 flowers. Nuclear microsatellite data suggests very high levels of genetic diversity ($H_E=0,806$; $H_O=0,74$). Contrastingly, all chloroplast regions were monomorphic, with no haplotype differentiation. Genetic structure was very low ($F_{ST}=0,031$) and isolation-by-distance hypothesis was refuted. Moderated signs of recent bottleneck events were detected in four out of 13 populations, suggesting that most populations were demographically stable since the last glacial maximum. Controlled pollination experiments showed complete self-incompatibility in *T. aeranthos*. In conclusion, our results show high levels of genetic diversity and demographic stability in the species, with gene flow occurring freely without evidence of geographic barriers across the species geographic distribution.

Keywords: Phylogeography, microsatellites, population genetics, Pampas, Bromeliaceae

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Introdução geral

Filogeografia na América do Sul

A América do Sul é o subcontinente mais biodiverso da Terra, e nela são contidos cinco dos *hotspots* de biodiversidade mundial (Myers 2000). Fisicamente, uma vasta extensão latitudinal e geomorfologia complexa permitem a ocorrência de gradientes variáveis de perfis climáticos e, consequentemente, muitos perfis vegetais (Turchetto-Zolet 2013). Historicamente, eventos climáticos e orogênicos moldaram a paisagem do continente e seus oceanos de forma contínua durante diversas eras geológicas; criando um mosaico complexo e dinâmico de condições às quais a biota foi submetida. Assim, houve atuação de uma vasta gama de processos evolutivos responsáveis pela biodiversidade atual do continente (Turchetto-Zolet 2013).

Apesar de sua exuberância singular, pouco se sabe sobre os processos evolutivos e padrões de diversificação responsáveis pela extraordinária biodiversidade do continente Sul Americano. O período atual, Antropoceno, é caracterizado por rápidas mudanças ambientais inflingidas na biota (Steffen *et al.* 2011). Portanto estudos filogeográficos podem nos fornecer informações valiosas sobre a influência que processos históricos (climáticos e orogênicos) tiveram sobre a dinâmica evolutiva dos organismos. Apesar de estudos que integram informações genéticas à teoria ecológica da radiação adaptativa serem uma tendência crescente entre biólogos evolutivos da atualidade (Schluter 2000), estes ainda são escassos para a América do Sul em relação às regiões temperadas do globo (Hewitt 2004, Turchetto-Zolet 2013).

Filogeografia dos Pampas

Apesar de estudos envolvendo ambientes sul-americanos serem uma tendência crescente entre biólogos evolutivos da atualidade, alguns biomas se encontram sub-representados na bibliografia principalmente em relação biomas como a Floresta Amazônica, a Mata Atlântica, o Cerrado e a Patagônia (Turchetto-Zolet *et al.* 2013, Leal *et al.* 2016). Um deles é a região dos Pampas.

Os Pampas são caracterizados principalmente por extensas planícies dominadas por gramíneas alternadas por longas faixas de mata de galeria e afloramentos rochosos, compreendendo a extremidade sul do Brasil, parte do centro-leste da Argentina e praticamente todo o território do Uruguai (Bilenca & Miñarro, 2004). A geomorfologia da região é complexa. No oeste, região de limite com o domínio do Chaco, predominam planícies de deposição no Quaternário. Ao leste do rio Uruguai, deposições sedimentares ocorreram do Devoniano ao Holoceno sobre e dentro do Escudo Brasileiro, conferindo à paisagem planícies intercaladas com afloramentos rochosos datados do pré-Cambriano. Sua extremidade leste, a Planície Costeira Sul Americana, foi intensamente moldada por transgressões e regressões do oceano Atlântico promovidas por oscilações climáticas do Pleistoceno e pela transgressão flandriana no Holoceno, que depositaram sedimentos sucessivamente criando uma nova linha costeira formada por sistemas lagunares (Brea & Sucol, 2011; Fregonezi *et al.*, 2012). Em suma, a região dos Pampas apresenta um mosaico de perfis edáficos e formações geológicas de idades distintas que culminaram em uma heterogeneidade singular da flora (Grela, 2004; Boldrini, 2009).

Atualmente o clima da região dos Pampas é subtropical a temperado, com um gradiente de pluviosidade maior ao leste (precipitação anual > 1600mm), e ambientes semi-áridos a áridos no oeste e sudoeste (<400mm) (Speranza *et al.* 2007). Estudos paleoclimáticos realizados na Planície Costeira Sul Americana e áreas adjacentes indicam que florestas e campos de gramíneas expandiram e contraíram alternadamente durante oscilações climáticas do Pleistoceno, com expansão de vegetação xérica do sudoeste em direção ao nordeste durante períodos mais secos do fim do Pleistoceno e do Holoceno (Iriando, 1999; Fregonezi *et al.* 2012; Mäder *et al.* 2013).

Trabalhos de filogeografia, biogeografia ou genética de populações envolvendo clados oriundos dos Pampas são predominantes com animais (González *et al.* 1998; Johnson *et al.* 1999; Ojeda, 2010; Mapelli *et al.* 2012; Felappi *et al.* 2015,

Langone *et al.* 2016, Ramos-Fregonezi *et al.* 2017), com apenas três gêneros de plantas estudados até o presente momento (Solanaceae: *Calibrachoa* Cerv. e *Petunia* Juss. -Fregonezi *et al.* 2012, Mäder *et al.* 2013, Longo *et al.* 2014; Turneraceae: *Turnera* L. - Speranza *et al.* 2007). A maioria dos estudos encontrou altos níveis de estruturação filogeográfica e forte correlação entre a localização geográfica das populações e a distribuição da variabilidade genética, evidenciando a heterogeneidade e natureza fragmentada dos habitats no Pampas. Contudo, uma espécie de anuro (*Pseudopaludicola falcipes*– Langone *et al.* 2016) apresentou baixa estruturação genética devido principalmente ao mecanismo de dispersão passiva da espécie ao longo da hidrografia pampeira e a suas grandes densidades demográficas, demonstrando a importância de caracteres ecológicos na diversidade de padrões filogeográficos dentro do mesmo bioma.

A família Bromeliaceae

A família Bromeliaceae possui cerca de 3.140 espécies de distribuição geográfica restrita à região neotropical, com a exceção de uma espécie oeste-africana (Porembski & Barthlott 2000, Givnish *et al.* 2011). Apresenta diversidade ecológica e funcional notável entre suas espécies, colonizando assim grande parte dos ambientes neotropicais (Palma-Silva *et al.* 2016). Densas populações de bromeliáceas podem ser encontradas habitando desde ambientes xéricos como escarpas andinas e restingas até ambientes de umidade acentuada como florestas tropicais de montanha e a Floresta Atlântica (Benzing 2000). Adaptações-chave para tal radiação adaptativa envolvem: (1) diversidade de vias fotossintéticas na família (C3 ou CAM) que proporciona mecanismos para resistir à dessecação em ambientes xéricos ou epifíticos; (2) tricomas foliares especializados que permitem absorção foliar de nutrientes e água; e (3) hábito de tanque, que proporciona uma via alternativa de armazenamento hídrico independente de solo ou sistema radicular (Benzing 2000; Crayn *et al.* 2004; Silvestro *et al.* 2014).

A família se originou há cerca de 100 milhões de anos no Escudo das Guianas, com divergência entre linhagens atuais datada em aproximadamente 19 milhões de anos (Givnish *et al.* 2011). Bromeliaceae é tradicionalmente dividida em três subfamílias: Bromelioideae, Pitcairnioideae e Tillandsioideae (Smith & Downs, 1974, 1977, 1979). Contudo, estudos envolvendo marcadores plastidais constataram parafilias em Pitcairnioideae, resolvidas pela divisão desta em seis novas subfamílias e propondo a seguinte relação filogenética em oito subfamílias de Bromelaceae: (Brocchinioideae, (Lindmanioideae, (Tillandsioideae, (Hechtioideae, (Navioideae, (Pitcairnioideae, (Puyoideae, Bromelioideae)))))) (Givnish *et al.* 2007, 2011). A maior subfamília de Bromeliaceae, Tillandsioideae, é de monofilia confirmada com base em caracteres genéticos (Givnish *et al.* 2011) e tem sua origem há aproximadamente 14.2 milhões de anos na região andina, sofrendo subsequente radiação adaptativa explosiva com o soerguimento dos Andes e posterior invasão do Escudo Brasileiro e Serra do Mar, possibilitada por períodos de maior pluviosidade durante o Pleistoceno (Givnish *et al.* 2011).

Estudos de filogeografia e genética de populações em Bromeliaceae se concentram principalmente em espécies da Floresta Atlântica brasileira (Barbará *et al.*, 2007, 2009; Palma-Silva *et al.*, 2009, 2011; Hmeljevski *et al.*, 2011; Zanella *et al.*, 2011; Neri, 2017; Goetze *et al.*, 2016, 2017, 2018; Meirelles & Manos 2018, Soares

et al., 2018); com alguns estudos realizados em florestas semi-decíduas da América Central (Soltis, 1987; Murawski & Hamrich, 1990; Izquierdo & Piñero, 2000, González-Astorga *et al.*, 2004; Pinzón *et al.*, 2016), escarpas andinas (Sgorbati *et al.*, 2004; Castello *et al.*, 2016, Gallegos & Sebastián, 2016), e Cadeia do Espinaço (Cavallari *et al.* 2006; Aoki-Gonçalves *et al.*, 2013; Lavor *et al.*, 2014); e uma espécie estudada oriunda de afloramentos rochosos da Guiana Francesa (Sarhou *et al.*, 2003; Boisselier-Dubayle *et al.*, 2010). Até o presente momento, nenhuma bromeliácea dos Pampas teve seus padrões de diversidade genética e estrutura filogeográfica estudados.

