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DIEGO SOTERO DE BARROS PINANGÉ

Filogenia molecular e estudos populacionais no gênero *Dyckia* Schult. & Schult.f. (Bromeliaceae)

RECIFE
AGOSTO/2013

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Tese apresentada ao curso de Doutorado do Programa de Pós Graduação em Ciências Biológicas da Universidade Federal de Pernambuco, como parte dos requisitos obrigatórios para obtenção do título de Doutor em Ciências Biológicas, na área de concentração Biotecnologia/Biologia Celular e Molecular.

Orientador: Profa. Dra. Ana Maria Benko-Iseppon

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Aprovada em 30/08/2013

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Resumo

O gênero *Dyckia* Schult & Schult.f. (Pitcairnioideae, Bromeliaceae) compreende aproximadamente 150 espécies, de natureza xerofítica/rupícola, e caracteriza-se por apresentar uma considerável variação morfológica e ocorrência de micro-endemismos, com cerca de 80% de sua ocorrência no território brasileiro. Os estudos filogenéticos (morfológicos e moleculares) prévios apontaram o monofiletismo no grupo, todavia, com relações inter-específicas ainda não esclarecidas. Assim, a reconstrução filogenética em 101 espécimes (ca. 60 spp.) do gênero foram conduzidos, a partir da geração dos marcadores do tipo AFLP (*Amplified Fragments Lenght Polymorphism*). Quanto às análises populacionais, com o objetivo de revelar os primeiros insights nas populações de Dyckia nos brejos pernambucanos, foram desenvolvidos *primers* de microssatélites e aplicados em quatro populações de *Dyckia pernambucana* e uma de *D. limae* ocorrentes nos Brejos de Altitude do Planalto da Borborema (Pernambuco, Brasil), bem como, três populações de *D. dissitiflora,* localizadas na região da Chapada Diamantina, totalizando 87 indivíduos. Assim, dados acerca da variabilidade genética e padrões de fluxo gênico, foram obtidas, mediante uso dos marcadores de microssatélites (nucleares e plastidiais) e AFLP. As inferências filogenéticas confirmaram o monofiletismo do grupo, com topologia apresentando elevado suporte na maioria dos nós terminais do dendrogama. Adicionalmente, os dados revelaram a estreita relação de *Dyckia* com o grupo-irmão *Encholirium,* no entanto, os índices de consistência e de retenção (CI e RI) revelaram níveis significantes de homoplasia, onde politomias e baixa resolução foram observados, principalmente nos nós mais basais. No entanto o padrão genético/geográfico encontrado corroborou as hipóteses, de irradiação recente, no grupo. A associação dos marcadores revelaram níveis de variabilidade consideráveis para as populações de Pernambuco, provavelmente modeladas por deriva genética, apresentando acentuada riqueza alélica e haplotípica, quando comparadas a outros trabalhos populacionais em Bromeliaceae. Os dados de diferenciação genética revelaram estruturação populacional acentuada, com definida separação entre as populações de *D. dissitiflora* e do Grupo Pernambuco. Adicionalmente, os dados moleculares em conjunto com os morfológicos não puderam estabelecer a separação de *D. limae* das demais populações de *D. pernambucana,* sugerindo que este grupo Pernambuco é composto por apenas uma espécie.

Palavras-chave: Pticairnioideae, Floresta Atlântica, Planalto da Borborema, variabilidade genética, evolução, marcadores moleculares.

Abstract

The xeromorphic genus *Dyckia* Schult.f. (Pticairnioideae subfamily, Bromeliaceae) currently comprises about 150 species, exhibiting an enormous morphological variability and occurrence of micro-endemisms with about 80% of their occurrence in the Brazilian territory. Previous phylogenetic studies (morphological and molecular) have reported the monophyly of this group, nevertheless, with still unclear interspecific relationships. Thus, the phylogenetic reconstruction, in 101 specimens (ca. 60 spp.), in Dyckia was performed from the amplification of AFLP (Amplified Fragments Lenght Polymorphism) marks. As for population analysis microsatellite primers were developed and apllied in one and four populations of *D. limae* and *D. pernambucana* respectively, occurring in the "*Brejos de Altitude*" of the Borborema Plateau (Pernambuco, Brazil), in order to reveal the first insights in populations of *Dyckia* in Pernambuco State. Also, populations of *D. dissitiflora* (considered the closest species of the Pernambuco Group) located in the "*Chapada Diamantina*", were collected, with a total of 87 individuals. In order to understand the patterns of gene flow and the genetic variability among populations, both microsatellite loci (nuclear and plastidial) and AFLP markers were employed. The phylogenetic inference has confirmed the monophyly in the genus with high supported topology at terminal nodes of the tree, as well as, a clear genetic/geographic pattern. Additionally, the data has revealed a close relationship with the genus *Encholirium*, corroborating previous studies. On the other hand the significant levels of homoplasy and politomies, especially at basal nodes, were achieved, in the present work. The association of these markers revealed considerable levels of variability in the Pernambuco populations, probably modeled by genetic drift, presenting accentuated allelic and haplotypic richness when compared to other population's studies in Bromeliaceae. In relation to the genetic differentiation data, the combined informations of Bayesian clustering, phenetics and inbreeding indices displayed strong population structure with a defined separation between populations of *D. dissitiflora* and the Pernambuco Group. Moreover, the molecular data together with morphological aspects could not establish the clear separation of *D. limae* from other populations of *D. pernambucana*, suggesting that the Pernambuco Group, possibly, consists of a single species.

Keywords: Pticairnioideae, Atlantic Rainforest, Borborema Plateau, genetic variability, evolution, molecular markers.

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Sumário

1 Introdução Geral

O gênero *Dyckia* Schult.f. pertence à subfamília Pitcairnioideae (Bromeliaceae) e compreende aproximadamente 150 espécies, destacando-se por exibir uma considerável plasticidade morfológica, que, sob o ponto de vista taxonômico, dificulta a delimitação das espécies descritas (LEME & KOLLMANN, 2011; LEME *et al*., 2012). São plantas predominantemente rupícolas e terrícolas, de natureza xerofítica, podendo ser encontradas no Brasil nos domínios do Cerrado, da Caatinga e da Floresta Atlântica (SMITH & DOWNS, 1974; FORZZA, 2001). Algumas espécies são consideradas reofíticas, desempenhando um papel ecológico relevante a partir do acúmulo de matéria orgânica e favorecendo o estabelecimento de outras espécies vegetais e animais (ROGALSKI *et al*., 2007). Estudos cladísticos apontam tanto o monofiletismo do grupo, dentro de Pticairnioideae, como a estreita relação com o gênero irmão *Encholirium*, legitimando também as análises morfológicas existentes (FORZZA, 2001; GIVNISH *et al.*, 2011; KRAPP, 2013a). Adicionalmente, de acordo com Krapp (2013a), *Dyckia* e *Encholirium* compreendem grupos de diversificação e irradiação recentes com prováveis ocorrências significativas de hibridização e introgressões, sugerindo a necessidade de maiores estudos (tanto em níveis inter quanto intra-específicos), no intuito de oferecer mais informações acerca do histórico de colonização do grupo, tanto sob ponto de vista macro, como micro evolutivos.

Desta forma, a diversidade filogenética configura-se como um índice da biodiversidade que mensura o tamanho dos caminhos evolutivos que conectam os táxons (FAITH, 1992; VANE-WRIGHT *et al*., 1991), tendo em vista que a aplicação de estudos filogenéticos é de grande utilidade para a identificação de regiões ambientais chave, tanto para a continuidade da evolução da vida sobre a terra como em benefício da sociedade (FOREST *et al*, 2007). Com base nisso, o potencial evolutivo de determinada espécie pode ser relacionado a seus índices de variabilidade genética. Trata-se, por conseguinte, da matéria-prima que modula as taxas de extinção e diversificação dos organismos (FLEISHMAN *et al*., 2001; JONES *et al*., 2001; MAMURIS *et al.*, 2001; PITHER *et al.*, 2003).

O acesso à diversidade genética via marcadores moleculares de DNA, associados a sofisticadas ferramentas analíticas, têm colaborado significativamente para o entendimento dos processos históricos (bióticos e abióticos) que modelam a diversificação intra e interespecífica da biota (TEMPLETON 1998; AVISE 1994, 2000; POSADA & CRANDALL, 2001; ALI *et al.*, 2004). Dentre os marcadores moleculares amplamente utilizados em

vegetais, o AFLP tem demonstrado eficiência tanto nos estudos de filogenia molecular quanto em estudos populacionais. Em Bromeliaceae, tais marcadores têm revelado quais foram os principais fatores que modelaram a história e adaptação das populações de bromélias, tais como restrição de fluxo gênico e endogamia, em determinados grupos, bem como evidências de hibridização e introgressão (REX *et al.*, 2007; SHULTE *et al.*, 2010). Do mesmo modo, marcadores de microssatélites têm demonstrado relevância inquestionável nos estudos populacionais e filogeográficos (FALEIRO, 2007). A maioria dos trabalhos disponíveis sobre diversidade genética em bromélias utilizou tais marcadores como ferramenta analítica, permitindo inferências sobre padrões de fluxo gênico, níveis de estruturação e identificação de tendências quanto ao histórico de expansão e irradiação adaptativa das populações estudadas (BARBARÁ *et al*., 2007, 2008a, 2008b, PALMA-SILVA *et al*., 2009, 2011; ZANELLA *et al*., 2012).

O presente estudo teve como objetivo obter novas informações que permitam a reconstrução das relações filogenéticas dentro do gênero *Dyckia*, baseando-se em marcas do tipo AFLP, bem como dados referentes à diversidade e estrutura genética das populações, através da associação de marcadores dominantes (AFLP) e codominantes (microssatélites), de espécies ocorrentes nos denominados brejos nordestinos. De posse destas ferramentas moleculares, pretende-se lançar luz às relações filogenéticas e à diversidade do grupo nos níveis infragenérico, inter e intra-populacional.

2 Revisão bibliográfica

2.1 A Família Bromeliaceae Juss.

Os Neotrópicos apresentam-se como um cenário único no mundo por possuir uma fauna e flora extremamente diversa, modulados por processos evolutivos singulares, com elevados níveis de endemismo, com aproximadamente 100.000 espécies de angiospermas estimadas (GOVAERTS, 2001; KIER *et al*., 2009). Nesse contexto, a família Bromeliaceae (com cerca de 3.200 espécies distribuídas em 58 gêneros), destaca-se por ser um dos grupos mais diversos, com ampla variabilidade morfológica, fisiológica e por sua adaptabilidade ecológica, apresentando diferentes hábitos (litofítico, terrícola, epifítico etc.) dentre as plantas vasculares neotropicais. Tal plasticidade tem permitido a colonização de diferentes habitats,

desde florestas tropicais úmidas a regiões semidesérticas (BENZING, 2000; LUTHER, 2008; GIVNISH *et al*., 2011).

Bromeliaceae foi classicamente dividida em três subfamílias: Bromelioideae, Pitcairnioideae e Tillandsioideae, de acordo com Harms (1930) e subsequentemente por Smith & Downs (1974, 1977, 1979). No entanto, a partir dos estudos de filogenia molecular – com sequências oriundas de oito regiões plastidiais – realizados por Givnish *et al.* (2011), a família foi subdividida em oito subfamílias: Brocchinioideae, Lindmanioideae, Tillandsioideae, Hechtioideae, Navioideae, Pitcairnioideae, Puyoideae e Bromelioideae, conforme ilustrado na Figura 1.