O gênero *Tillandsia*

Tillandsia L. é o maior gênero e de mais ampla distribuição geográfica em Bromeliaceae, compreendendo cerca de 610 espécies habitando toda a região Neotropical e avançando até o sul do estado da Virgínia - EUA (e.g. *Tillandsia usneoides* L.) (Smith 1977; Bennet 1986; Luther 2010). Tradicionalmente, seis subgêneros são reconhecidos: *Tillandsia* L., *Phytarrhiza* (Vis.) Baker, *Allardtia* (A. Dietrich) Baker, *Diaphoranthema* (Beer) Baker, *Pseudoalcantarea* Mez e *Anoplophytum* (Beer) Baker (Benzing, 2000), contudo, estudos filogenéticos recentes (Barfuss 2005, 2016) revelaram polifilias e parafilias entre todos os subgêneros. Dentre *Tillandsia* que ocorrem no Brasil, espécies de *Anoplophytum* possuem grande representatividade, incluindo muitos casos de endemismo estrito no sul e sudeste do Brasil (Martinelli *et al.* 2008). Uma região com alta riqueza de espécies e altos níveis de endemismo é considerada uma arena para a especiação, e disponibiliza um excelente sistema modelo para o estudo de hipóteses sobre evolução do isolamento reprodutivo e especiação (Van der Niet *et al.* 2006; Wendt *et al.* 2008).

Apesar de sua diversidade singular, poucos estudos de filogeografia e genética de populações contemplam espécies de *Tillandsia* (Soltis *et al.*, 1987; González-Astorga *et al.*, 2004; Pinzón *et al.*, 2016; Castello *et al.*, 2016), e apenas uma espécie subtropical foi estudada (Castello *et al.*, 2016). Maiores esforços são necessários para a compreensão dos padrões filogeográficos do gênero uma vez que sua conservação é delicada, principalmente devido à perda de habitat (García & Chocamo, 2008). Por exemplo, muitas espécies de *Tillandsia* possuem grande potencial ornamental e comercial no mundo todo: a exportação massiva de exemplares selvagens de *T. xerographica*, por exemplo, já causou impacto significativo em populações naturais da espécie (García & Chocamo, 2008).

Tillandsia aeranthos (Lois.) L.B. Smith, a espécie modelo deste estudo, é uma epífita métrica de distribuição ampla que habita matas ciliares ao longo da região dos Pampas no Brasil, Argentina, Paraguai e Uruguai (*SpeciesLink*). Possuem inflorescências vistosas, com brácteas róseas e pétalas púrpura, provavelmente polinizadas por beija-flores e abelhas, apesar de nenhum estudo específico de biologia da polinização ter sido realizado com esta espécie. Seus frutos são do tipo cápsula e as sementes possuem apêndices plumosos de dispersão anemocórica, os indivíduos ocorrem em densas populações tanto em ambientes conservados quanto em localidades de urbanização acentuada.

Objetivos

A presente dissertação teve como objetivo geral avaliar processos microevolutivos atuantes em *Tillandsia aeranthos*, como fluxo gênico e deriva genética, através de dados genéticos e ecológicos. Para tal, foram estipulados os seguintes objetivos específicos:

1. Testar a amplificação heteróloga de marcadores microssatélites nucleares descritos para outras espécies de bromeliáceas em *Tillandsia aeranthos* (**Capítulo 1**);
2. Avaliar padrões de variação demográfica, variabilidade e estrutura genética em *T. aeranthos* (**Capítulo 2**);
3. Descrever o sistema reprodutivo de populações brasileiras de *T. aeranthos* e relacioná-lo com padrões filogeográficos encontrados (**Capítulo 2**);
4. Testar hipóteses de como possíveis barreiras/corredores ao fluxo gênico influenciam a coesão da espécie (**Capítulo 2**).

Capítulo 1

Cross-amplification of microsatellite markers in two common atmospheric bromeliads with contrasting reproductive systems

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Cross-amplification of microsatellite markers in two common atmospheric bromeliads with contrasting reproductive systems

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Introduction

Tillandsia L. is the most diversified genus of Bromeliaceae (ca. 600 species; Luther 2012) and a recurrent element in canopy communities, representing one of the most specialized cases of vascular epiphytism (i.e., ‘atmospheric bromeliads’; Benzing 2000, 2012). These plants can form massive populations on dry and wet forests (e.g. Pittendrigh 1948; Johansson 1974; Benzing 2000; Reyes-García *et al.* 2008; Einzmann *et al.* 2015), and also in anthropogenic transformed landscapes, as silvicultures, urban trees, and even on telephone wire cables (e.g. Martin *et al.* 1986; Wester & Zotz 2010; Benzing 2012). Such great species diversity and ecological specialization make the *Tillandsia* genus an interesting model for evolutionary and population genetics studies. However, until now, one single study isolated molecular markers for a *Tillandsia* species (Boneh *et al.* 2003) and a few attempts have been made in order to disentangle evolutionary processes acting at the population-level (e.g. Cascante-Marín *et al.*; 2006; 2014)

Microsatellites markers (i.e. simple sequence repeat; SSRs) are widely used for a variety of genetic studies due to their hypervariability, codominant inheritance, reproducibility, relative abundance, and extensive genome coverage (Powel *et al.* 1996; Varshney *et al.* 2005). Since designing species-specific markers for every target

species is limited by time and resources, cross-amplification of microsatellite markers is a feasible approach especially in plant families with a large number of developed markers (Barbará *et al.* 2007).

Here we report the cross-transferability of SSR markers previously designed for other bromeliad species in two *Tillandsia* species: *T. recurvata* (L.) L. and *T. aeranthos* (Lois.) L.B. Smith Desf. These species are well distributed in the Neotropics, being highly abundant in natural and anthropogenic modified environments, and present contrasting reproductive systems (Bianchi & Vesprini 2014, Soltis *et al.* 1987).

Material and Methods

Studied species

Tillandsia recurvata, a species of *Diaphoranthema* Beer subgenus, is one of the most abundant and widespread bromeliads, occurring from the north of Argentina and northeast of Brazil to the south of USA (GBIF 2012) and has an autogamous reproductive system with cleistogamous flowers (Soltis *et al.* 1987; Bianchi and Vesprini 2014; Orozco-Ibarrola *et al.* 2015). *Tillandsia aeranthos*, in its turn, is a species of *Anoplophytum* Beer subgenus which thrives in mesic areas of the South American region surrounding the Plata River basin, occurring in Argentina, Brazil, Paraguay and Uruguay; it has an allogamous reproductive system with self-incompatible flowers (Bianchi & Vesprini 2014).

We characterized population diversity in two populations of each species. Individuals of *T. recurvata* were sampled from populations located in an anthropogenic-transformed area of Jaboticabal-SP (21°14'S, 48°17'W) and a natural landscape in Pedregulho-SP (20°15'S, 47°27'W), while *T. aeranthos* individuals were sampled in anthropogenic-transformed and natural environments located in Viamão-RS (30°05'S, 51°05'W) and Arambaré-RS (30°57'S, 51°22'W), respectively

DNA extraction and PCR amplification

Genomic DNA extraction was carried out according to Tel-Zur *et al.* (1999) from silica gel dried leaves. We tested the cross-amplification of 27 microsatellite markers (Table 1) in *Tillandsia recurvata* (L.) L. and *T. aeranthos* (Lois.) L.B. Smith. The microsatellite loci were PCR amplified in a reaction volume of 10 µL containing ~1 ng DNA template, 5 × GoTaq Master Mix (Promega Corporation), 5 pmol forward

primer, 10 pmol reverse primer, and 1 pmol universal M13 primer tagged with distinct fluorochromes (FAM, VIC, PET, or NED). PCRs were performed in a Veriti 96-Well Thermal Cycler (Applied Biosystems) using a touchdown program as described by Palma-Silva *et al.* (2007). Loci were genotyped on an ABI 3730 DNA Analyzer Sequencer and sized against a GeneScan LIZ molecular size standard (Applied Biosystems) using GeneMarker software v. DEMO (SoftGenetics).

Data analysis

The Micro-Checker 2.2.3 program (van Oosterhout *et al.*, 2004) was used to assess genotyping errors due to the presence of null alleles, stuttering, and allele dropout. For each population and locus, we estimated the number of alleles (A), observed (H_O) and expected (H_E) heterozygosities according to the Hardy–Weinberg equilibrium (HWE), within-population inbreeding coefficient (F_{is}), fixation index (F_{ST}), and allelic richness (AR) using the R-package “hierfstat” (Goudet 1995) (Table 2). Deviations from Hardy–Weinberg equilibrium (HWE) were evaluated using exact tests, as implemented in GENEPOP on the Web (Raymond & Rousset 1995). Linkage disequilibrium (LD) between all pairs of loci in each population was also tested in GENEPOP on the Web (Raymond & Rousset 1995). The statistical significance was adjusted for multiple testing using a sequential Bonferroni correction (Rice, 1989).

Results and Discussion

In *T. recurvata*, 18 microsatellite markers amplified well and six were polymorphic with a scorable pattern (Table 1). The number of alleles per locus ranged from four to 11, allelic richness ranged from 3.989 to 12, H_E and H_O ranged from 0.224 to 0.860 and 0 to 0.339, respectively. The inbreeding coefficient values were high for most loci (Table 3), and five of them showed significant departures from HWE. The F_{ST} value was 0.0308 ($p < 0.0001$). LD was significant ($p < 0.05$) for five out of 42 pairwise locus comparisons. MICRO-CHECKER software (van Oosterhout *et al.* 2004) found evidence for null alleles in 3 loci. Such indications of null alleles might be due to the Wahlund effect, in which a set of populations is being considered erroneously as one population; however they are probably due to the excess of homozygotes promoted by the highly endogamic nature of the species (Sicard & Lenhard 2011).

In *T. aeranthis*, nine microsatellite loci amplified well and seven were polymorphic with a scorable pattern (Table 1). The number of alleles per locus ranged from seven to 15, allelic richness ranged from 7.404 to 14.253, H_E and H_O ranged from 0.798 to 0.896 and from 0.454 to 0.889, respectively (Table 2). The inbreeding coefficient values were moderate for all of the loci (Table 2), and one of them showed significant departure from HWE. The F_{st} value was 0.0128 ($p < 0.0001$). LD was significant for one out of 42 pairwise locus comparisons and MICRO-CHECKER software (van Oosterhout *et al.* 2004) found no evidence for null alleles and scoring error caused by stuttering or large allele dropout.

The two species present opposite reproductive strategies: while *T. recurvata* performs only self-pollination (Soltis *et al.* 1987), *T. aeranthis* is completely self-incompatible (Bianchi & Vesprini 2014); and such differences clearly reflect in the estimated parameters. The larger departures from HWE and LD in *T. recurvata* imply that it performs mostly self-pollination, while the larger observed heterozygosity and lower departures from HWE and LD in *T. aeranthis* are in accordance to its self-incompatibility (Loveless & Hamrick 1984). These data, and the distinct distribution pattern of both species, are in accordance with the predictions of Baker's law (Baker, 1955), which states that self-compatible species have higher chances to colonize new environments. So, this could be a key factor that led to the broader distribution of *T. recurvata* and the narrower distribution of *T. aeranthis*.

The percentages of successful amplification and polymorphic loci (81.2% and 27.3% in *T. recurvata*, 60% and 46.7% in *T. aeranthis*, respectively) are in accordance to other studies with monocots (Barbará *et al.* 2007) and other bromeliads (Ferreira *et al.* 2017; Goetze *et al.* 2013, Lavor *et al.* 2013, Miranda *et al.* 2012, Neri *et al.* 2015, Paggi *et al.* 2008, Palma-Silva *et al.* 2006, Wöhrmann & Weising 2011; Zanella *et al.* 2012). Comparing the populations in anthropogenically transformed and natural environments on both species, we did not see significant differences between them (Table 3).

Atmospheric bromeliads are an important group of plants due to their drought adaptation to epiphytic or rupicolous niches, which could be a key factor for their great diversity and large distribution over the American continent (Benzing 2000, 2012). However, despite being closely related (Benzing 2012), each atmospheric bromeliad species has a particular distribution, and studies on topics from phylogeny and phylogeography to ecology and population genetics could disentangle the

processes underlying these patterns. In this way, the microsatellite markers that were successfully tested in both species of our study, with distinct reproductive strategies, are a helpful tool and starting point for promising studies

Table 1. Microsatellite markers tested in each one of the species and amplification/polymorphism results.

Primer	Size (bp)	<i>T. recurvata</i>		<i>T. aeranthos</i>		Reference
		Amplification	Polymorphism	Amplification	Polymorphism	
Op13	208-216	No	No	No	No	Aoki-Gonçalves et al. 2013
Op69	108-136	Yes	No	No	No	Aoki-Gonçalves et al. 2013
Op77	151--168	-	-	No	No	Aoki-Gonçalves et al. 2013
Op89	135-139	-	-	No	No	Aoki-Gonçalves et al. 2013
E6	148	Yes	Yes	Yes	Yes	Boneh et al. 2003
E6B	151	Yes	Yes	Yes	Yes	Boneh et al. 2003
E19	137	Yes	No	-	-	Boneh et al. 2003
P2P19	204	Yes	No	Yes	Yes	Boneh et al. 2003
CT5	189	Yes	Yes	No	No	Boneh et al. 2003
VS1	181-266	No	No	-	-	Neri et al. 2015
VS2	239-258	Yes	No	-	-	Neri et al. 2015
VS8	203-229	Yes	No	-	-	Neri et al. 2015
VS9	306-329	Yes	No	-	-	Neri et al. 2015
VS10	187-196	Yes	Yes	-	-	Neri et al. 2015
PaA10	146-149	Yes	No	-	-	Paggi et al. 2008
PaD07	233-239	Yes	Yes	-	-	Paggi et al. 2008
VgA04	200-225	-	-	Yes	Yes	Palma-Silva et al. 2007
VgB01	156-162	No	No	No	No	Palma-Silva et al. 2007
VgB06		-	-	Yes	Yes	Palma-Silva et al. 2007
VgC01	208-218	Yes	Yes	Yes	Yes	Palma-Silva et al. 2007
VgF02	176-204	Yes	No	-	-	Palma-Silva et al. 2007
VgG05	152-204	Yes	No	-	-	Palma-Silva et al. 2007
Acom12.12	196	Yes	No	Yes	No	Wörhmann&Weising 2011
Acom78.4	193-205	-	-	Yes	Yes	Wörhmann&Weising 2011
NgFos11	162	No	No	-	-	Wörhmann et al. 2012
NgFos12	178	Yes	Yes	Yes	No	Wörhmann et al. 2012
Dd10	248-254	Yes	No	-	-	Zanella et al. 2012

Table 2. Mean values of number of alleles (A), allelic richness (Ar), expected heterozygosity (H_E), observed heterozygosity (H_O) and inbreeding coefficient (F_{IS}) for each locus in each species. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ H_E and H_O

Locus	<i>Tillandsia recurvata</i>					<i>Tillandsia aeranthos</i>				
	A	Ar	H_E	H_O	F_{IS}	A	Ar	H_E	H_O	F_{IS}
CT5	7	6.973	0.756	0.022	0.972***	-	-	-	-	-
Acom78.4	-	-	-	-	-	11	12	0.890	0.861	0.457
E6	11	12	0.860	0	1***	12	7.404	0.896	0.889	-0.018
E6B	4	4.663	0.224	0.070	0.626**	11	8.613	0.832	0.454	-0.057
NgFos12	5	4.707	0.522	0.045	0.912***	-	-	-	-	-
P2P19	-	-	-	-	-	11	13.798	0.863	0.731	0.154
PaD07	4	3.989	0.403	0.339	0.078	-	-	-	-	-
VgA04	-	-	-	-	-	15	14.253	0.857	0.665	0.036
VgB06	-	-	-	-	-	9	13.974	0.835	0.880	0.228*
VgC01	11	10.929	0.693	0	1***	7	12.466	0.798	0.812	0.009

Table 3. Mean values of expected heterozygosity (H_e), observed heterozygosity (H_o), inbreeding coefficient (F_{IS}), fixation index (F_{ST}) and number of alleles (A) for both populations of each species.* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Species	Population	Status	A	H_e	H_o	F_{IS}	F_{ST}
<i>T. recurvata</i>	Jaboticabal	Anthropogenically transformed	4.703	0.561	0.047	0.767	0.0308***
<i>T. recurvata</i>	Pedregulho	Natural	6.884	0.706	0.095	0.823	
<i>T. aeranθος</i>	Viamão	Anthropogenically transformed	8.492	0.877	0.780	0.107	0.0128***
<i>T. aeranθος</i>	Arambaré	Natural	6.981	0.837	0.732	0.124	

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Capítulo 2

High gene flow maintains wide-range species cohesion in a Neotropical riparian epiphyte (*Tillandsia aeranthos* Lois. L.B. Smith – Bromeliaceae)

High gene flow maintains wide-range species cohesion in a Neotropical riparian epiphyte (*Tillandsia aeranthos* Lois. L.B. Smith – Bromeliaceae)

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Abstract

Background and Aims Phylogeographic frameworks allow us to test hypothesis about organisms' evolutionary responses to fluctuations in habitat connectivity and availability. Yet, studies on patterns of genetic diversity, population structure, phylogeography and ecological data across species' geographic ranges are still scarce for South American clades. Here we present a study aiming to assess genetic diversity, population structure and breeding system across the geographic distribution of a subtropical epiphyte endemic to the Plata River basin, *Tillandsia aeranthos*(Lois.) L.B. Smith (Bromeliaceae).

Methods A set of seven nuclear microsatellite markers was genotyped in 203 individuals of *T. aeranthos* from 13 localities across Brazil and Argentina and 13 chloroplast regions were sequenced in a subset of the data. Additionally, we performed controlled pollination experiments in order to discuss correlations between the breeding system, the genetic diversity and the phylogeographic structure in the species.

Key results Nuclear diversity levels were high ($H_E=0.806$, $H_O=0.745$, Allelic richness = 5.860) with no haplotypic differentiation detected (~6kpb sequenced). Bayesian assignment analysis, supported by principal component analysis of genetic distances and analysis of molecular variance, show low phylogeographic structure

across the whole studied area ($F_{ST}=0.031$, $p<0.001$). Most populations were demographically stable during Quaternary climatic oscillations, with no bottleneck events detected. Controlled pollination experiments indicated complete self-incompatibility in all populations analyzed.

Conclusions *Tillandsia aeranthos* shows elevated levels of genetic diversity and effective gene flow across an extensive area in the Pampas biome. The self-incompatible breeding system adopted by these plants implicates in mandatory pollen flow, which relates directly to high levels of heterozygosity. The potential connectivity of the riparian forests the species inhabits allied with its anemochorous seeds and obligatory outcrossing may be the key factors that maintain pollen flow and seed dispersal efficiency, causing such nearly panmictic conditions.

Keywords: Population genetics, phylogeography, reproductive system, bromeliads.

Introduction

South America is the most biodiverse subcontinent of the planet, bearing five out of the 35 world's biodiversity *hotspots* (Myers *et al.*, 2000). Although the majority of phylogeographic studies is concentrated on the Northern Hemisphere, studies on South American biota are uprising in numbers (see reviews of Turchetto-Zolet *et al.*, 2013; Leal *et al.*, 2016.). While tropical biomes such as the Amazon, the Brazilian Atlantic Rainforest and the Cerrado are well represented in phylogeographic studies, there is little emphasis on subtropical areas outside the Patagonian region, including one of the largest grasslands in the world, the Pampas (Fregonezi *et al.*, 2013; Mäder *et al.*, 2013; Turchetto-Zolet *et al.*, 2013; Longo *et al.*, 2014; Leal *et al.*, 2016).

The Pampas biome is characterized by extensive areas of grassy vegetation alternated by long patches of riparian gallery forests and rocky outcrops (Rambo, 1956). It is located comprising the southernmost portion of Brazil, part of northeastern Argentina and the full extent of Uruguay's territory. The geomorphology of the Pampas region is very diverse and causes great soil heterogeneity in the biome (Rambo, 1956). The western portion is mainly derived from sediment depositions of the Quaternary. To the east of the Uruguay River, most sediment depositions occurred

from the Devonian to the Holocene over the Brazilian Shield, which is the most ancient formation of the Pampas dating back to the Pre-Cambrian and from which most rocky outcrops originate. Its easternmost portion, the South Atlantic Coastal Plain, was extensively molded by sea level regressions and transgressions promoted by the oscillatory glaciation cycles of the Pleistocene and by the Flandrian Transgression of the Holocene, resulting in a new coastal line formed from barrier-lagoon systems (Brea & Sucol, 2011).