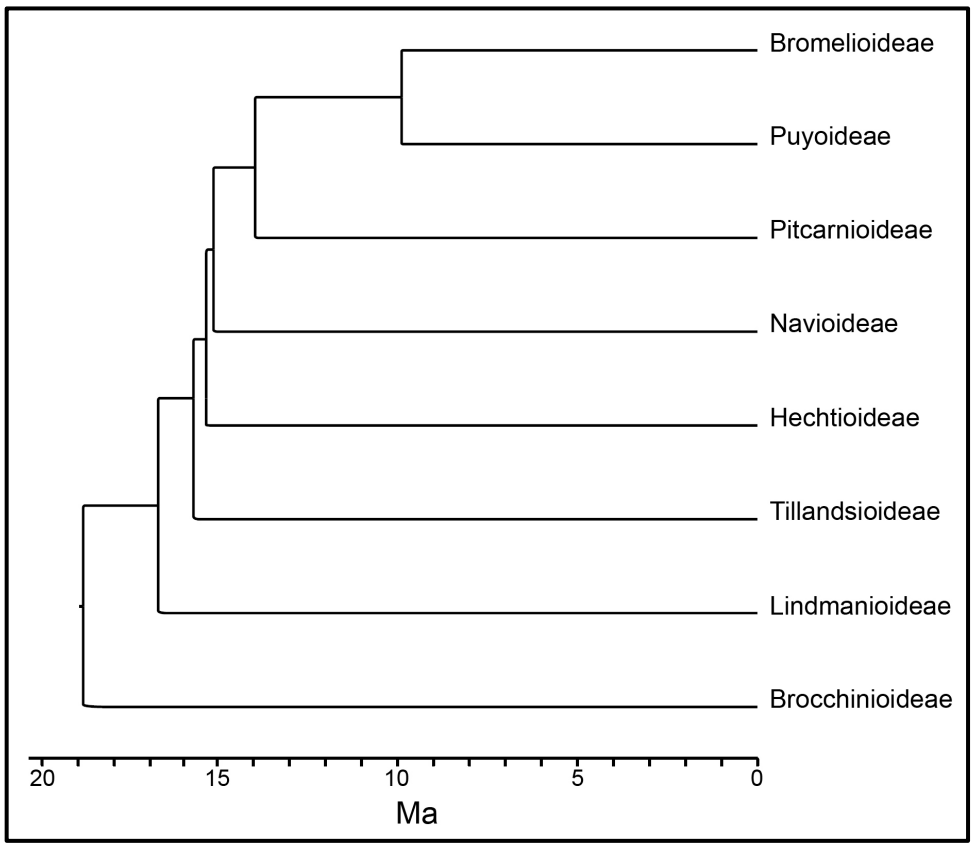

Figura 1: Relações filogenéticas e datação dos ramos das oito subfamílias em Bromeliaceeae. Baseado em Givnish *et al.* (2011).

A distribuição geográfica da família é quase que exclusivamente Neotropical, excetuando-se apenas a espécie *Pitcairnia feliciana* (A. Chev.) Harms & Mildbr., a qual ocorre na costa oeste da África (JACQUES-FELIX, 2000; GIVNISH *et al.,* 2004). Como limite setentrional, a ocorrência compreende os estados da Virgínia, Texas e Califórnia, no sul

dos Estados Unidos, e como limite meridional o norte da Patagônia, na Argentina (LEME & MARIGO, 1993). As bromélias provavelmente tiveram como centro de irradiação ancestral o norte da América do Sul, no Planalto das Guianas, ambiente que é geologicamente antigo e estável, onde sua diversificação massiva iniciou-se durante o Mioceno (~19 Ma) (BENZING, 2000; GIVNISH *et al*., 2004; BARFUSS *et al.,* 2005; GIVNISH *et al*., 2011).

A família caracteriza-se, primordialmente, por apresentar folhas em roseta, que frequentemente represam água ("hábito tanque" ou fitotelma), a via fotossintética do tipo CAM e presença de tricomas epidérmicos absortivos, características estas consideradas inovações-chave que permitiram a vasta diversificação adaptativa já comentada (CRAYN *et al*., 2004; GIVNISH *et al*., 2007; SCHULTE *et al*., 2009). Devido a sua formação fitotelma, as bromélias desempenham um papel relevante na formação da complexidade do habitat. Tal formação permite várias interações, com diversos grupos animais e vegetais, tais como: diversas espécies de insetos, anfíbios e répteis, formando micro-habitats e fonte alimentícia significativa para espécies de aves e morcegos, decorrente da retenção de grande quantidade de material orgânico, desta forma, caracterizando os componentes deste grupo como verdadeiros amplificadores de biodiversidade (BENZING 2000; VERSIEUX *et al*., 2008).

Em Bromeliaceae, duas estratégias reprodutivas são comumente encontradas: via produção de sementes (normalmente envolvendo a fusão de gametas), tanto por fecundação cruzada quanto por autofecundação, e via propagação clonal (BENZING, 2000; IZQUIERDO & PIÑERO, 2000; SAMPAIO *et al*., 2002; SGORBATI *et al*., 2004; PAGGI *et al*., 2007; PALMA-SILVA *et al.*, 2008; MATALLANA *et al*., 2010). A diversidade genética registrada dentro das populações pode ser considerada alta, se comparada a outras espécies que apresentam exclusivamente reprodução sexuada (SARTHOU *et al*., 2001; CAVALLARI *et al*., 2006, PALMA-SILVA *et al*., 2009; 2011). Adicionalmente, dada as características florais especializadas encontradas no grupo, tais como: a hercogamia - quando há uma barreira física separando os filetes curtos e estiletes longos, assim como a dicogamia – amadurecimento dos órgãos reprodutores em épocas diferentes - mecanismos reprodutivos diferenciados têm sido relatados, contribuindo para a manutenção da alta diversidade genética nas populações naturais (BENZING, 2000; WENDT *et al.*, 2002; CASCATE-MARIN *et al*., 2006; MATALLANA *et al*., 2010).

Quanto aos tipos de polinização, Bromeliaceae distingue-se por ser uma das poucas famílias onde a presença de vertebrados como visitantes predomina sobre a entomofilia (SAZIMA *et al.*, 2000). Além disso, dentre os grupos vegetais existentes, esta família ocupa

um papel de destaque uma vez que apresenta um número significativo de polinizadores ou combinação destes: ornitofilia, quiropterofilia, entomofilia, polinizador não específico e/ou associação entre os tipos e autogamia (KESSLER & KRÖMER, 2000; WENDT *et al*., 2008; SCHIMDT *et al.*, 2010). Grande parte do sucesso atrativo deste grupo deve-se às características particulares de sua composição floral, com as mais variadas formas, cores e aromas, assim como elevadas concentrações de açúcar no néctar (BENZING, 2000; KRÖMER *et al*., 2008).

2.2 O Gênero *Dyckia* Schult.f.

Dyckia é o segundo maior gênero da subfamília Pitcairnioideae, apresentando cerca de 150 espécies com elevada plasticidade morfológica, sendo superada somente pelo gênero *Pticairnia* (ca. 340 spp.) (LEME & KOLLMANN, 2011; LEME *et al.* 2012; KRAPP, 2013b). Sua distribuição compreende todo o sudeste da América do Sul, com registros de ocorrência na Argentina, Brasil, Bolívia, Paraguai e Uruguai (Figura 2). Aproximadamente 80% das espécies descritas ocorrem em território brasileiro, sendo as regiões central e sudeste consideradas os seus centros de dispersão, nos Campos Rupestres do Cerrado (SMITH & DOWNS, 1974; FORZZA, 2001).

Morfologicamente, *Dyckia* apresenta uma ampla diversidade fenotípica (Figura 3), sendo caracterizada majoritariamente por não formar o hábito "tanque central", formando uma roseta com folhas usualmente coriáceas e suculentas além da presença de acúleos marginais geralmente bem desenvolvidos. As inflorescências, sempre laterais, podem ser simples ou ramificadas, com pigmentação floral variando entre amarelo, laranja ou avermelhado, embora alguns registros para os tipos esverdeados e castanho-vináceos tenham sido reportados. Os estames são geralmente inclusos com filamentos espessos, frequentemente recurvados, de livre a extremamente conados na base (assim como as pétalas) acima do tubo comum (FORZZA, 2001; LEME *et al.*, 2012). Segundo Varadarajan & Brown (1988), os estigmas são classificados como espiral-conduplicado e os grãos de pólen são sulcados com exinas geralmente microrreticuladas. As sementes são aladas, tratando-se de uma sinapormofia da subfamília como um todo, essencialmente dispersas pelo vento, podendo ser classificadas em dois tipos (I e II), sendo o primeiro essencialmente classificado como longo-ovalado, não achatado de aspecto triangular e o segundo ovalado, achatado e discoide (STREHL & BEHEREGARAY, 2006).

Figura 2: Mapa de distribuição geográfica no gênero *Dyckia* (adaptado de Givnish *et al*., 2011).

Como estratégias reprodutivas o grupo exibe o tipo sexuado ou opcionalmente propagação vegetativa por estolões (LUTHER, 2006, ROGALSKI *et al.,* 2007, 2009). As espécies de *Dyckia* são de natureza predominantemente xerofítica, podendo ser terrícolas ou rupícolas, onde a ocorrência deste último tipo se dá em diversos tipos de afloramentos rochosos (graníticos, quartzíticos, sedimentares e afloramentos de rochas ferruginosas – denominados "cangas"). Assim sendo, são comumente encontrados em ambientes secos, com forte exposição solar, do Domínio Mata Atlântica, Cerrado e Caatinga (LEME *et al*., 2012). Possuem um sistema radicular bastante desenvolvido, cumprindo funções de absorção de água e sais minerais, bem como de fixação da planta no substrato (PITTENDRIGH, 1948).

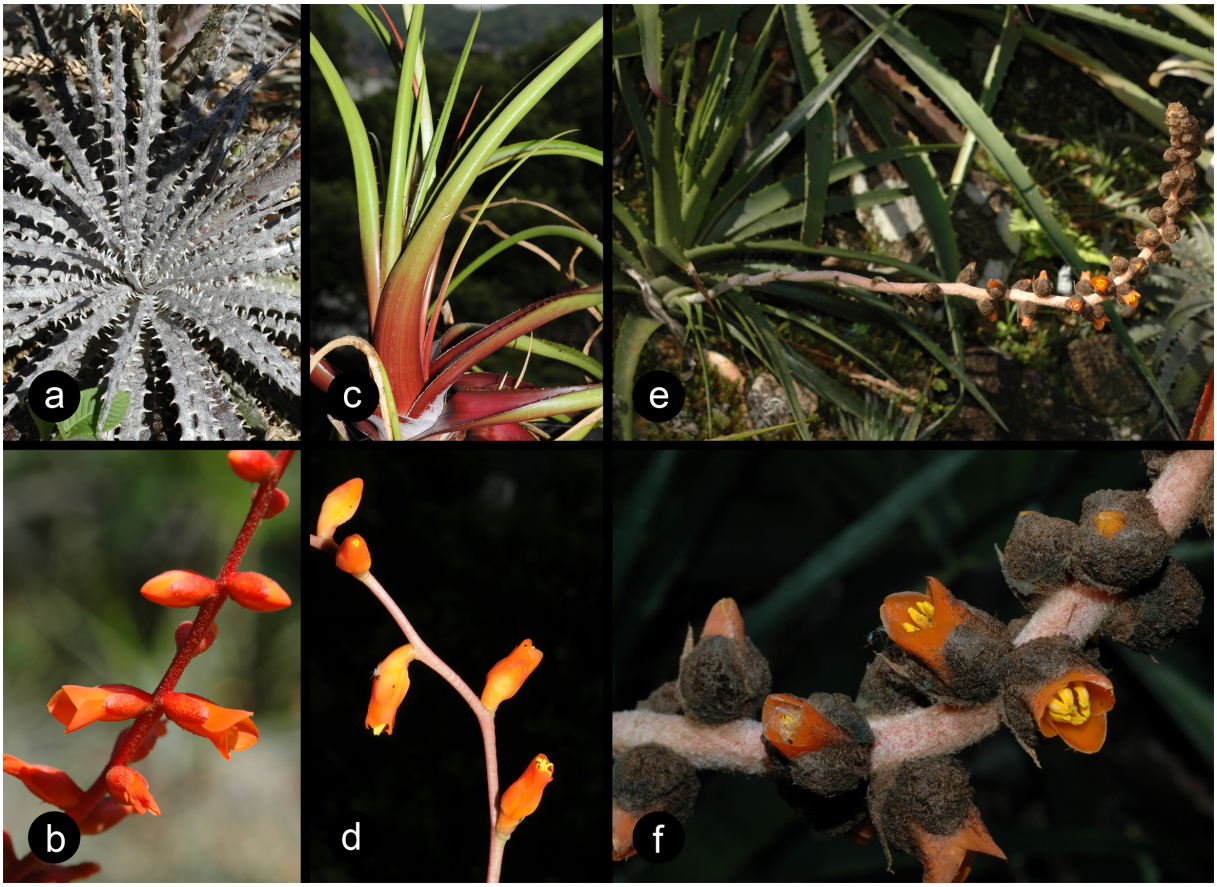

Figura 3: Hábito e detalhe das inflorescências de representantes do gênero *Dyckia,* exibindo sua diversidade morfológica. (a) *e* (b): *Dyckia fosteriana* L.B.Sm., (c) e (d): *Dyckia parensis* Leme e (e) *e* (f): *Dyckia ursina* L.B.Sm. Fotos gentilmente cedidas por Elton Leme.

Algumas espécies deste gênero são consideradas reofíticas, tais como *Dyckia brevifolia* Baker e *Dyckia distachya* Hassler, sendo caracterizadas por possuírem distribuição associada a ambientes mais úmidos ou que periodicamente inundam, como por exemplo, os leitos dos rios nos estados de Santa Catarina e do Rio Grande do Sul (VAN STEENIS, 1981; STREHL, 1994). Deste modo, as características adaptativas registradas apresentam estreita relação ao tipo de ambiente, formando grupos densos que acumulam grandes quantidades de matéria orgânica, massiva produção de sementes que são dispersas pelas correntezas dos rios, germinando sob condições de extrema umidade e/ou inundações (WIESBAUER *et al*., 2007).

No que diz respeito aos tipos de polinização no grupo, os recursos florais associados a estratégias adaptativas quanto à reprodução sugerem beija-flores como polinizadores, embora, várias espécies de insetos (abelhas e borboletas) também têm sido documentadas como polinizadores para *Dyckia* (VARADARAJAN & BROWN, 1988; BERNARDELLO *et al*., 1991; VOSGUERITCHIAN & BUZATO, 2006; ROGALSKI *et al*., 2007).

As primeiras análises filogenéticas no grupo, baseando-se em caracteres morfológicos, apontaram o monofiletismo do grupo, o qual é suportado por quatro sinapomorfias: inflorescência axial, brácteas do pedúnculo diferente das folhas, presença de nectários nas sépalas e anel pétalo-estaminal, características estas que distinguem *Dyckia* do gênero irmão *Encholirium*, bem como dos demais grupos de Pitcairnioideae (FORZZA, 2001). Posteriormente, a partir do uso dos marcadores moleculares na reconstrução das relações filogenéticas da família conduzidas por Givnish *et al.*, (2011), confirmou-se o referido gênero como monofilético. Dados moleculares recentes dentro do grupo apontam *Dyckia* como um grupo de diversificação recente, onde o ancestral comum de *Dyckia* e *Encholirium* provavelmente tenha ocorrido no leste do Brasil antes das diferenciações de linhagens dos gêneros citados (KRAPP, 2013a).

2.2.1 *Dyckia limae* L.B.Sm

Esta espécie (Figura 4) foi descrita por Lyman Smith em 1970 [Phytologia 20(3): 179, pl 2.] e caracteriza-se por ser uma planta anual de hábito rupícola, podendo ser encontrados desde poucos indivíduos até a formação de verdadeiros agrupamentos (touceiras). Sob o aspecto morfológico, os indivíduos são caracterizados por exibirem folhas verdes rígidas extremamente coriáceas, suculentas, estreitamente triangulares e unilateralmente recurvadas, com tricomas de densamente a grosseiramente branco-lepidotos e acúleos parcialmente retrorsos. Sua inflorescência é ereta usualmente simples a composta na base com flores alaranjadas sub-eretas a secundas, laxamente arranjadas em número de 20, no ramo terminal (SIQUEIRA-FILHO & LEME, 2006). Os espécimes são endêmicos dos Inselbergs sedimentares da Serra de Jerusalém (localidade Tipo), Chapada de São José, Serra das Torres e Serra Branca, localizados no Parque Nacional Vale do Catimbau (Buíque – PE) (SIQUEIRA-FILHO & LEME, 2006). De acordo com Machado & Lopes (2004) e Santos (2005) os indivíduos de *D. limae* são polinizados por duas espécies de beija-flores: *Chlorostilbon aureoventris* e *Chrysolampis mosquitos* com floração dos indivíduos no período seco de setembro a dezembro.

Figura 4: *Dyckia limae*, localidade Serra de Jerusalém, PARNA Vale do Catimbau, Buíque, Pernambuco (Foto do autor).

2.2.2 *Dyckia pernambucana* L.B.Sm

Esta espécie (Figura 5), também descrita por Lyman Smith na mesma publicação em 1970, é endêmica dos Inselbergs dos Brejos de Altitude do Estado de Pernambuco, apresentando hábito rupícola e quando florida, pode atingir um metro de altura. Suas folhas arqueadas exibem coloração escura de esverdeado a vináceo, sendo fortemente coriáceas e suculentas com bainhas sub-ereniformes, castanho-escuras em direção à base e acúleos antrorsos. Inflorescência sub-ereta simples a distintamente composta na base, com flores alaranjadas sub-eretas a levemente secundas, com cerca de 40, em número, no ramo terminal (SIQUEIRA-FILHO & LEME, 2006).

Notadamente esta espécie possui uma estreita relação morfológica com *D. limae*, o que diferencia esta última de *D. pernambucana* são algumas nuances que levantam dúvidas quanto à delimitação das mesmas, como: porte da planta, disposição dos acúleos, encurvamento das folhas e tamanho das inflorescências (SIQUEIRA-FILHO & LEME, 2006). Além disso, *D. pernambucana* exibe uma maior distribuição geográfica, onde populações podem ser encontradas em diversos Inselbergs das zonas de transição entre Caatinga e Floresta Atlântica (Brejos de Altitude) de Pernambuco. De acordo com Veloso *et al*. (1991) estas conformações vegetacionais ocupam as áreas mais elevadas do Planalto da Borborema

(geralmente acima de 600 m), sendo também denominadas florestas montanas. O surgimento dos Brejos em pleno semiárido dá-se especialmente em função da altitude supracitada, bem como das características intrínsecas do relevo, criando uma situação particular denominada "precipitação oculta", propiciando uma intensa condensação noturna, especialmente nos meses mais frios (ANDRADE & LINS, 1965; ANDRADE-LIMA, 1981).

Fenologicamente, esta espécie é classificada como sub-anual, onde há sobreposição de frutificação e floração, as quais ocorrem mais de uma vez ao ano (entre os meses de janeiro e agosto). Ademais, segundo Siqueira-Filho & Leme (2006) e A. M. Wanderley (informação pessoal, agosto, 2011), este táxon apresenta a ornitofilia (beija-flores) como síndrome de polinização, do mesmo modo como registrado para *D. limae*.

Figura 5: *Dyckia pernambucana*, Fazenda Bitury, Brejo da Madre de Deus, Pernambuco. Foto do autor.

2.3 Análises Filogenéticas na Família Bromeliaceae

Originalmente, Bromeliaceae era apontada como único grupo dentro da ordem Bromeliales, condição esta decorrente das características morfológicas intrínsecas do grupo (CRONQUIST, 1981). No entanto, de acordo com estudos subsequentes que incluíram análises moleculares às investigações sistemáticas nas Angiospermas, (APG, 1998; APG II,

2003 APG III, 2009) além de estudos mais específicos dentro do grupo, levaram ao reposicionamento de Bromeliaceae na ordem Poales, ratificando também seu monofiletismo (GILMARTIN & BROWN, 1987; CRAYN *et al.*, 2004; JANSSEN & BREMER, 2004; GIVNISH *et al*., 2007, 2010, 2011).

A classificação dos gêneros dentro da família configurou-se como um desafio para os taxonomistas do grupo por muito tempo. Como citado anteriormente, a clássica divisão da família em três subfamílias, baseada fundamentalmente em caracteres morfológicos como sementes, fruto e posição do ovário (SMITH & DOWNS, 1974, 1977, 1979; SMITH & TILL, 1998), foi indicada como uma classificação artificial devido à constatação de altos índices de homoplasias e plesiomorfias nos diversos estudos morfológicos que foram conduzidos, especialmente dentro de Pticairnioideae (VARADARAJAN & BROWN, 1988; VARADARAJAN & GILMARTIN, 1988a; VARADARAJAN & GILMARTIN, 1988b; BROWN & TERRY, 1992; GRANT, 1993b; BROWN & LEME, 2005).

Desta forma, os estudos moleculares recentes auxiliaram na resolução das incongruências existentes nas análises morfológicas. Os primeiros estudos em Bromeliaceae utilizaram regiões de DNA plastidial para inferir quanto às relações filogenéticas dentro das bromélias (RANKER *et al*., 1990; CLARK *et al.*, 1993). Os resultados confirmaram a origem monofilética, embora as relações entre as três subfamílias não puderam ser claramente resolvidas devido à limitação amostral. Assim, duas análises posteriores publicadas por Terry *et al*. (1997a, 1997b), nas quais foram utilizados marcadores plastidiais diferentes, focaram nas relações entre as subfamílias, onde, apesar da baixa resolução das árvores obtidas, o monofiletismo da subfamília Tillandsioideae foi confirmado, a passo que surgiram os primeiros questionamentos acerca do parafiletismo em Pticairnioideae.

Trabalhos subsequentes utilizando outras regiões plastidiais de evolução rápida – *trnL*, *matK* (HORRES *et al*., 2000; CRAYN *et al*., 2000, 2004) mostraram baixa divergência entre as linhagens de Bromeliaceae, além de também haver uma baixa representatividade amostral de acessos das subfamílias. Somente com a associação de diferentes regiões de DNA, assim como um esforço amostral focando as subfamílias Tillandsioideae (BARFUSS *et al*., 2005) e Bromelioideae (SCHULTE & ZIZKA, 2008), muito dos questionamentos remanescentes na determinação dos principais clados entre e dentro as subfamílias puderam ser esclarecidos.

No entanto, somente com a inclusão dos marcadores nucleares e, principalmente, a combinação de diferentes tipos de marcadores organelares e nucleares, bem como marcadores multilocus como o AFLP, as resoluções filogenéticas aumentaram consideravelmente

(SHULTE *et al.*, 2005, 2009; REX *et al.*, 2007, 2009; HORRES *et al*., 2007; SHULTE & ZIZKA, 2008; SHULTE *et al*., 2010). Com o acúmulo dos estudos e evidências levantadas pelos trabalhos citados, Givnish *et al.* (2007, 2011) propuseram a reorganização das subfamílias, subdividindo Pticairnioideae em seis grupamentos (ver Figura 1). Apesar dos notáveis avanços dos estudos filogenéticos no referido grupo, relações ainda não esclarecidas ainda se fazem presentes nas principais subfamílias, particularmente em gêneros que apresentam grande diversidade morfológica como *Aechmea*, *Vriesea* e *Dyckia*, por exemplo.

2.4 Marcadores Moleculares

Uma marca genética, essencialmente pode ser classificada: com base visualmente em características avaliáveis (morfológicas e agronômicas, por exemplo); aquelas baseadas em produtos gênicos (marcadores químicos) e as que se baseam em ensaios de DNA (marcadores moleculares). Os marcadores moleculares, desde sua descoberta e aplicação, tem exercido um papel fundamental em diversas áreas das ciências naturais, tais como: taxonomia, fisiologia, biologia das populações, ecologia comportamental, evolução de organismos e filogenia. Dentre as diversas características desejáveis na construção dos marcadores, a neutralidade em relação às mudanças ambientais configura-se como ponto de partida essencial no que diz respeito a ampla aplicação e sucesso das diferentes técnicas existentes (AVISE, 1994; SEMAGN *et al*., 2006; SHARMA *et al.*, 2008).