Currently the Pampean region has a subtropical to temperate climate, with moisture gradient from humid in the east (annual precipitation > 1600 mm) to semi-arid and arid (< 400mm) in the west and south-west (Speranza *et al.*, 2007). Paleoenvironment studies on the western portion of the Pampas evidence relatively stable persistence of grasslands since the Last Glacial Maximum (LGM) (Behling, 2005). In the Pampean east, South Atlantic Coastal Plain and adjacent areas, woody vegetation and grasslands alternately expanded and contracted influenced by Pleistocene climatic oscillations with xeric vegetation expanding from the southwest to the northeast during the late Pleistocene and mid-Holocene dry periods (Iriondo & García, 1993; Iriondo, 1999; Behling, 2004). Population history effects are especially significant for species that have withstood the Quaternary climate oscillations (Hewitt, 2000). Fluctuations in habitat availability due to climate changes may cause great shifts in species distribution and the genetic consequences of such events can help us to interpret evolutionary history (Hewitt, 2004). Phylogeographic studies corroborate both the importance of Pleistocene climatic oscillations and ecological factors such as the biome's characteristic soil heterogeneity to the current genetic structure of native Pampean plants (Fregonezi *et al.*, 2012; Mäder *et al.*, 2013; Longo *et al.*, 2014) and animals (González *et al.*, 1998; Johnson *et al.*, 1999; Fernández-Stolz *et al.*, 2007; Ojeda, 2010; Mapelli *et al.*, 2012; Roratto *et al.*, 2015; Felappi *et al.*, 2015; Langone *et al.*, 2015; Bruno *et al.*, 2016; Mora *et al.*, 2016; Ramos-Fregonezi *et al.*, 2017).

In conjunction with paleoclimatic and orogenic events, patterns of genetic variation in natural plant populations are also determined by several factors such as gene flow, drift, selection, mutation and the spatial arrangement of individuals (Wright 1943; Doligez *et al.*, 1998; Levin *et al.*, 2003). As in sexually reproducing plants gene flow occurs through seed dispersal and pollen flow, the breeding system

adopted by a species is a major ecological factor influencing the spatial distribution of genetic variability (Loveless & Hamrick 1984).

Bromeliaceae is a plant family characteristic to the Neotropical region. It presents key adaptations that allowed exemplar adaptive radiation (Benzing 2000). Population genetics and phylogeography studies performed so far with bromeliads show a wide range of genetic diversity and structure patterns (Zanella *et al.*, 2012). While species native to *inselbergs* and other rocky outcrops show generally accentuated genetic structure with limited gene flow between populations (Sarhou *et al.*, 2003, Palma-Silva *et al.* 2011, Zanella *et al.*, 2012, Aoki-Gonçalves *et al.*, 2013, but see Lator *et al.*, 2014); species from forest environments tend to be less structured with higher levels of gene flow (Palma-Silva *et al.*, 2009, Zanella *et al.*, 2012, Goetze *et al.*, 2016, Neri *et al.*, 2017). Comparatively fewer bromeliads native to open areas such as grasslands and deserts have been used in studies concerning patterns of genetic diversity and structure (González-Astorga *et al.*, 2004, Sgorbati *et al.*, 2004; Castello *et al.*, 2016, Gallegos, 2016).

Tillandsia is the most diverse and wide-ranged genus of the family and originated in the Andean region (Tillandsioideae – 14.2 million years, Givnish *et al.*, 2011). The Brazilian Atlantic Forest bears great diversity of the subgenus *Anoplophytum* (Martinelli *et al.*, 2008). *Tillandsia aeranthos* Lois. L.B. Smith is a mesic *Anoplophytum* epiphyte native to Argentina, Brazil, Paraguay and Uruguay. It is found in dense populations predominantly in riparian environments of the Pampas grasslands, and marginally in the Atlantic Forest and Chaco biomes. It has showy inflorescences, dry capsule-shaped fruits and entangling anemochorous seeds with plumose appendages.

Here we present a phylogeography study concerning an *Anoplophytum* species endemic to the Río del Plata basin, *Tillandsia aeranthos* Lois L.B. Smith. Here we aimed to assess genetic diversity, genetic structure and signs of demographic changes in the species to answer the following questions: (1) how are levels of intra and interpopulational genetic diversity distributed across the species geographical range? (2) What is the current extent of genetic structure, and which past demographic processes may have shaped its phylogeographical patterns? (3) What is the breeding system in Brazilian populations of the species and how does it influences patterns of genetic diversity and structure?

Methods

Sampling and DNA extraction

The geographic range of the species was estimated by consulting occurrence registrations in herbarium databases (GBIF, SpeciesLink and Documenta Florae Australis) and by visiting herbariums (Corrientes and Córdoba). In this study, we sampled a total of 203 individuals from 13 localities across most of the species distribution (Fig 1, Table 1). Genomic DNA extraction was performed following protocol developed by Tel-Zur *et al.*, (1999) from leaf fragments stored in silica gel and DNA quantification was carried in agarose electroforesis.

Genetic markers

Seven informative cross-amplified nuclear microsatellite markers (nSSR) previously tested and optimized (Chapter 1) were genotyped. Fragment amplification was conducted in a reaction volume of 10 μ L containing $\sim$ 1 ng DNA template, 5 $\times$ GoTaq Master Mix (Promega Corporation, Madison, WI, USA), 5 pmol forward primer, 10 pmol reverse primer, and 1 pmol universal M13 primer tagged with distinct fluorochromes (FAM, VIC, PET, or NED). Polymerase chain reactions (PCRs) were performed in a Veriti 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA) using a touchdown program as described in other bromeliad species (Palma-Silva *et al.*, 2007). Microsatellite loci were genotyped on an ABI 3730 DNA Analyzer Sequencer and sized against a GeneScan LIZ molecular size standard (Applied Biosystems) using GeneMarker software v.DEMO (SoftGenetics).

Amplification and polymorphisms were tested using a set of 28 universal plastid regions and 12 individuals from four distal localities of species distribution (Viamão, Taim, Uruguiana and Arambaré, Table 1) (Appendix S1). PCR was carried in a reaction volume of 30 μ L containing $\sim$ 2 ng DNA template, 5 $\times$ GoTaq Master Mix (Promega Corporation), 10 pmol forward primer and 10 pmol reverse primer using a standard program as described by Palma-Silva *et al.*, (2009). Reaction products were sequenced in Macrogen inc. (Seoul, South Korea) and sequences were edited and ensembled in Geneious 6.0.6.

Data Analysis

Genetic diversity

Nuclear microsatellite diversity was estimated within populations by calculating the following parameters: number of alleles (A); allelic richness (A_R) (El Mousadik & Petit 1996); observed heterozygosity (H_O) and expected heterozygosity (H_E) under the Hardy-Weinberg equilibrium (HWE) (Nei, 1978); estimated using Fstat 1.2 (Goudet, 1995). GENEPOP on the Web (Raymond & Rousset 1995) was used to calculate the inbreeding coefficient (F_{IS}) (Weir & Cockerham 1984), to detect any deviations from the Hardy-Weinberg equilibrium (HWE) and to test linkage disequilibrium (LD) between all pairs of loci in each population. The full set of microsatellite markers was tested for null alleles, stuttering, and large allele dropout using Micro-Checker 2.2.3 (van Oosterhout *et al.*, 2004).

Genetic structure

Genetic structure was assessed using model-based Bayesian clustering analysis performed in STRUCTURE 2.3.4 (Pritchard *et al.*, 2000), which probabilistically assigns individuals to genetic clusters (K). To estimate the number of clusters most likely to fit our data (ΔK), we ran STRUCTURE considering K values from 1 to 10, with a burn-in of 100,000 Markov chain Monte Carlo generations discarded and 500,000 steps per run, and 10 iterations per K to confirm stabilization of the summary statistics (Pritchard *et al.*, 2000). Then, ΔK (Evanno *et al.*, 2005) was calculated using STRUCTURE-HARVESTER 0.6.94 (Earl, 2012). We also used GenAlEx6.5 (Peakall & Smouse, 2001) to perform a principal component analysis (PCoA), an ordination method that computes genetic distances between all individuals and plots them in a two-dimensional graphic proportionally (Rohlf 1974). Analysis of molecular variance (AMOVA) were used to estimate the fixation index between populations (F_{ST}). Additionally, a Mantel test (Mantel, 1967) was performed comparing matrices of genetic distances (pairwise F_{ST} – Appendix S2) and geographical distances between populations to test for isolation-by-distance (Wright, 1965) using GENEPOP on the Web (Raymond & Rousset, 1995).

Demography

To detect signs of recent past population contraction events (i.e. genetic bottlenecks) we used heterozygosity excess test implemented in the software BOTTLENECK 1.2.02 (Piry *et al.*, 1999) using the two-phase mutation model (TPM) as recommended for microsatellite data by the user manual (Piry *et al.*, 1999).

Statistical significance was assessed in 10,000 replicates using a one tailed Wilcoxon signed-rank test of heterozygosity excess for each population. These tests are able to detect recent and pronounced reduction in effective population size due to genetic bottlenecks. Populations which have experienced a recent reduction of their effective population size exhibit a reduction of allele numbers and heterozygosities at polymorphic loci, but allelic diversity is reduced faster than heterozygosity (Piry *et al.*, 1999). So heterozygosities higher than the expected, or in excess, for a specific value of allelic diversity constitute a strong sign of bottleneck events.

Additionally, the past genetic bottlenecks were estimated by the *M*-ratio test calculated using the software *M_P_Val.exe* for each population (Garza & Williamson, 2001). The *M-value* represents the ratio between the total number of alleles and the range in allele size for each locus (Garza & Williamson, 2001). When random alleles are lost due to a reduction in effective population size, the number of alleles tends to reduce faster than the overall allelic range (Garza & Williamson, 2001). Significance for each population was assessed by comparing between *M*-ratios and critical values of M_c obtained by simulating the distribution of *M*-ratios under specific demographic and mutational conditions using the *CRITICAL_M.EXE* software (<http://swfsc.noaa.gov/textblock.aspx?Division=FED&id=3298>). The program simulated the genetic diversity of a constant-sized population at a microsatellite locus evolving under a single-step mutation model, under specific mutational and demographic conditions, estimating the critical *M*-value (M_c – Table 5). *M*-ratios below the critical *M* represent a strong sign of recent past bottleneck events.