Segundo Semagn *et al.* (2006) os marcadores moleculares não devem ser considerados como genes, uma vez que os mesmos não possuem efeito biológico particular. Adicionalmente, sabe-se que há a tendência por parte dos genes e marcas de segregarem ligados em um cromossômo, seguindo regras básicas de herança, possibilitando o acesso irrestrito a estrutura genômica de determinada espécie e/ou grupo alvo, tendo em vista a gama de técnicas existentes (Tabela 1). Os primeiros registros, no que diz respeito à utilização dos conceitos referente aos marcadores, surgiram no começo do século passado (SAX, 1932; WEXELSEN, 1933), mas, somente com o desenvolvimento de técnicas moleculares, a evolução analítica neste campo das ciências biológicas se solidificou, intensificando a publicação de vários trabalhos. As diferentes técnicas podem ser classificadas fundamentalmente em três grupos principais: a) o modo de herança (dominante ou codominante); b) o modo de transmissão da característica (biparental, herança materna ou paterna e oriunda de organela ou nuclear) e c) o método de análise em si (baseada em PCR ou hibridização). Assim, os marcadores moleculares configuram-se como uma das mais eficazes

formas de mensurar os polimorfismos existentes nos diferentes níveis taxonômicos. Logo, tais técnicas possibilitaram avanços significativos, nos mais diversos campos da biologia, comumente utilizando a diversidade genética como matéria-prima (BOTSTEIN *et al*., 1980; LITT & LUTY, 1989; WILLIAMS *et al*., 1990; CAETANO-ANOLLES *et al*., 1991; JORDAN & HUMPHRIES, 1994; ZIETKIEWICZ *et al*., 1994; VOS *et al*., 1995; WEISING & GARDNER, 1999; BORNET & BRANCHARD, 2001).

Tabela 1: Comparação entre os marcadores moleculares mais utilizados em vegetais. Adaptado de Mueller & Wolfenbarger (1999) e Semagn *et al.* (2006)

	Microssatélites	**RAPD**	**AFLP**	**ISSR**
Abundância genômica	Média	Elevada	Elevada	Média
Quantidade de DNA requerida	Baixa	Média	Média	Baixa
Tipo de polimorfismo [a]	Mudanças no comprimento das repetições	*Indels* [b], mudanças únicas de base	*Indels,* mudanças únicas de base	*Indels,* mudanças únicas de base
Níveis de polimorfismos	Alta	Alta	Muito alta	Alta
Herança	Codominante	Dominante	Dominante	Dominante
Detecção de alelos	Sim	Não	Não	Não
Resolução das diferenças geneticas	Elevada	Moderada	Elevada	Moderada a elevada
Complexidade metodológica	Difícil	Fácil	Moderado a Difícil	Fácil
Reprodutibilidade	Elevada	Variável	Média a elevada	Média
Tipo de *primers*	Sequências únicas específicas	Sequência randômica de 10 pb	Região específica – enzimas de restrição	Sequência repetitiva específica

[a]Média de heterozigozidade como probabilidade de detecção de dois alelos escolhidos ao acaso;

[b]Inserções e deleções.

2.4.1 AFLP (*Amplified Fragment Length Polymorphism*)

A técnica do polimorfismo no comprimento de fragmentos amplificados (AFLP), classificada entre marcadores multilocos ou *fingerprinting* de DNA, caracteriza-se

primordialmente por possuir associação com sítios de restrição, onde o genoma-alvo é clivado em diversos fragmentos mediante a presença de enzimas de restrição. Logo, tal metodologia distingue-se por combinar a replicabilidade do RFLP (*Restricted Fragment Length Polymorphism*) com a flexibilidade das tecnologias baseadas em PCR. Consequentemente, diferenciações causadas por alterações em bases nos sítios de restrições ou deleções, inserções e rearranjos que alterem o comprimento dos fragmentos de restrição podem ser visualizadas (VOS *et al*., 1995; LYNCH & WALSH, 1998; LUO *et al.*, 2007).

O AFLP tem se destacado em estudos multidisciplinares, envolvendo diferentes subáreas da biologia, a partir do efetivo acesso à diversidade genética dos organismos. Deste modo, elucidações de questões evolutivas em diferentes grupos têm sido relatadas primordialmente utilizando métodos de distância e análises heurísticas de parcimônia (KIERS *et al*., 2000; HODINKSON *et al.*, 2002; PELSER *et al*., 2003; DESPRES *et al*., 2003; KOOPMAN *et al*., 2005). Contudo, este marcador tem sido mencionado como sendo mais adequado em menores níveis hierárquicos (infragenérico e/ou intraespecífico), tendo em vista que os fragmentos de restrição podem apresentar incongruências quanto a duas premissas filogenéticas básicas, decorrente do elevado polimorfismo gerado: evolução independente dos fragmentos e falta de homologia de marcas do mesmo tamanho (BLACK, 1993; KARP *et al.*, 1996; MECHANDA *et al*., 2004; JACOBS *et al*., 2008). Por outro lado, estudos recentes indicaram a efetividade do uso das marcas AFLP em inferências filogenéticas, desde que as divergências nas análises de sequências sejam consideravelmente baixas, para o grupo em questão, assim como a topologia dos dendrogramas gerados não sejam significativamente assimétricos, sugerindo ainda que os dados de AFLP comportam-se como caracteres neutros (BONIN *et al*., 2007; GARCÍA-PEREIRA *et al*., 2011).

No entanto, vale salientar que a maior aplicação deste tipo de marcador tem sido registrada para análises de variação genética em nível populacional, permitindo uma visão da estrutura de populações e em maior escala, auxiliando desvendar padrões filogeográficos de populações naturais (inclusive envolvendo espécies crípticas), identificação de *locus* sob seleção, principalmente se o equilíbrio de Hardy-Weinberg e a ausência de endogamia forem assumidos nos cálculos das frequências alélicas (LYNCH & MILLIGAN, 1994; ARENS *et al*., 1998; ZHIVOTOVSKY, 1999; LUIKART *et al*., 2003; HILL & WEIR, 2004; BONIN *et al*., 2007; FOLL *et al*., 2010).

2.4.2 Microssatélites

Existem três tipos de sequências repetidas em tandem em organismos superiores: DNA satélite, minissatélites e microssatélites, que se distinguem entre si basicamente quanto ao comprimento de seus fragmentos repetidos. Os microssatélites, também conhecidos como SSR (*Simple Sequence Repeat*), são elementos polimórficos abundantes em genomas eucariotos, apresentando natureza codominante, sendo organizados em motivos curtos (de 1 a 6 pb) e repetidos várias vezes, configurando-se como uma importante ferramenta no estudo das relações em nível populacional (POWELL *et al*., 1996; ARMOUR *et al.*,1999; CHAMBERS & MACAVOY, 2000). Esta classe de DNA repetitivo pode ser encontrada tanto em regiões codificantes quanto não-codificantes, sendo as variações existentes oriundas de mutações designadas "*slipped-strand mispairing*", ou seja, pareamento incorreto das fitas decorrente de deslizes das enzimas no processo de síntese de DNA. Quando este tipo de falha no mecanismo de replicação ocorre, deleções e inserções das unidades de repetições ocorrem, resultando na ampla variação alélica (JANE & LAGORDA, 1996; POWELL *et al*., 1996; EISEN, 1999; HANCOCK, 1999).

Para se obter marcadores de microssatélites faz-se necessário em geral o desenvolvimento de *primers* espécie-específicos, isto é, a construção de bibliotecas genômicas para determinada espécie-alvo, conferindo custo e demanda de tempo consideráveis (LITT & LUTY, 1989; ZIETKIEWICZ *et al*., 1994; EISEN, 1999). Por outro lado, a natureza codominante, herança Mendeliana, a ocorrência de alelos múltiplos em um único *locus* e o alto potencial de transferabilidade de marcadores para organismos relacionados, tem conferido um poder analítico inquestionável à referida ferramenta (BARBARÁ *et al.*, 2007; PALMA-SILVA *et al*., 2007, 2011; PINHEIRO *et al*., 2009; KRAPP, *et al.*, 2012; WÖHRMANN *et al.*, 2012).

Além da região nuclear, a utilização dos microssatélites encontrados nos genomas mitocondriais (mtDNA) e cloroplastidiais (cpDNA) em eucariotos vem agregando conhecimento no entendimento de questões relacionadas à ecologia, evolução e estrutura genética. Por apresentar uma maior conservação de suas sequências, os genomas plastidiais vêm sendo utilizados e testados em organismos taxonomicamente mais distantes, a partir da hibridização heteróloga das técnicas moleculares existentes (OLMSTEAD & PALMER, 1994; PROVAN et al., 2004; AGARWAL et al., 2008). Além disso, algumas características intrínsecas como a não recombinação, origem predominantemente materna (no caso das angiospermas) e natureza haplotípica conferem um cenário analítico diferente em análises

populacionais e/ou filogenéticas (OLMSTEAD & PALMER, 1994; AZEVEDO *et al*. 2008; EBERT & PEAKALL, 2009; RAMOS *et al*., 2009).

2.5 Variabilidade Genética e Estrutura Populacional em Bromeliaceae

Pela sua história adaptativa e padrões de distribuição geográfica no Novo Mundo, Bromeliaceae constitui-se como um excelente modelo em estudos de genética de populações. Contudo, poucas espécies apresentam informações aplicadas a análises de diversidade genética ou estruturação populacional na literatura. Dentre todas as espécies do grupo, apenas 20 espécies pertencentes a 10 gêneros (*Aechmea*, *Alcantarea*, *Bromelia*, *Dyckia*, *Encholirium*, *Pticairnia*, *Puya*, *Guzmania*, *Tillandsia* e *Vriesea*) possuem dados prévios neste sentido, utilizando em sua maioria marcadores microssatélites (ZANELLA *et al.*, 2012).

O primeiro registro de análise genética, encontrado em bromélia diz respeito às análises conduzidas por Brewbaker & Gorrez (1967), onde estudos de segregação genética (mediante cruzamentos) identificaram locos relacionados a autoincompatibilidade em *Ananas comosus* (Bromelioideae). Os autores apresentaram evidências referentes ao controle gametofítico no fenótipo do pólen (com inibição do tubo polínico) para espécie em questão. Posteriormente, os primeiros registros, utilizando os marcadores isoenzimáticos foram empregados no intuito de estimar a variabilidade genética em Bromeliaceae (SOLTIS *et al*., 1987; MURAWSKI & HAMRICK, 1990; IZQUIERDO & PIÑERO, 2000, SARTHOU *et al*., 2001; GONZALEZ-ASTORGA, 2004; HMELJEVSKI *et al.*, 2011).

No gênero *Dyckia*, os dados apresentados por Hmeljevski *et al.* (2011) são, até o momento, o único registro na literatura sobre dados populacionais no referido grupo. Por meio do uso de nove *loci* isoenzimáticos, os autores analisaram os efeitos da deriva genética em subpopulações da espécie micro-endêmica *Dyckia ibiramensis* Reitz. Adicionalmente, foi constatada uma alta estruturação genética (*Gst* = 0,674) e variabilidade (*He* = 0,219) quando comparados a outros estudos anteriormente citados. Tais resultados foram de extrema importância, por se tratar de uma espécie rara e ameaçada que ocorre em uma área de interesse de empreendimentos hidroelétricos, identificando-se *hot-spots* de diversidade, impedindo a construção de uma usina hidroelétrica nos pontos considerados prioritários para conservação.

Além das análises com isoenzimas, marcadores moleculares baseados em PCR também têm sido bastante utilizados no grupo em pauta. Neste contexto, alguns trabalhos

utilizando marcadores dominantes do tipo RAPD e AFLP foram conduzidos (RUAS *et al*., 1995, SGORBATI *et al*., 2004; CAVALLARI *et al*., 2006; SCHULTE *et al*., 2010; DOMINGUES *et al*., 2011). Assim sendo, dentre os registros de tais inferências, destaca-se o de Cavallari *et al*. (2006) que avaliaram populações de três espécies de *Encholirium* (*E. pedicellatum*, *E. subsecundum* e *E. biflorum*) , o qual é considerado grupo irmão de *Dyckia* (FORZZA, 2001). Neste trabalho, a partir das marcas de RAPD, foi possível acessar diversidade genética nas espécies analisadas, revelando diferentes padrões na distribuição da diversidade e fluxo gênico.

Por fim, marcadores de microssatélites começaram a ser utilizados mais tardiamente com espécies de Bromeliaceae. Os primeiros *loci* desenvolvidos para família são oriundos de dois trabalhos: Boneh *et al*. (2003) e Sarthou *et al.* (2003). Assim sendo, Cascate-Marín *et al.* (2006) utilizaram tais marcadores em duas espécies: *Tillandsia fasciculata* e *Guzmania monostachia* (Tillandsioideae), encontrando disparidade quanto à diversidade intraespecífica (com baixos níveis para a primeira e moderado para a segunda). Tal condição discrepante foi associada à condição poliploide das espécies.

Desta forma, os trabalhos referentes as análises populacionais em Bromeliaceae, utilizando regiões de microssatélites, especialmente nos dados de variabilidade genética e fluxo gênico em populações de inselbergs graníticos (BARBARÁ et al., 2007; 2008; 2009; PALMA-SILVA *et al*. 2009; BOISSELIER-DUBAYLE *et al.*, 2010; DOMINGUES *et al.*, 2011), indicaram uma tendência à forte estruturação genética, com baixo fluxo gênico e restrita dispersão de sementes, que juntamente com a observação de endogamia foram mencionados comos os principais fatores que modelaram a estrutura das populações estudadas.

Por fim, em outro trabalho recente conduzido por Palma-Silva *et al*. (2011), espécies simpátricas do gênero *Pticairnia* provenientes de *inselbergs* da Floresta Atlântica do Rio de Janeiro foram analisadas através do uso de microssatélites nucleares e plastidiais. Os resultados revelaram evidências de introgressão e retenção de polimorfismo ancestral ao longo do tempo nas populações amostradas, com baixos níveis de fluxo gênico intraespecífico. A relevância e o alcance dos dados apresentados possibilitaram elucidar aspectos fundamentais nos padrões biogeográficos das populações e espécies de bromélias ocorrentes em tais Inselbergs, fornecendo também dados relevantes quanto às evidências e fatores que modelaram os processos de especiação ao longo do tempo.

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Capítulo I

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Molecular phylogeny of the genus *Dyckia* (Bromeliaceae) based on AFLP markers

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Abstract

- *Premise of the study*: *Dyckia* (Bromeliaceae) is a xeromorphic genus of Pitcairnioideae subfamily with 150 spp. distributed throughout southeastern South America and center of diversification in the Brazilian territory. The available phylogenetic studies based on nuclear and plastid sequences, in this genus, revealed in general low support towards species composition. Therefore, here we present a molecular phylogeny of *Dyckia* based on AFLP markers, in order to provide a better outlook regarding the infra-generic relationships in this genus.
- *Methods*: We amplified 522 AFLP markers in 100 samples of *Dyckia*, covering 58 species, also including eight samples of the related genera *Encholirium*, *Deuterocohnia* and *Fosterella*, as outgroups. For this purpose, the binary data matrix was subjected to different phylogenetic reconstruction methods, such as Maximum Parsimony, Maximum Likelihood and Bayesian Inference.
- *Key results*: The phylogenetic analyses revealed only partially resolved trees, with constraints especially at the basal nodes of the dendrograms, showing significant levels of homoplasy (CI=0.049 and RI=0.167). On the other hand the AFLP study provided better support at terminal nodes, providing in general monophyletic groups among specimens of the same species, as well as clear geographic pattern in the main observed clusters.
- *Conclusions*: The results provided the first insights regarding the relationships with a clear association among branches and the geographic distribution of the taxa. Thus, it seems that analyzing the species in a population framework along with a detailed taxonomic revision are the reasonable ways to test the available hypotheses.

Key words: Pitcairnioideae, genetic relationships, evolution, xerophytic, Bayesian Inference.

1 Introduction

The family Bromeliaceae, with nearly 3200 species distributed in 58 genera, emerges as one of the most diverse groups, showing wide variability in morphology, physiology, and ecological traits among Neotropical vascular plants (Luther, 2010; Givnish et al., 2011). Similarly to several groups of the Neotropics, this family is largely recognized because of particular key innovations, such as succulence, water absorbing trichomes, tank habit and CAM (Crassulacean Acid Metabolism) photosynthesis, that have supported the diversification of this family found in the life forms or growing habit (Benzing, 2000; Crayn et al., 2004; Schulte et al., 2009).

In early reports, Bromeliaceae intrafamiliar classification included three subfamilies – Bromelioideae, Pitcairnioideae and Tillandsioideae, based essentially on morphological characters as seeds, fruits and ovary position (Smith and Downs, 1974, 1977, 1979; Smith and Till, 1998). However, this division has been considered as an artificial classification due to the high levels of homoplasy, especially within Pitcairnioideae, also indicated by molecular phylogenetic studies (Varadarajan and Gilmartin, 1988a; Varadarajan and Gilmartin, 1988b; Brown and Terry, 1992; Terry et al., 1997a; Horres et al., 2000; Givnish et al., 2007). The most recent comprehensive molecular study by Givnish et al. (2011) was based on eight plastid loci and supported a new classification of the Bromeliaceae with eight subfamilies. In Pitcairnioideae, this analysis was responsible for promoting a reorganization in the subfamily, represented, therefore, by five genera: *Pitcairnia, Fosterella, Deuterocohnia, Encholirium* and *Dyckia* (details see Givnish et al., 2011).

Dyckia is a xeromorphic genus currently comprising 150 described species, being distributed throughout southeastern South America, with center of diversification in the Brazilian territory (Smith and Downs, 1974; Leme and Kollman 2011; Leme et al., 2012). *Dyckia* species can be found mostly dwelling in sun-exposed niches such as "*Campos*

Rupestres", "*Caatinga*" and "*Cerrado*" vegetations. Morphologically, this group is characterized by non-tank habit formation, coriaceous and succulent leaves, well developed marginal spines and a lateral racemose inflorescence (Smith and Downs, 1974; Forzza, 2001; Leme et al., 2012).

The first phylogenetic study in *Dyckia*, which was based on morphological characters, indicated its monophyly, being mainly supported by four synapomophies: an axial inflorescence; peduncule bracts different from leaves, presence of nectaries on the sepals and a petal-staminal ring (Forzza, 2001; Forzza and Silva, 2004). Subsequently, in the molecular studies, *Dyckia* remained in Pitcairnioideae subfamily, with *Encholirium* as sister group (Crayn et al., 2004; Givnish et al., 2004, 2007, 2011).

According to sequencing analysis performed by Krapp (2012), *Dyckia* and *Encholirium* have been confirmed as sister groups, with recent diversification and irradiation and a likely occurrence of both hybridization and introgression, supporting the hypothesis of the existence of a common ancestor before the complete differentiation of the *Dyckia* lineage. Hence, among several reasons, which account for the difficulty in determining properly the species delimitation in these related studies, the hypothesis of a recent diversification appears to be the key explanation for these genera.

In groups with low resolution after sequencing phylogenetic informative regions, as in the case of *Dyckia*, as highlighted by many authors (Despres et al., 2003; Mechanda et al., 2004; Jacobs et al., 2008; Krapp, 2013a), the AFLP (Amplified Fragments Length Polymorphism – Vos et al., 1995) method has been considered as a viable option for analyzing relationships in a lower taxonomic level (i.e. infrageneric). The AFLP method can be suitable in all organisms, often showing significant reproducibility, as already shown in analysis with Bromeliaceae groups, in which this approach was powerful in providing insights regarding both evolutionary and biogeographic histories (Rex et al., 2007; Schulte et al.,

2010). Therefore, here we present a molecular phylogeny of *Dyckia* based on AFLP markers, in order to provide a better outlook regarding the infra-generic relationships in this genus.

2 Materials and methods

2.1 Taxon sampling and DNA extraction

For this study, a total of 100 *Dyckia* samples were analyzed, comprising 56 of the approximately 150 described species for the genus (Leme et al., 2012; Florian, 2013), in our molecular data set (Table 1). Additionally, seven unidentified species were incorporated in the analyses, where three of them were assigned as morphospecies *D.* sp. 1 to 3, while four "*affinis* status" were assigned to the remaining accessions (Table 1). For the composition of the outgroup, we used six species of the sister genus *Encholirium*, as well as one species each of the related genera *Deuterocohnia*, *Fosterella* and a putative new genus (Wanderley, M.G. unpublished data) within the subfamily Pitcairnioideae (Table 1). The material was obtained either from the wild in collecting expeditions or in botanical living collections (Table 1). Fresh young leaf tissues were stored in sodium chloride saturated aqueous solution of cetyl trimethylammonium bromide (CTAB), in a proportion of 20 g of CTAB and 350 g of NaCl per liter of solution (Rogstad, 1992) until the DNA extraction.

The genomic DNA was isolated using the CTAB procedure, described by Doyle and Doyle (1987) with modifications introduced by Weising et al. (2005). In addition, aiming the purification of the extracted DNA, the protocol for precipitation of polysaccharides (Michaels et al., 2004) was employed in the entire sample set. Hence, final DNA concentrations and purity levels were achieved by optical density in a spectrophotometer ultrasensitive, NanoDrop® 2000c (Thermo Scientific, Wilmington, USA).

Table 1. Localities and accessions numbers of the plant material used in the present work.