Breeding system

To verify the breeding system adopted by *T. aeranthos* populations, controlled pollination experiments were carried out in 74 transplanted individuals from seven populations (Table 1), with a total of 543 flowers. The individuals were transplanted to a greenhouse three months prior to flowering and inflorescences were bagged before flower development. Four treatments were applied: (1) cross-pollination (flowers hand-pollinated with pollen from other individuals); (2) autonomous self-pollination (bagged inflorescences left undisturbed); (3) self-pollination (flowers hand-pollinated with pollen from the same inflorescence); and (4) free pollinator access (individuals were transplanted to open environment and unbagged). Fruit

development was checked weekly until maturity and only fruits that produced seeds were counted.

From the ratio between treated flowers and the number of healthy fruits formed in each treatment we calculated the indices of autogamy or capacity for autonomous self-pollination (AI) and self-incompatibility (ISI) (Lloyd & Schoen, 1992). AI represents the ratio between the percentage of fruit set from autonomous self-pollination and the percentage of fruit formed from hand cross-pollination. AI values higher than 0.30 indicate full or partial autonomous self-pollination. ISI represents the ratio between percentage of fruit formed from hand self-pollination and the percentage of fruit set from hand cross-pollination. ISI values equal or higher than 0.30 indicate full or partial self-compatibility, and values below 0.30 are a sign of self-incompatibility (Ramirez & Brito 1990).

Results

Genetic diversity

From the seven loci previously tested in *T. aeranthos*, three did not amplify in the Argentinean populations and MICRO-CHECKER found strong evidence for presence of null alleles in one (Acom78.4 - Wörhmann, 2011). Per locus, the number of alleles ranged from 10 to 19, allelic richness ranged from 4.998 to 7.102, H_e and H_o ranged from 0.768 to 0.844 and from 0.716 to 0.778, respectively (Table 2). The inbreeding coefficient values were moderate for most loci and three of them showed significant departures from HWE (Table 2). LD was significant for two out of 231 pairwise locus comparisons.

Per locality, the number of alleles ranged from eight to 25, allelic richness ranged from 3.349 to 6.678, H_e and H_o ranged from 0.616 to 0.870 and from 0.576 to 0.880, respectively (Table 3). The inbreeding coefficient values were low to moderate and nine localities showed significant departures from HWE.

From the 28 universal chloroplast regions tested, 13 amplified satisfactorily and were sequenced (Appendix S1). All sequenced regions were monomorphic among tested populations.

Genetic structure and demography

Results from STRUCTURE analysis (Figure 2) show similar probabilities of any individual to belong to any number of genetic clusters. STRUCTURE-HARVESTER resulted in a ΔK of four groups (Figure 4). PCoA showed the same lack of clustered pattern (Figure 3) and AMOVA resulted in $F_{ST}=0.031$ ($p\text{-val}<0.001$). Finally, the Mantel test did not find correlation between genetic and geographical distances between populations ($r=0.035$ $p\text{-val}=0.258$), rejecting the hypothesis of isolation-by-distance (Wright 1965).

Evidence for genetic bottlenecks (a significant heterozygosity excess compared to equilibrium heterozygosity $H_e > H_{eq}$) was found in four populations: Arambaré, Taim, Nova Prata, and Uruguaiana (Table 3). M-ratio values ranged from 0.616 and 0.835 across all localities and loci (Table 3). The simulations conducted with different θ and pg values generated a threshold critical M value (M_c) of 0.6175 (Table 5). At this M_c threshold value, only one population (Buenos Aires) showed signs of past genetic bottleneck events (Table 3).

Breeding system

The percentage of fruit set was very elevated in the hand cross-pollination treatment (84.44%) and moderated in the free pollinator access treatment (32.86%). No viable fruit were set from autonomous selfing or hand auto-pollination treatments. Consequently, both indices AI and ISI were equal to zero, indicating complete self-incompatibility. Results for each controlled pollination treatment are disclosed in Table 4.

Discussion

High levels of nuclear genetic diversity were observed in all populations across the species range (Table 3). Mean heterozygosity values were the highest for bromeliad species studied so far (Zanella *et al.*, 2012; Aoki-Gonçalves *et al.*, 2013; Lavor *et al.*, 2014; Gallegos, 2016; Goetze *et al.*, 2016; Neri *et al.*, 2017; Meirelles & Manos, 2018). High genetic diversity is considered advantageous for demographic stability (Vrijenhoek & Lerman, 1982) and overall fitness of individuals (Bush *et al.*, 1987). Contrastingly, haplotypic diversity could not be detected in any of the 13

universal chloroplast regions sequenced, although chosen markers were selected from the most polymorphic regions in monocots (Shaw *et al.*, 2005, 2007) and in Bromeliaceae (Barfuss *et al.*, 2005, Castello *et al.*, 2016) (Appendix S1). Mutation rates in chloroplast are expectedly low compared to mitochondrial or nuclear genomes (Wolfe *et al.*, 1987). However, the lack of polymorphisms in over 6kbp sequenced indicates a possible lack of haplotype differentiation in the species.

Bayesian assignment analysis, supported by principal component analysis of genetic distances and analysis of molecular variance, show low of genetic structure across the whole studied area and lack of isolation-by-distance. These results indicate high levels of gene flow occurring throughout the species distribution range and suggest large population sizes. In accordance, the F_{ST} value (0.031) was one of the lowest among studies on Bromeliaceae (Zanella *et al.*, 2012; Aoki-Gonçalves *et al.*, 2013; Lator *et al.*, 2014; Gallegos, 2016; Goetze *et al.*, 2016; Neri *et al.*, 2017). In *Tillandsia ionantha*, a species native to Central America with resembling habitat to *T. aeranthos* genetic structure was similar ($F_{ST}=0.04$; Soltis *et al.*, 1987). The high connectivity of riparian habitats (Hampe, 2004), along with adaptations such as anemochory in *Tillandsia* are probable key factors to the low genetic structure, as they facilitate long distance seed dispersal. Moreover, epiphytism may cause independence from direct soil conditions (Benzing, 1990) preventing population fragmentation due to the heterogeneous edaphic conditions that characterize the Pampas (Baldi *et al.*, 2006). Noticeably, one Pampean frog species, *Pseudopaludicola falcipes*, shows coincident high genetic diversity with low population genetic structure due to passive water dispersion mechanisms associated with riparian forests, habitat homogeneity and high population densities (Langone *et al.*, 2016). Contrastingly, studies performed with *Petunia* and *Calibrachoa*, terrestrial and herbaceous plants which are highly soil-associated and narrow endemics showed much higher levels of haplotypic divergence and elevated genetic structure between populations (Lorenz-Lemke *et al.*, 2010; Fregonezi *et al.*, 2012; Mäder *et al.*, 2013; Longo *et al.*, 2014).

Demographic contractions were detected in few populations (Table 3), suggesting that most populations were demographically stable during Quaternary climatic oscillations. Moreover, genetic bottlenecks signs might be artifact caused by limited loci and individual sampling (Williamson-Natesan, 2005). In addition, prominent levels of gene flow over large distances may act recovering alleles lost in

local bottleneck events, diluting the genetic consequences of past population contractions. Riparian forests are considered important tropical refugia during the oscillatory cycles of the Pleistocene as they act as humidity buffers during drier periods (Meave *et al.*, 1991, Kellman *et al.*, 1996). Hence, dense populations inhabiting moisture-stable environments with great connectivity (Hampe, 2004) may explain the high nuclear genetic diversity and demographic stability in *Tillandsia aeranthos*.

Moderate and significant inbreeding coefficient values (F_{IS}) observed for most populations are possibly due to biparental inbreeding promoted by the high density of seedlings that clump near mother plants (field observation) and trapline foraging by hummingbird pollinators (Ohashi & Thomson, 2009). Despite no information on pollination biology of *Tillandsia aeranthos* is available, we believe hummingbird and bees are the most probable pollinators. Moreover, field pollinator observations in closely related Argentinean species of *Anoplophytum* with similar habits and morphology (*T. ixioides* and *T. tenuifolia*) showed predominance of hummingbirds as floral visitors (Bernardello *et al.*, 1991); expectedly according to the showy floral morphology, and presence of saccharose-rich nectar in their flowers (Kromer *et al.* 2008).

Our controlled pollination experiments indicate all seven populations of *T. aeranthos* analyzed are completely self-incompatible (Tables 1 and 4), in accordance with one previously evaluated Argentinean population (Bianchi & Vesprini, 2014). Together, these results reinforce the homogeneity of the self-incompatible breeding system likely across the whole geographic distribution. Self-incompatibility implicates in mandatory outcrossing, enforcing pollen to travel and contributing to the mixing between populations, which makes genetic sub-divisions less likely (Loveless & Hamrick 1984) and relates directly to the low phylogeographic structure found. For instance, two sympatric closely related bromeliad species, *Vriesea scalaris* and *V. simplex*, differ significantly in genetic structure due to distinctive breeding systems adopted, stressing how this ecological trait may influence the phylogeographic patterns of natural populations (Neri *et al.* 2016). Breeding systems in bromeliads show variations among subfamilies, genera, species (Gilmartin & Brown, 1985; Martinelli, 1995; Wendt *et al.*, 2001, 2002, 2008; Sgorbati *et al.*, 2004; Cascante-Marín *et al.*, 2005; Barbará *et al.*, 2007, 2008, 2009; Morillo *et al.*, 2009; Matallana *et al.*, 2010; Orozco-Ibarrola *et al.*, 2015; Neri *et al.*, 2017; Lavor *et al.*, 2016; Bastos *et*

al., 2017), and among populations (Cascante-Marín *et al.*, 2006; Paggi *et al.*, 2007, 2013; Zanella *et al.*, 2011; Schmid *et al.*, 2011; Hmeljevski *et al.*, 2011; Palma-Silva *et al.*, 2015). In *Tillandsia*, as expected for a highly diverse genus, no trend in breeding system is clear. The genus presents completely or partially self-incompatible species normally showing vibrant colored inflorescences (Till, 1992; Morillo *et al.*, 2009; Orozco-Ibarrola *et al.*, 2015), as well as many self-compatible species, such as the whole subgenus (*Diaphoranthema*) with autogamous/cleistogamous flowers and mostly inconspicuous inflorescences (Till, 1992; Martinelli, 1995; Orozco-Ibarrola *et al.*, 2015), and other *Tillandsia* species (Till, 1992; Matallana *et al.* 2010; Martinelli, 1995; Orozco-Ibarrola *et al.*, 2015) including *T. tenuifolia* and *T. stricta*, species closely related to *T. aranthos* (Matallana *et al.* 2010).