Species	Accession no.[a]	DNA no.	Locality[b]
Dyckia areniticola Leme	RG 6964 (RB)	D76	Brazil, MT, Chapada dos Guimarães
D. aurea L.B.Sm.	RG 6445 (LC)	D53	Brazil, GO, Cristalino
D. beateae E. Gross & Rauh	RG 1961 (LC)	D136	Brazil, MT, Coxim
D. brachyphylla L.B.Sm	Braun 836 (BN)	F45	Brazil, MG, Itacambira
D. braunii Rauh	Braun 690 (BN)	F42	Brazil, GO
D. brevifolia Baker	RG 6484 (LC)	D56	Brazil, SC, Indaial
D. brevifolia Baker	Kassel s.n. (LC)	F67	Brazil, SC
D. cinerea Mez	Bonn s.n. (LC)	F47	-
D. dawsonii L.B.Sm.	Bonn s.n. (LC)	F43	-
D. delicata Larocca & Sobral	RG 6492 (LC)	D43	Brazil, RS, Barros Cassal
D. densiflora Schult. f.	RG 4249 (LC)	D78	Brazil, MG, Serra da Piedade
D. dissitiflora Schult.f.	AMBI 1605 (UFP)	D20	Brazil, BA, Morro do Chapéu
D. dissitiflora Schult.f.	AMBI 1605 (UFP)	D21	Brazil, BA, Morro do Chapéu
D. dissitiflora Schult.f.	AMBI 1605 (UFP)	D23	Brazil, BA, Morro do Chapéu
D. dystachia Hassl.	Zanella et al. s.n.	D155	Brazil, RS, Derrubadas
D. dystachia Hassl.	Zanella et al. s.n.	D156	Brazil, RS, Derrubadas
D. elisabethae S. Winkl.	RG 4461 (LC)	D85	Brazil, RS, Barra do Ribeiro
D. elisabethae S. Winkl.	RG s.n. (LC)	D134	-
D. encholirioides Mez	Zanella et al. s.n.	D150	Brazil, PR, Ilha do Mel
D. encholirioides Mez	Zanella et al. s.n.	D151	Brazil, PR, Ilha do Mel
D. encholirioides Mez	Zanella et al. s.n.	D153	Brazil, SC, Florianópolis
D. encholirioides Mez	Zanella et al. s.n.	D154	Brazil, SC, Florianópolis
D. espiritosantensis Leme & A.P.Fontana	RG 6930 (LC)	D72	Brazil, ES, São Roque do Canaã
D. espiritosantensis Leme & A.P.Fontana	RG 6655 (LC)	D65	Brazil, ES, Coltaina
D. estevesii Rauh	RG 1888 (LC)	D86	Brazil, GO, Caiapônia
D. estevesii Rauh	Pereira s.n. (HD)	F05	Brazil, GO, Caiapônia
D. ferox Mez.	RG 6696 (LC)	D60	-
D. ferox Mez.	Rauh 64237 (HD)	F20	Argentina, CR, Cerro Colorado
D. ferox Mez.	W. & Till 6016 (UW)	F56	Paraguay, PG, Cordillera de los Altos
D. ferruginea Mez in C.DC.	RG 1958 (LC)	D139	Brazil, MT
D. floribunda Griseb.	W. &Till 5069 (UW)	F52	Argentina, RO, Patquia
D. floribunda Griseb.	W. & Till 5144 (UW)	F108	Argentina
D. fosteriana L.B.Sm.	RG 6461 (LC)	D148	Brazil, PA, Singéis
D. grandidentata P.J.Braun & Esteves	RG 6840 (LC)	D42	Brazil, MT, Rio Verde do M. Grosso
D. granmogulensis Rauh	Rauh 56468 (HD)	F07	Brazil, MG, Grão-Mogol
D. hebdingii L.B.Sm.	RG 4470 (LC)	D44	Brazil, RS, Barra do Ribeiro
D. hebdingii L.B.Sm.	RG 4471 (LC)	D45	Brazil, RS, Barra do Ribeiro
D. hohenbergioides Leme & Esteves	RG 1959 (LC)	D73	Brazil, BA, Botuporã
D. jonesiana Strehl	RG 2959 (LC)	D82	Brazil, RS, Caçapava do Sul
D. leptostachya Baker	RG 1992 (LC)	D57	Brasil
D. limae L.B.Sm.	A. Wanderley s.n. (LC)	D183	Brazil, PE, Vale do Catimbau
D. limae L.B.Sm.	A. Wanderley s.n. (LC)	D184	Brazil, PE, Vale do Catimbau
D. limae L.B.Sm.	A. Wanderley s.n. (LC)	D185	Brazil, PE, Vale do Catimbau
D. lindevaldae Rauh	RG 6468 (LC)	D55	Brazil, GO, Alto Paraíso
D. lindevaldae Rauh	Braun 691 (HD)	F19	Brazil, GO, Alto Paraíso
D. lunaris Leme	RG 4951 (LC)	D52	Brazil, GO, Alto Paraíso
D. macedoi L.B.Sm.	Louzada et al. 151 (SP)	D120	Brazil, MG, Santana do Riacho
D. macedoi L.B.Sm.	Louzada et al. 151 (SP)	D121	Brazil, MG, Santana do Riacho
D. macedoi L.B.Sm.	Louzada et al. 151 (SP)	D122	Brazil, MG, Santana do Riacho
D. marnier-lapostollei L.B.Sm	RG 6437 (LC)	D80	Brazil, GO, Cristalina
D. martinelli B.R. Silva & Forzza	RG 6555 (LC)	D59	Brazil, RJ, Paraty
D. maritima Baker	RG 3319 (LC)	D135	Brazil, RS, Torres
D. maritima Baker	Zanella et al. s.n.	D158	Brazil, RS, Viamão
D. maritima Baker	Zanella et al. s.n.	D160	Brazil, RS, Viamão
D. maracasensis Ule	RG 274 (LC)	D133	Brazil, BA, Maracás
D. machrisiana L.B.Sm	RG 3291 (LC)	D48	Brazil, GO
D. mezii Mez.	Louzada et al. 159 (SP)	D144	Brazil, MG, Tiradentes
D. mezii Mez.	Louzada et al. 159 (SP)	D145	Brazil, MG, Tiradentes

Cont. Table 1.

Species	Accession no.[a]	DNA no.	Locality[b]
D. microcalyx Baker	W. & Till 6020 (UW)	F54	Paraguay, PG, Acahay
D. microcalyx Baker	W. & Till 6066 (UW)	F111	-
D. milagrensis Leme	RG s.n (LC)	D138	Brazil, BA, Milagres
D. mirandana Leme & Z.J.G.Miranda	RG 6379 (LC)	D63	Brazil, GO, Alto Paraíso
D. monticola L.B.Sm. & Reitz	RG 1664 (LC)	D132	Brazil, SC, Campo Alegre
D. nana Leme & O.B.C. Ribeiro	RG 7485 (LC)	D50	Brazil, MG, Diamantina
D. niederleinii	Marburg s.n. (LC)	F103	-
D. parensis L.B.Sm.	RG 7647 (LC)	D49	Brazil, PA, Guarantan do Norte
D. pectinata L.B.Sm. & Reitz	RG 6490 (LC)	D47	Brazil, MG, Conceição do R Verde
D. pectinata L.B.Sm. & Reitz	RG 7271 (LC)	D87	Brazil, MG, Conceição do R Verde
D. pernambucana L.B.Sm.	Pinangé s.n. (LC)	D90	Brazil, PE, Brejo da Madre de Deus
D. pernambucana L.B.Sm.	Pinangé s.n. (LC)	D91	Brazil, PE, Brejo da Madre de Deus
D. pernambucana L.B.Sm.	Pinangé s.n. (LC)	D92	Brazil, PE, Brejo da Madre de Deus
D. platiphyla L.B.Sm.	RG s.n (LC)	D74	-
D. pulquinensis Wittm.	RG 2415 (LC)	D58	Bolivia
D. pumila L.B.Sm.	RG 4706 (LC)	D147	Brazil, GO, Caipônia
D. rariflora Shult. f.	RG 6543 (LC)	D46	Brazil, MG, Mariana
D. rariflora Shult. f.	RG 6545 (LC)	D81	Brazil, MG, Mariana
D. reitzii L.B.Sm.	RG 1505 (LC)	D77	Brazil, RS, Cambará do Sul
D. remotiflora	Horst. s.n. (HD)	F11	Brazil
D. rojasii Mez.	RG 6465 (LC)	D54	Brazil, PA, Rio Branco do Ivaí
D. saxatilis Mez.	Louzada et al. 168 (SP)	D09	Brazil, MG, Serra da Piedade
D. saxatilis Mez.	Louzada et al. 168 (SP)	D11	Brazil, MG, Serra da Piedade
D. secunda L.B.Sm.	RG 3682 (LC)	D51	Brazil, BA, Contendas do Sincorá
D. sordida Baker.	Louzada et al. 156 (SP)	D100	Brazil, MG, Jaboticatubas
D. tobatiensis Hassl.	W. & Till 6050 (HD)	F18	Paraguay, COR, Tobati
D. tomentella	Wien s.n (LC)	F114	-
D. ursina L.B.Sm.	RG 1837 (LC)	D137	Brazil, MG, Jaboticatubas
D. velascana Mez	Wien s.n.	F104	Argentina, CR, Ascochinga
D. vestita Hassl.	W. & Till 6018	F32	Paraguay, PG, Paraguari
D. aff. *aurea*	RG 4426 (LC)	D66	Brazil, GO, Serra da Mesa
D. aff. *brasiliana*	RG 4513 (LC)	D88	Brazil, GO, Anápolis
D. aff. machrisiana	RG 7879 (LC)	D84	Brazil, GO
D. aff. *tuberosa*	RG 6837 (LC)	D68	Brazil, PR, São Gerônimo da Serra
D. sp. (1)	Louzada et al. 173 (SP)	D15	Brazil, MG, São Thomé das Letras
D. sp. (1)	Louzada et al. 173 (SP)	D16	Brazil, MG, São Thomé das Letras
D. sp. (1)	Louzada et al. 173 (SP)	D17	Brazil, MG, São Thomé das Letras
D. sp. (2)	Louzada et al. 172 (SP)	D11	Brazil, MG, Ouro Preto
D. sp. (2)	Louzada et al. 172 (SP)	D12	Brazil, MG, Ouro Preto
D. sp. (2)	Louzada et al. 172 (SP)	D13	Brazil, MG, Ouro Preto
D. sp. (3)	Louzada et al. 171 (SP)	D140	Brazil, MG, Brumadinho
D. sp. (3)	Louzada et al. 171 (SP)	D141	Brazil, MG, Brumadinho
D. sp. (3)	Louzada et al. 171 (SP)	D142	Brazil, MG, Brumadinho
Encholirium brachypodum L.B.Sm. & Read	EB1-RJ (LC)	D205	Brazil, BA
E. erectiflorum L.B.Sm	ERA-FR1 (LC)	D206	Brazil, PE, Afrânio
E. horridum L.B.Sm	EH1-RJ (LC)	D207	Brazil, MG, Jaboticatubas
E. spectabile Mart. ex Schult. &. Schult.f.	ES-CF18 (LC)	D208	Brazil, SE, C. de São Francisco
Deuterocohnia brevispicata Rauh & L.Hrom	N. Schütz 06/028 (LC)	F71	Bolivia, SZ, Samaipata
Gen.sp.Nov.	M. Wanderley & Souza 2630 (SP)	D162.2	Brazil, PI
Fosterella vilosulla (Harms) L.B.Sm	J. Peters 06.0105 (LC)	F76	Bolivia, CO, Cochabamba

Abbreviations: [a] BN: University of Bonn, HD: Botanical garden of Heidelberg; LC: Living collection, RB: Herbarium of Jardim Botânico do Rio de Janeiro, RG: Recanto dos Gravatás, SP: Instituto de Botânica de São Paulo, UFP Universidade Federal de Pernambuco; UW: University of Vienna. [b] BA: Bahia, CO: Cochabamba, COR: Cordillera, CR: Cordoba, ES: Espírito Santo, GO: Goiás, MG: Minas Gerais, MT: Mato Grosso, PA: Pará, PE: Pernambuco, PG: Paraguari, PI: Piauí, PR: Paraná, RJ: Rio de Janeiro, RO: La Rioja, RS: Rio Grande do Sul, SC: Santa Catarina, SE: Sergipe, SZ: Santa Cruz.

2.2 AFLP amplifications and scoring

The AFLP analyses were performed in accordance with the protocol originally described by Vos et al. (1995) and subsequently adjusted by Debenet and Mattiesh (1999), with some modifications. In short, 30 ng of genomic DNA were digested in a final volume of 25 µL at 37 °C with simultaneous restriction using the endonucleases *Hind*III and *Mse*I, as well as their respective adapters, for 12 h. The pre-selective and selective amplifications were carried out using primers with one (+1) and three (+3) selective nucleotides at their 3' ends. For the pre-selective PCR reactions, 2 µL of 1:10 diluted restriction-ligation product, 0.5 µM of the unlabeled *Mse*I +1 primer, 1 µL of the PCR buffer 10× (Peqlab blue), 25 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate (dNTP) and 0.025 U of Taq polymerase, were used (SawadyTaq, Peqlab, Germany).

In order to identify the most informative primers, an initial screening of selective primers using 30 primer combinations was performed in a randomic sub-sample of eight accessions. Out of these, nine primer combinations (ACC/ACA, ACA/ACA, ATC/AAC, ATC/AGC, AGG/AGC, CAC/AAC, CAG/AAC, CTG/ACA, CTA/AGC) were chosen for the final analysis, as they yielded well scorable polymorphic patterns. The PCRs were performed with 2.5 µL of the 1:20 diluted pre-amplification product and different combinations of the *Mse*I (+3) primer (Carl Roth, Karlsruhe, Germany) (0.25 µM) and the fluorescence-labeled *Hind*III (+3) primer (WellRED- D2, -D3, -D4, Sigma Aldrich, Munich, Germany) (0.05 µM) (Table 2). Final products of the selective amplifications were run on an automated sequencer (CEQ8800, Beckman Coulter, Krefeld, Germany) as a multiplex of three primer combinations labeled and an internal size standard (GenomeLab DNA Size Standard Kit 600, Beckman Coulter, Krefeld, Germany).

AFLP banding patterns were scored semi-automatically as presence or absence through the software Genemarker 1.9 (SoftGenetics, State College, PA, USA). The

reproducibility test was also performed with approximately 15% of the total data set that had also been taken randomly for this purpose. The intensity of each individual peak was normalized on the basis of a fixed threshold, with cutoff in less than 10% of the second highest signal intensity.

2.3 Phylogenetic analysis

Initially, a maximum parsimony analysis with the binary matrix was carried out using the program PAUP* v. 4.0b10 (Swofford, 2002), with heuristic searches of 1.000 replicates and 10.000 random addition sequence (RAS), as well as branch swapping via tree bisection reconnection (TBR). The consensus tree was obtained by combining the multiple parsimony trees in both forms, the strict consensus and the 50% majority rule consensus tree. The evaluation of the statistical support in the tree topology was assessed with bootstrap analyses by performing 1000 pseudo-replicates each with 10 RAS and TBR branch swapping. The degree of homoplasy was estimated using both consistency (CI) and retention (RI) indexes.

The maximum likelihood analysis (ML) were performed using RAxML (v. 7.2.8; Stamakis, 2006) through the graphical fron-end raxmlGUI (Silvestro and Michalak, 2011). Thus, 10 independent ML searches were conducted under the BINGAMMA model with a proportion of invariable sites. The rapid bootstrap of 1,000 pseudo-replicates was employed in order to obtain support values in the topology, displaying the highest likelihood tree value found.

Bayesian inference (BI) was conducted with MrBayes 3.2 (Ronquist and Huelsenbeck, 2003). Four simultaneous and independents Markov Chains Monte Carlo (MCMC) were run for 10,000,000 generations, with or without gamma distribution for rate heterogeneity (Yang, 1994). In both models, four simultaneous chains were run for 10,000,000 generations, each. Subsequently, the software Tracer (Rambaut and Drummond, 2007) was employed to

examine the efficiency of the MCMC sampling as well as the burnin-in phase. Therefore, the first 2 million generations were discarded as burnin-in phase and the remaining trees were used to construct an all-compatible consensus tree with posterior probabilities values as the measure of the statistical support of the nodes.

3 Results

The well-resolved banding patterns generated from the nine primer combinations produced different AFLP profiles of the 108 samples, with 522 characters including the outgroup. The numbers of scorable band positions ranged from 90 to 350 base pairs, whereas the numbers of scored characters per primer combinations varied between 45 and 90. In relation to the percentage of variation, from the 522 scored band positions, 97% were polymorphic.

The different phylogenetic analysis (maximum parsimony, maximum likelihood and Bayesian inference), in general, resulted in poorly resolved trees, especially at the basal nodes of the dendrograms (Figure 1; supplementary Figure S1). The maximum parsimony analysis (MP) resulted in 47 shortest trees of 8008 steps length with consistency index (CI) of 0.049 and retention index (RI) of 0.167, denoting a very high level of homoplasy. Additionally, from 522 amplified characters, 515 (98.7%) were parsimony-informative.

The Maximum Likelihood (ML) and the Bayesian inference (BI) trees (Supplementary Figure S1; Figure 1) had, in general, congruent topologies, with the main differences affecting only different levels of statistical support of branches (bootstrap and posterior probabilities values, respectively), with higher support values in the BI tree. The obtained marginal likelihood values -29836.05 (model + gamma) and -30813.80 (simple model) indicated a strong support for the first model. Hence, the first model was considered as the most reliable in the BI.

The BI consensus tree revealed, with a strong support, the monophyly of *Dyckia* (posterior probabilities - PP of 0.99) with accessions of *Encholirium* presenting the closest relationship (sister group position), in comparison with the remaining accessions of the outgroup – *Deuterocohnia brevispicata*, the putative new genus, and *Fosterella vilosulla* (Figure 1). On the other hand, the BI tree displayed a basal polytomy of various weakly supported clades, which exhibited a low resolution, especially the first six nodes in the tree, comprising 27 species and 30 accessions mostly from the mid-west region of Brazil, as well as species from Argentina and Paraguay (Figure 1).

In the BI tree, four main clusters, with various levels of PP support could be recognized (Figure 1). The weakly supported cluster *A* is composed by 7 species and 21 accessions, all of them presenting a distribution in the state of Minas Gerais. Furthermore, all specimens of *Dyckia mezii* (D144 and D145), *D. rariflora* (D46 and D41), and *D. pectinata* (D47 and D87) as well as the unclassified *D.* sp1 (D15, D16 and D17), *D.* sp2 (D11, D12 and D13) grouped together as monophyletic groups with significant support. This cluster also presented some subclades with polytomies, especially in the basal position with three split species: *D. macedoi* (D120, D121 and D122), the unclassified *D.* sp3 (D140, D141 and D142) and *D. saxatilis*.

The cluster *B* united most of the species from northeastern Brazil, which mostly occur in the inselbergs of *Chapada Diamantina, Caatinga* and Atlantic Forest Domain, composed by 11 species and 18 accessions. Most of the terminal PP values were high, where the specimens of *D. limae* (D183, D184 and D185), *D. pernambucana* (D90, D91 and D92), *D. dissitiflora* (D120, D121 and D122) and also *D. milagrensis*, *D. maracasensis*, *D. granmogulensis,* with *D. secunda* as sister groups, constituted a well-defined monophyletic group. Additionally, all *D. espiritosantensis* accessions (D65 and D72) were grouped together, forming a basal sub-clade with the species *D. ursina*, *D. nana*, *D. brachyphylla*.

Furthermore, the cluster B is found as a sister group of the cluster A, forming a well-defined monophyletic group (PP 0.90, Figure 1).

The weakly supported cluster *C* harbored species of *Dyckia* which occur in southern Brazil, comprising a group of ten species and 18 accessions. The specimens of *D. encholirioides* (D134, D150, D151, D153 and D154) and *D. fosteriana* (D148) comprised a well-supported monophyletic group, as this latter displaying a sister position group (Figure 1). The remaining nodes of the cluster *C* exhibited polytomies and received low posterior probabilities, except for the terminal clades which had species with more than one specimens available, grouped together with good PP values – *D. dystachia* (D155 and D156) *D. maritima* (D135, D158 and D160) and *D. hebding*ii (D44 and D45) (*Pryonophilum* group) (Figure 1).

The weakly supported Cluster *D* is represented mostly by species from Argentina, Bolivia and Paraguay. The species *D. brevifolia* (F67), which was present in the cluster C, was also found in this clade. In general, the subclades presented high levels of polytomy and low resolution. Nevertheless, although presenting a polytomy, the subclade composed by the *D. floribunda* (F52 and F108), *D. velascana*, *D. nierderleinii* and *D. brevifolia* received a high support value (PP 0.98). In the clusters C and D, as well as for the remaining species in the analyzed tree, the statistical values were low, mostly forming weakly supported groups (Figure 1).

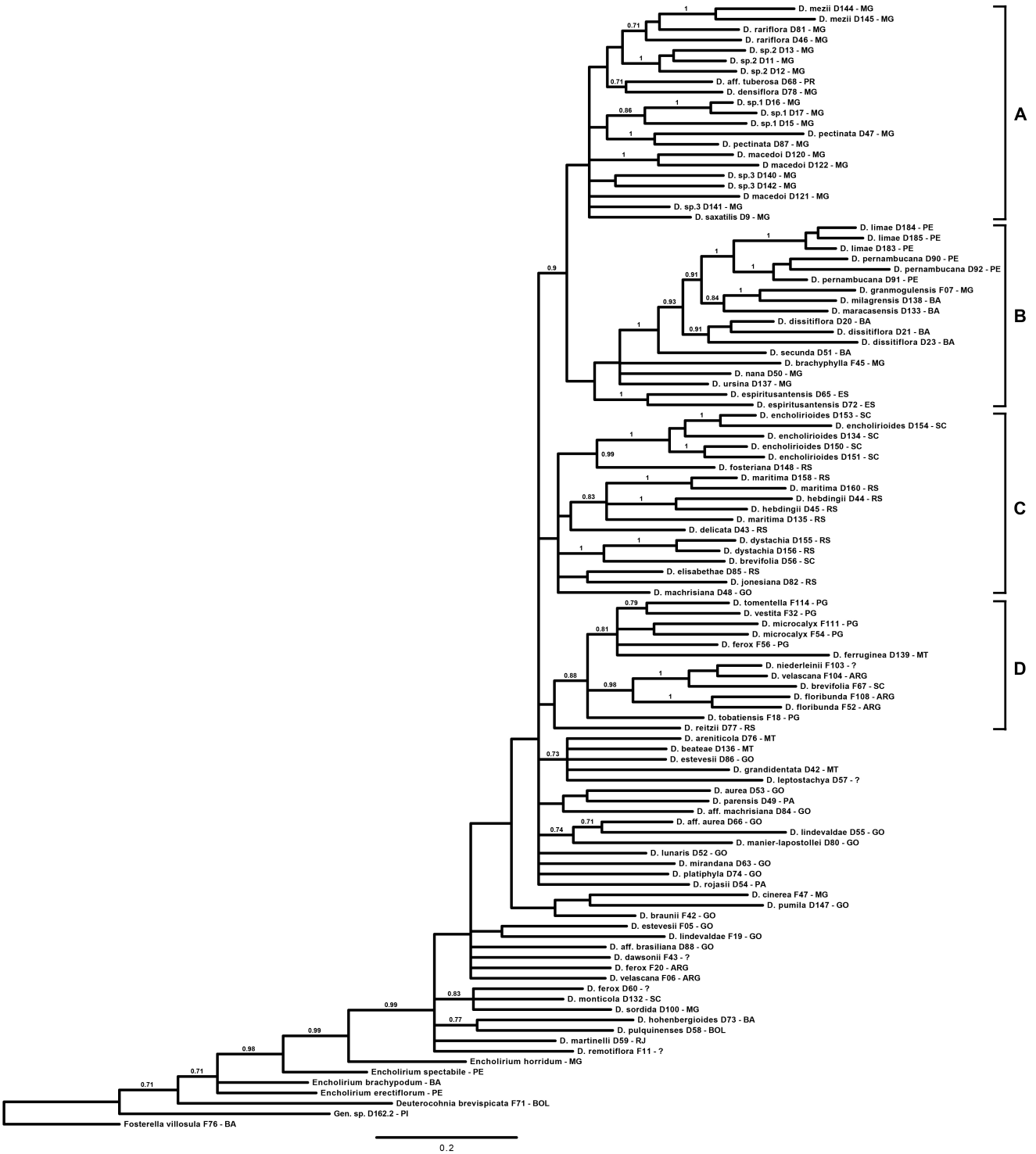

Figure 1: Phylogenetic reconstruction based on Bayesian inference in 101 *Dyckia* accessions, from 522 AFLP characters, and seven accessions that comprised representatives of *Encholirium*, *Deuterocohnia*, a putative new genus and *Fosterella,* as outgroup. Posterior probabilities (PP) > 70 are given above the branches. Clusters A to D are referred to in the text.

4 Discussion

In previous molecular phylogenetic analysis in Bromeliaceae, the genus *Dyckia* has always been underrepresented (Terry et al., 1997; Crayn et al., 2004; Givnish et al., 2004; 2007, 2011; Horres et al., 2007), thus raising questions regarding its monophyly, as mentioned by Horres et al. (2000), for instance. However, this hypothesis has been refuted by recent wide phylogenetic inferences in the family (Givnish et al., 2004; 2007, 2011) and properly tested in the analysis performed by Krapp (2012) and in the current study. Furthermore, *Dyckia* is known by its extreme morphological variation, which has been hindering the definition of species boundaries. In this regard, Krapp (2012) provided the first insights on the infra-generics relationships within *Dyckia*, using nuclear (*phyC*) and plastid sequences (*rpl*32-*trn*L, *rps*16-*trn*K, *mat*K, *rps*16-intron, *pet*D-intron and *trn*D-*trn*T). Therefore, the current study is the first molecular study based on multilocus markers and a significant sampling, being as an attempt to elucidate the relationships in *Dyckia*, despite the lack of a taxonomic revision for this group.

Besides ratifying the monophyly of *Dyckia*, the AFLP data also confirmed the close relationship with *Encholirium*, as shown in previous molecular studies (Crayn et al., 2004; Givnish et al., 2004, 2007, 2011; Klein 2012; Schütz, 2012) and corroborating the existing morphological inferences (Forzza and Wanderley, 1998; Forzza, 2001). The genus *Fosterella*, as a sister group of all xerophytic genera (*Deuterocohnia*, "Gen. sp.", *Encholirium* and *Dyckia*) corroborates the phylogenetic analysis based on nuclear and chloroplast sequences performed by Krapp (2012). However, as shown by other molecular phylogenetic studies (Klein, 2012; Schütz, 2012; Krapp 2012), as well as by morphological approaches used by Forzza (2001), *Encholirium* appeared as a paraphyletic group.