Conclusions

Tillandsia aeranthos represents a case in which diverse factors resulted in high gene flow between populations and maintenance of very elevated levels of genetic diversity. Divergences between phylogeographic histories of other Pampean plants and *T. aeranthos*, as well as similarities with a frog species show how complex relationships between organisms' traits and the environment they live affect species evolutionary histories. Further sampling is necessary, especially in the Uruguayan territory, however chloroplast undifferentiation and lack of nuclear genetic structure over a very large sampled area suggest this species might represent a rare case of panmixia. High levels of nuclear genetic diversity and low population genetics structure found in *T. aeranthos* suggest that habitat fragmentation and consequent genetic drifts have not yet impacted within-population genetic diversity, despite the natural and human-induced fragmentation characteristic to the Pampas (Baldi *et al.*, 2006).

Table 1. Localities visited with control numbers, GPS coordinates and number of sampled individuals of *T. aeranthos* in each area.

Location	Country	control number	latitude	longitude	altitude	Sampled <i>T. aeranthos</i>
*Arambaré°	Brazil	1	-31,0515849	-52,5183906	5m	22
*Taim	Brazil	2	-32,4915358	-52,5930826	5m	11
Osório	Brazil	3	-29,8943185	-50,2675958	16m	8
Nova Prata	Brazil	4	-28,7861337	-51,6274196	820m	8
Caçapava do Sul°	Brazil	5	-30,515779	-53,485431	440m	21
*Uruguaiana°	Brazil	6	-30,2722833	-57,42765	70m	33
Santa Maria°	Brazil	7	-29,6887402	-53,8183952	113m	20
Itapeva	Brazil	8	-29,3595405	-49,7453957	989m	20
*Viamão°	Brazil	9	-30,0973692	-51,085067	9m	17
Santana do Livramento°	Brazil	10	-30,877285	-55,54142	208m	21
Bagé°	Brazil	11	-31,3313827	-54,108228	212m	21
Mercedes	Argentina	12	-29,162	-58,223	38m	12
Buenos Aires	Argentina	13	-34,614097	-58,351415	5m	8
Corrientes	Argentina	14	-27,461231	-58,830212	52m	not found
Santa Ana	Argentina	15	-27,460098	-58,664954	50m	not found
Iberá	Argentina	16	-28,853431	-58,739078	30m	not found
Córdoba	Argentina	17	-31,3992544	-64,2643837	352m	not found
Capilla del Monte	Argentina	18	-30,8573503	-64,5672209	979m	not found
Cruz del Eje	Argentina	19	-30,7218026	-64,8484294	449m	not found

* locations with sequenced individuals

° locations with individuals used in controlled pollination experiments

Table 2. Results of diversity parameters per locus: expected and observed heterozygosities (H_E and H_O , respectively), number of alleles (A), inbreeding coefficient (F_{IS}) and allelic richness (A_R).

Locus	H_E	H_O	A	F_{IS}	A_R	Primers's reference
VgA04	0.865	0.756	19	0.134	7.273	Palma-Silva, 2007
VgB06	0.874	0.702	20	0.206***	7.384	Palma-Silva, 2007
VgC01	0.885	0.845	17	0.047	7.273	Palma-Silva, 2007
E6	0.788	0.784	13	0.004	7.193	Boneh, 2003
E6B	0.831	0.785	12	0.059	5.635	Boneh, 2003
P2P19	0.768	0.658	14	0.152*	5.326	Boneh, 2003
Acom78.4	0.756	0.264	12	0.660***	5.185	Wörhmann, 2011

Departures of within-population inbreeding coefficients (F_{IS}) from HWE are indicated by asterisks:

* $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$.

Table 3. Results of diversity parameters per locality: Sampled individuals (N), expected and observed heterozygosities (H_E and H_O , respectively), mean alleles per locus (A), inbreeding coefficient (F_{IS}) and allelic richness (A_R).

Locality	N	A	A_R	H_E	H_O	F_{IS}	M-ratio ¹
Arambaré	22	8.00	6.037	0.839	0.756	0.155*	0.813 ²
Taim	9	6.75	5.805	0.852	0.797	0.181	0.729 ²
Osório	8	7.75	6.678	0.870	0.808	0.216*	0.656
Nova Prata	8	5.25	6.105	0.803	0.740	0.132	0.820 ²
Caçapava	20	8.75	5.791	0.824	0.788	0.215***	0.758
Uruguaiana	25	9.00	5.476	0.810	0.685	0.259***	0.765 ²
Santa Maria	11	7.50	6.126	0.821	0.731	0.163*	0.701
Itapeva	19	7.50	5.621	0.826	0.749	0.144*	0.835
Viamao	17	7.75	6.267	0.827	0.880	0.082***	0.815
Santana do Livramento	21	6.75	5.325	0.761	0.735	0.184***	0.648
Bagé	19	7.50	5.550	0.785	0.693	0.198***	0.793
Mercedes	12	4.00	3.349	0.616	0.576	0.068	0.751
Buenos Aires	8	6.25	5.684	0.840	0.750	0.113	0.616

Departures of within-population inbreeding coefficients (F_{IS}) from HWE are indicated by asterisks: * $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$.

¹ A population is considered to have undergone a bottleneck if its M-ratio value falls below the lower threshold of critical M-ratio ($M_c = 0.618$) showed on Table 5.

²Populations where significant bottlenecks were detected: $p < 0.05$.

Table 4. Results from controlled pollination experiments showing no fructification from any self-pollination treatment.

Treatment	Individuals	Flowers	Produced fruits	Fructification	Standard deviation
Hand cross-pollination	23	212	180	84.44%	14.1%
Autonomous selfing	8	94	0	0%	0%
Hand auto-pollination	17	167	0	0%	0%
Free pollinator access	6	70	23	32.86%	12.75%

Table 5. Parameters for the two-phased mutation model (T.P.M.) used to generate critical values of M-ratio (Mc). Theta (Θ), proportion of single-step mutations (pg), and average size of mutations larger than one-step (Δg).

Parameters	Θ	pg	Δg	Mc
1	0.5	0.1	3.5	0.7857
2	0.5	0.3	3.5	0.6335
3	2.0	0.1	3.5	0.7385
4	2.0	0.3	3.5	0.6175
5	10.0	0.1	3.5	0.8270
6	10.0	0.3	3.5	0.7512

Figure 1. Map of the Pampean region showing visited locations with presence or absence of *T. aeranthos*, topography of the studied area and the South American Dry Diagonal (S.A.D.D.) Localities control numbers are disclosed in Table 1.

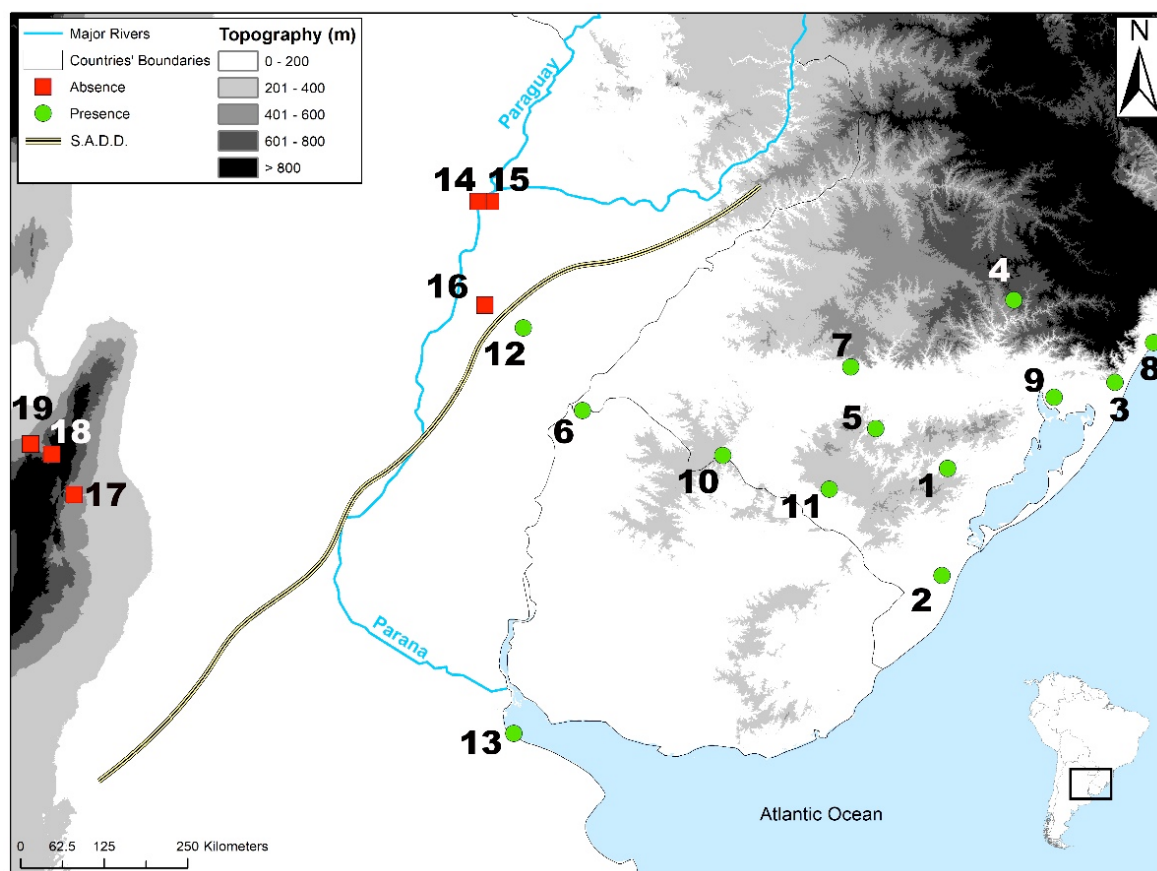


Figure 2. STRUCTURE results for $k=2, 3$ and 4 , respectively. The horizontal pattern observed between clusters indicates lack of genetic structure.

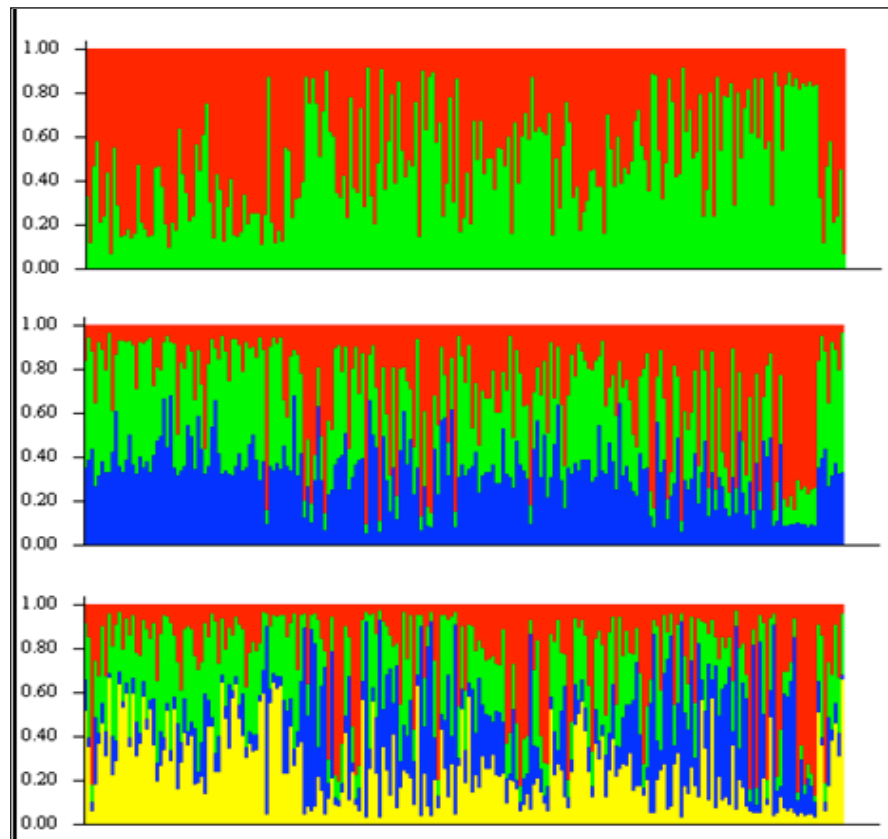
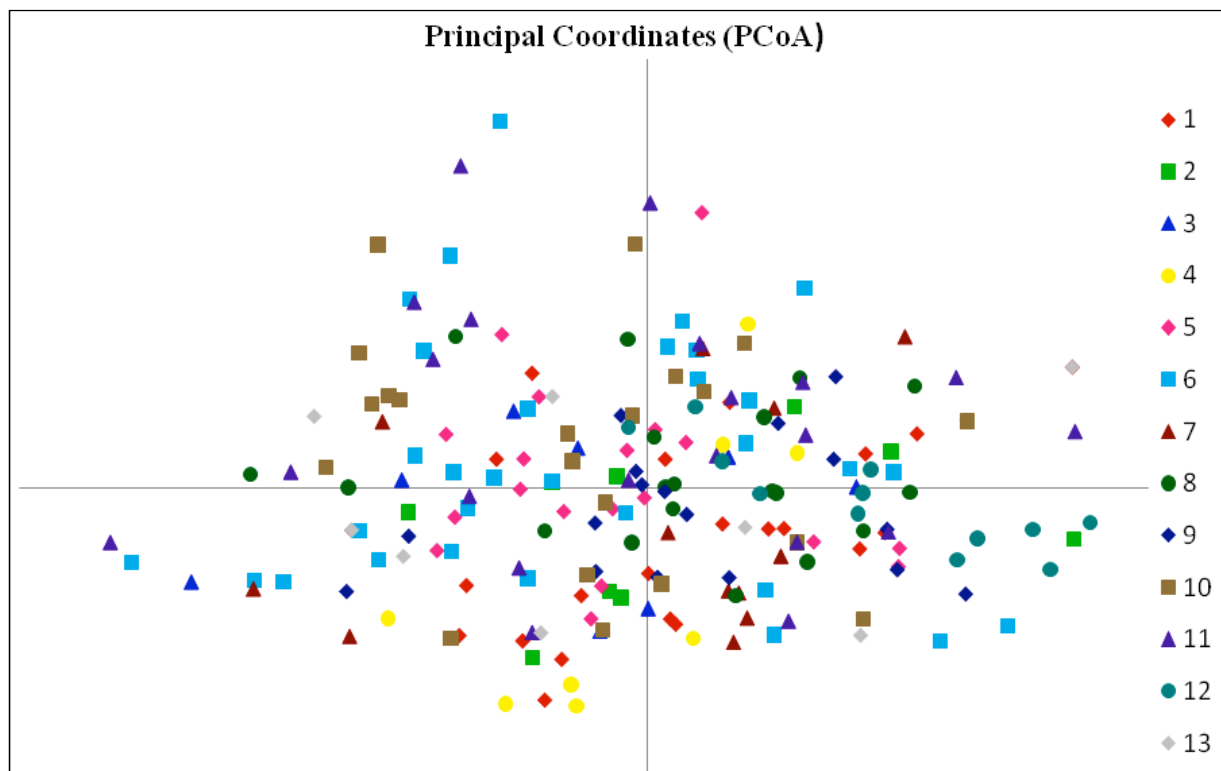
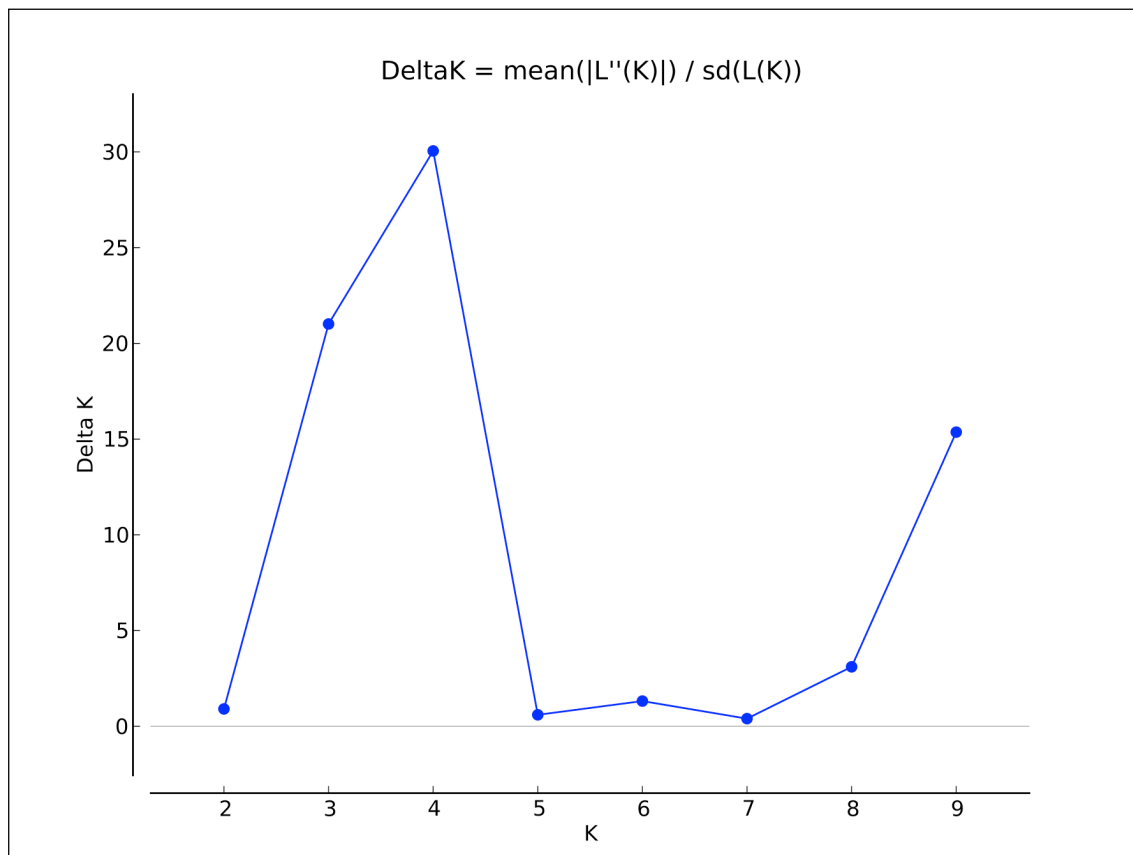


Figure 3. Principal Component Analysis (PCoA) showing no clustering between individuals from the same localities.



*Numbers represent locations disclosed on Table 1.

Figure 4. STRUCTURE-HARVESTER results for ΔK showing a most likely $K=4$ that fits our data.



Appendix S1: Chloroplast regions tested and sequenced in *T. aeranthos*.

Region	Primers	Reference	Results in <i>T. aeranthos</i>
trnC-petN	trnC petN_BR	Versieux <i>et al.</i> , (2012)	Monomorphic
petG-trnP	petG-trnP_F petG-trnP_R	Hwang <i>et al.</i> , (2000)	Monomorphic
trnV_intron	trnV_intron_F trnV_intron_R	Hwang <i>et al.</i> , (2000)	Monomorphic
matK	BROmatK_F BROM1_R Matk 5 (R) MatK 2.1A (F)	Schulte <i>et al.</i> , (2005) Schulte <i>et al.</i> , (2005) Schulte <i>et al.</i> , (2005) Schulte <i>et al.</i> , (2005)	Did not amplify
trnL	trnL-Fc (F) trnL- Fd (R)	Shaw <i>et al.</i> , (2005)	Monomorphic
psba-trnH	psbA (F) trnH (R)		Monomorphic
trnD/T	trnD_F trnT_R	Shaw <i>et al.</i> , (2005)	Did not amplify
rpl32-trnL	rpl32 (F) trnL (R)	Shaw <i>et al.</i> , (2007)	Monomorphic
SfM-trnS- trnFM	trnFM (R) trnS (F)	Demesure <i>et al.</i> , (1995)	Did not amplify
trnL	trnL b (R) trnT a (F)	Taberlet <i>et al.</i> , (1991)	Did not amplify
trnC-ycf6	ycf6 (R) trnC (F)		Did not amplify
psbM-trnD	trnD (R) psbM (F)	Shaw <i>et al.</i> , (2007)	Monomorphic
atpI-atpH	atpI (F) atpH(R)		Did not amplify
rps16-trnK	rps16_x_2F2 (F) trnk_x1 ®	Castello <i>et al.</i> , (2016)	Monomorphic
ycf 1	ycf1-1113f-br_(F) ycf1-2660r-br_(R)		Monomorphic
ycf 2	ycf1-2567f-br_(F) ycf1-4104r-br_(R)		
ycf 3	ycf1-3872f-br_(F) ycf1-5440r-br_(R)		
rpob-trnC- petN 1	rpob-3165f-br_(F) petn-5337r-br_(R)	Shaw <i>et al.</i> , 2014	
rpob-trnC- petN 2	rpob-3950f-br_(F) trnc-4127r-dp_(R)		

Appendix S1: sequel: Chloroplast regions tested and sequenced in *T. aeranthos*.

Region	Primers	Reference	Results in <i>T. aeranthos</i>
rpS16-trnQ	trnQ rpS16x1	Shaw <i>et al.</i> , 2014	Monomorphic
ndhA	ndhAx1 ndhAx2		
rpL32-ndhf	rpL32-R ndhF		
petA-psbj	psbJ petA		
ndhC-trnV	ndhC-f trnV-r		
rpL16 Intron	F71 R1661	Unpublished	Monomorphic
P000	P000 F P000 R		
P004	P004 F P004 R		

Appendix S2 Pairwise F_{ST} values between populations, values in bold are significant ($p < 0.05$). Localities control numbers are listed on Table 1.

Localities	1	2	3	4	5	6	7	8	9	10	11	12
1												
2	-0.01											
3	-0.02	0.00										
4	-0.01	0.01	-0.01									
5	0.01	0.01	0.01	0.04								
6	0.01	0.02	0.00	0.02	0.00							
				-								
7	0.00	0.01	-0.04	0.02	0.01	0.00						
8	0.02	0.03	0.03	0.05	0.03	0.01	0.04					
				-								
9	-0.01	0.01	-0.01	0.01	0.03	0.04	0.01	0.06				
10	0.05	0.06	0.04	0.09	0.01	0.02	0.05	0.04	0.08			
11	0.07	0.09	0.08	0.12	0.04	0.05	0.09	0.02	0.11	0.00		
12	0.07	0.09	0.05	0.06	0.11	0.09	0.08	0.11	0.10	0.10	0.11	
							-					
13	-0.03	-0.03	-0.04	0.00	0.01	0.01	0.01	0.02	-0.01	0.04	0.07	0.07

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Conclusões gerais

Esta dissertação faz parte de um projeto mais amplo que engloba estudos que visam elucidar mecanismos de diversificação e especiação na família Bromeliaceae e que fornecerão modelos importantes para o entendimento da dinâmica evolutiva de plantas da América do Sul e em geral. O conjunto de dados apresentado neste trabalho pode contribuir para o melhor entendimento sobre relações existentes entre sistemas reprodutivos, diversidade genética e padrões filogeográficos em Bromeliaceae e nos Pampas. Para formulação de estratégias de conservação do bioma pampeiro, o acúmulo de conhecimento sobre a história evolutiva de sua biota é essencial. A abordagem deste estudo avaliando sistema reprodutivo e genética de populações auxiliará no esclarecimento de aspectos da história evolutiva do gênero *Tillandsia* e da dinâmica de populações de *Tillandsia aeranthos* (Lois.) L.B. Smith na região dos Pampas.

O organismo modelo deste estudo, *Tillandsia aeranthos*, foi escolhido devido à observação em campo de suas grandes populações de floração sincronizada e muito conspicua ocorrendo mesmo de áreas muito urbanizadas ao longo do estado do Rio Grande do Sul. Dentro da mesma população pode-se observar variações morfológicas como cor de pétalas e brácteas, comprimento e cor de folhas e caulescência. Tais variações poderiam decorrer de plasticidade fenotípica em resposta a fatores ambientais dada a natureza altamente plástica de algumas *Tillandsia* (Haslam *et al.* 2003) e/ou da grande variabilidade genética na espécie, iniciando-se assim a formulação de perguntas e hipóteses sobre sua dinâmica evolutiva ao longo da ampla área de ocorrência.

Apesar de não haver marcadores microsatélites desenvolvidos especificamente para *Tillandsia aeranthos*, muitos destes marcadores foram desenvolvidos para outras espécies de bromeliáceas nos últimos anos (Sarhou *et al.* 2003, Boneh *et al.* 2003, Barbará *et al.* 2007, Palma-Silva *et al.* 2007, Kinsuat & Kumar 2007, Paggi *et al.* 2008, Zanella *et al.* 2012, Wöhrmann *et al.* 2012 e 2013, Aoki-Gonçalves *et al.* 2014, Neri *et al.* 2015). A amplificação heteróloga de microsatélites em bromélias possui vários exemplos de sucesso (Palma-Silva *et al.* 2007, Paggi *et al.* 2008, Zanella *et al.* 2012, Aoki-Gonçalves *et al.* 2014, Neri *et al.* 2017, Ferreira *et al.* 2017) e por esse motivo foram elaborados os experimentos de amplificação cruzada que constituíram o primeiro capítulo desta dissertação. Um conjunto de sete regiões de microsatélites nucleares apresentou amplificação e níveis de polimorfismo satisfatórios nas duas populações analisadas de *T. aeranthos*. Cálculos iniciais de fatores de diversidade genética e estrutura populacional foram realizados e os resultados mostraram uma boa resolução da informação genética provida pelos marcadores.

No capítulo 2 foi realizado um estudo dos padrões de diversidade nuclear e plastidial em *Tillandsia aeranthos* ao longo de sua distribuição na região dos Pampas. Um total de 19 localidades foram visitadas no Brasil e Argentina. Em algumas localidades na Argentina apesar de registros de herbário indicarem a presença da espécie, nós não encontramos nenhum indivíduo de *Tillandsia aeranthos* e sim indivíduos de outras espécies, como por exemplo (listas as outras que foram encontradas). O que nos faz pensar que nos registros anteriores de ocorrência da espécie podem ter ocorrido erros de identificação. Plantas foram amostradas vivas em sua maioria, retirando-se uma roseta de uma colônia saudável em locais de menor influência antrópica possível e com uma distância mínima de 15 metros entre cada

indivíduo. Na maioria das localidades nas quais *T. aeranthos* foi encontrada, as populações eram grandes e se mostraram densas e aparentemente saudáveis. Apesar da magnitude da área estudada (amplitude: 608km Norte-Sul e ~828km Leste-Oeste) não foram encontrados indícios de estruturação genética ou correlação entre variabilidade genética e localização geográfica nas localidades amostradas (STRUCTURE, AMOVA, PCoA, isolamento por distância). Altos níveis de diversidade gênica nuclear contrastaram com diferenciação haplotípica indetectável no sequenciamento de 13 regiões universais do cloroplasto de alto polimorfismo entre bromeliáceas e monocotiledôneas (Shaw *et al.* 2005 e 2007, Barfuss *et al.* 2005, Castello *et al.* 2016) mas de acordo com as baixas taxas evolutivas para o genoma plastidial já observado para a Bromeliaceae, em especial em espécies da sub-família Tillandsioideae.

Os estudos prévios envolvendo plantas dos Pampas apresentam padrões filogeográficos distintos ao encontrado neste trabalho, com altos níveis de diferenciação haplotípica e correlação estrita entre haplótipos e localização geográfica das populações (Fregonezi *et al.* 2012, Mäder *et al.* 2013, Longo *et al.* 2014). Notavelmente, *Calibrachoa heterophylla* apresenta auto-incompatibilidade polínica assim como *Tillandsia aeranthos* (Tsukamoto *et al.* 2002), evidenciando que as diferenças nas distribuições de variabilidade genética entre estas espécies podem estar relacionadas a outros fatores.

Dentre os padrões filogeográficos evidenciados até então para organismos dos Pampas, o que mais se assemelha ao encontrado em *Tillandsia aeranthos* é o de um anuro (*Pseudopaludicola falcipes* – Langone *et al.* 2016) que apresentou altos valores de diversidade genética dentro das populações e pouca estruturação entre elas. O trabalho faz análises demográficas detalhadas e infere que grandes tamanhos efetivos

populacionais e hidrocoria fluvial garantiram fluxo gênico intenso na espécie que manteve baixos níveis de estruturação genética.

Matas ciliares são ambientes de elevada conectividade (Hampe, 2004), que associada às sementes anemocóricas de *T. aeranthos* e sua grande densidade populacional são potencialmente fatores chave na manutenção do fluxo gênico e consequente baixa diferenciação entre populações da espécie. Estudos demográficos mais aprofundados são necessários para determinar o número efetivo populacional de *T. aeranthos*, assim como o número de migrantes por geração entre as populações. Além disso, uma amostragem mais ampla em territórios argentinos e uruguaios garantiria uma melhor cobertura da área de ocorrência total de *T. aeranthos*.

A diversidade de padrões filogeográficas encontrada em clados dos Pampas (González *et al.* 1998, Johnson *et al.* 1999, Speranza *et al.* 2007, Ojeda 2010, Fregonezi *et al.* 2012, Mapelli *et al.* 2012, Mäder *et al.* 2013, Longo *et al.* 2014, Felappi *et al.* 2015, Langone *et al.* 2015, Mapelli *et al.* 2016, Ramos-Fregonezi *et al.* 2017) demonstra o quão complexa uma paisagem aparentemente homogênea de campos dominados por gramíneas pode ser. O bioma é considerado vulnerável, com atividades antrópicas promovendo rápida erosão e empobrecimento do solo em áreas de difícil recuperação posterior (Krapovickas & Giacomo, 1998), reforçando a necessidade de mais trabalhos que visem elucidar os processos evolutivos responsáveis pela sua diversidade atual. Assim, esforços mais eficientes podem ser tomados para a conservação da biota local.

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