The high degree of uncertainty within *Dyckia* was also observed in the dendrogram, especially in the reconstruction of basal nodes. According to Després et al. (2003), a low

support at this level of phylogenetic inference is expected, in view of the application of such markers in large sample sets. This could also be detected in the AFLP analysis performed by Rex et al. (2007) with accessions of *Fosterella* (Pitcairnioideae) and in the phylogenetic reconstruction of *Cryptanthus* (Cruz et al., in prep) a genus of the subfamily Bromelioideae, which was based on the same marker system. Nevertheless, this method confirmed its effectiveness in the composition of terminal nodes in several analyses in lower taxonomic levels (Koopman et al., 2008; Schulte et al., 2010; Jabaily and Systma, 2013; Cruz et al. in prep.; Louzada et. al in prep.), a different picture from that found for *Dyckia* by Krapp (2012).

Besides the polytomies and low support values observed, the phylogenetic reconstruction presented here depicts a clear pattern association with the geographic distribution of *Dyckia*, a genus which is known by its vast diversity of isolated microhabitats of endemic and micro-endemic species (Smith and Downs, 1974). Similarly, Krapp (2012) has observed well-trimmed geographically associated clades for this genus, by using a six plastid loci phylogenetic network. Therefore, the four main recognized clusters (*A*, *B*, *C* and *D*) presented here, in general, were congruent with the plastid-based geographical groups (Krapp 2012). However in the present work, a higher resolution was observed for the "Central Brazil" clade (Clusters *A* and *B*) denominated by Krapp (2012). Hence, in those clades, three main acknowledged groups could be observed here: (1) species from Minas Gerais and northeastern Brazil; (2) species from the lowlands of Paraguay and Argentina; and (3) species from the south of Brazil (the reophytic species and the *Prionophylum* group).

The cluster *A* comprised the species that occurs in the quartzite-sandstone rocky outcrops of the Espinhaço Range, in Minas Gerais, where the greatest diversity of this genus can be found (Forzza et al., 2013). The species of this cluster are mainly characterized by orange to orange-reddish flowers (except for *D. rariflora*, with castaneous-wine flowers), glabrous to cinereum-lepidote bracts and sepals and saxicolous (rarely terrestrial) habit.

Although a moderately supported sister position to the cluster B and the mostly monophyletic conditions of terminal nodes in this cluster, the lack of statistical support and the presence of polytomies in some sub-clades makes the development of a consistent phylogenetic hypothesis very difficult, taking into account the relationships observed among the studied *Dyckia* species.

On the other hand, the species that comprised the cluster *B* yielded the best statistical support within the genus. This strongly supported monophyletic group was formed by species from Bahia (*D. secunda*, *D. dissitiflora*, *D. milagrensis*, *D. maracaensis*), the closely related species (*D. limae* and *D. pernambucana*) from the inselbergs of Atlantic Forest from the northern margin of São Francisco River in Pernambuco and *D. granmogulensis*, a micro-endemic species from the *Campos Rupestres* in northern Minas Gerais (geographically associated with the rocky outcrops of Bahia). The main features that describe the species from this monophyletic group rely on similar phylogenetic informative characters of habit (rupicolous or saxicolous), leaves (blades abaxially densely lepidote or white lepidote), inflorescences (glabrous to densely lepidote, simple or racemose), upper bract sepals (ovate and usually shorter than the sepals), orange petals (vs. yellow in *D. maracasensis*) and filaments (usually connate above the common tube vs. not connate in *D. pernambucana*) (Smith and Downs, 1974; Siqueira-Filho and Leme, 2006). Thus, there is a strong indication that the main morphological features, which validate the hypothesis of close relationships among species of this group, are not homoplasic. On the other hand, according to Siqueira-Filho and Leme (2006) and M. G. Wanderley (personal communication), *D. secunda*, *D. dissitiflora*, *D. limae* and *D. pernambucana* are morphologically related, in contrast to the molecular data presented here (Figure 1). These results suggest, therefore, that the intrinsic nature of morphological characters used to determine species in *Dyckia* still raises many questions.

In this regard and, as commonly observed, for many groups of *Dyckia* (Leme et al., 2012), some of these morphological characters raise questions about the status of some species. The closely related species *D. pernambucana* and *D. limae* (well supported as sister groups) are distinguished simply by slight differences on the orientation of the leaves, the arrangement of the spines in the leaf blades and the inflorescence length (details see Siqueira and Leme, 2006). Thus, it seems that, for the foremost questions in this genus, population genetics approaches may be very useful to disentangle existing difficulties in species boundaries within *Dyckia*, as it has been demonstrated in recent reports (Hmejelvski et al., 2011; Pinangé et al., in prep).

The cluster *C* covered species of *Dyckia* from southern Brazil, which displays a different set of morphological characters when compared with the species mentioned so far. The species of this group are reophyte and terrestrial, exhibiting a large plant size with a very dense rosette, numerous and relatively larger leaves, as well as inflorescences consisting of many flowers usually distinctly branched with few to many lateral branches. *D. fosteriana*, according to Forzza et al. (2013), exhibits a disjunction distribution (Pará and –Paraná, states from northern and southern Brazil, respectively) and although geographically related to *D. encholirioides* (found only in Paraná), these two species show differences in their morphological characters such as height (up to 50 cm - *D. fosteriana* vs. up to 3 m - *D. encholirioides*), arrangement of spines (laxly serrate with coarse curved spines vs. serrate recurved or antrorse), scape ornamentation (sparsely flocculose vs. ferrugineous-puberulous) and inflorescence (simple, densely lepidote vs. simple to paniculate, many lateral branches) (Smith and Downs, 1974). Therefore, we suggest that a deeper analysis should be addressed in order to provide additional informative morphological characters to explain the relationship between these two species.

The next less supported sub-clade of the southern group comprised species from the previous *Prionophyllum* group (*D. maritima* and the not included *D. selloa*), a current synonym for *Dyckia,* which was described by Karl Koch (1873) together with the related species *D. hebdingii*. This group is characterized by a tripinnate (or more divided) inflorescence, floral bracts and sepals reaching a maximum length of 3 and 5 mm, respectively, and the occurrence of unisexual flowers (Smith and Downs, 1974). Additionally, according to Strehl and Beheregaray (2006), the seed features are taxonomically informative in this southern group, dividing the species into two distinct clusters. Therefore, *Prionophylum* comprises the types I (long oval, non-flat and triangular seeds) and II (oval, flat and discoid seeds), containing species like *D. dystachia*, *D. elisabetahe* and *D. remotiflora*, for instance. Although the *Prionophyllum* group presents unique features that could influence its position in a phylogenetic reconstruction, the occurrence of natural hybridization in these species (E.M. Leme, personal communication) can possibly explain the relationships observed here and corroborated the previous hypothesis of hybridization events in *Dyckia* (Leme et al., 2012; Krapp, 2012). Furthermore, the related reophyte species (*D. dystachia* and *D. brevifolia*) formed an expected well-supported clade.

The weakly supported Paraguayan/Argentinian clade (Cluster *D*) provided only a single well-supported clade grouping species from Argentina (*D. nierderleinii*, *D. velascana*, *D. floribunda*) besides the split of *D. brevifolia*. This group was also identified by Krapp (2012), however with no evidence of monophyly in the terminal nodes, where admixture of species from different recognized geographical cluster was observed. The patterns found in the present work provided additional insights regarding the biogeographic hypothesis proposed in the previous *Dyckia* phylogenetic reconstruction (Krapp 2012). Briefly, the intense radiation in *Encholirium/Dyckia* began right after the split from *Deuterochonia* (around 4.6 ma) starting from Bolivia to the mid-western Brazil (states of Mato Grosso, Mato

Grosso do Sul and Espírito Santo). In relation to this, Forzza (2005) also provided some ideas regarding the putative origin of diversification in *Dyckia*. Around 4.0 ma, the first irradiation towards southern region (Clusters *C*) occurred with the second massive irradiation towards the denominated "Central Brazil" group (Clusters *A* and *B*) occurring around 2.9 ma.

5 Concluding Remarks

The results showed here for the genus *Dyckia* provided the first insights regarding the existing relationships in a diverse and recent bromeliad group. As mentioned before, it seems that analyzing the species from each cluster/clade in a population framework along with a detailed taxonomic revision is a reasonable way to test the available hypotheses. Furthermore, even within a clear monophyletic group, the reproductive barriers do not appear to be entirely defined, with a probable exchange of genetic material among species, mainly after the separation of individual species, which is indicated by the low statistical support for the observed groups in the present analysis.

The genetic/geographic distribution pattern observed here confirmed some of the previous morphological observations made for *Dyckia*. Furthermore, this geographic distribution pattern, characterized by several endemic and micro-endemics species, can also probably be correlated to seed characters, which are ineffectively dispersed by the wind and/or water, as also suggested by Rex et al. (2007) in *Fosterella*. Thus, as also hypothesized in this latter mentioned work, it seems that, among several factors, this seed features have been responsible for having modeled the geographic pattern found here.

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Capítulo II

Artigo publicado na *AJB Primer Notes & Protocols in The Plant Sciences* (2012) 99: e470-e473

A set of plastid microsatellite loci for the Genus *Dyckia* (Bromeliaceae) derived from 454 pyrosequencing

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AJB Primer Notes & Protocols in the Plant Sciences

A set of plastid microsatellite loci for the genus *Dyckia* (Bromeliaceae) derived from 454 pyrosequencing[1]

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- *Premise of the study:* Phylogeographical analyses of *Dyckia* (Bromeliaceae) suffer from low levels of sequence variation. Plastid microsatellite markers were developed to achieve a better-resolved genus-wide plastid genealogy of *Dyckia*.
- *Methods and Results:* Approximately 84% of the *D. marnier-lapostollei* plastome was sequenced using 454 technology. Flanking primer pairs were designed for 34 out of 36 chloroplast simple sequence repeats (cpSSRs) detected, and 12 loci were further characterized by genotyping *Dyckia* samples at the level of populations and species. Three, five, and six cpSSRs were polymorphic among four individuals of *D. limae*, 12 individuals of *D. dissitiflora*, and 12 of *D. pernambucana*, respectively, with two to three alleles per locus and species. All loci were polymorphic among 19 different *Dyckia* species, with three to 10 alleles per locus. Ten primer pairs cross-amplified with bromeliad genera from five subfamilies.
- *Conclusions:* The set of 12 cpSSR markers provides a toolbox to analyze phylogeographical patterns of *Dyckia* species.

Key words: Bromeliaceae; cpSSR; *Dyckia*; plastome; population genetics.

The genus *Dyckia* Schult. f. (Bromeliaceae) currently comprises 147 described species of xerophytic, terrestrial, or epilithic rosette plants with showy yellow, red, or orange flowers (Smith and Downs, 1974). The genus is distributed across eastern South America, with a center of diversity in the cerrado biome of Brazil and adjacent countries. Species of *Dyckia* and of its closest relative *Encholirium* Mart. ex Schult. f. typically inhabit azonal, arid, or rupicolous habitats that are characterized by poor soil, little water supply, high temperatures, and strong sun exposure. Pollination is mainly by hummingbirds and insects. Fruits are capsules that release winged, wind-dispersed seeds upon maturity (Smith and Downs, 1974).

Little is known about infrageneric relationships within *Dyckia*, the genetic structure and variation within its species, and the mechanisms of speciation. This paucity of information is in part due to the fact that many *Dyckia* species are rare and narrow endemics, which are barely represented in herbaria and living collections. Some species are even known from their type locality only. Another problem is the high degree of intraspecific morphological plasticity, which makes species delimitation in *Dyckia* notoriously difficult. We have initiated a genus-wide molecular phylogenetic study of *Dyckia*, based on plastid and nuclear DNA sequences. Our preliminary results indicate very low levels of plastid sequence divergence, suggesting a young age of the genus (Krapp, unpublished data). Whereas chloroplast haplotypes are often shared between species, haplotype networks based on plastid DNA show a pronounced geographical pattern across the distributional range of the genus. Chloroplast simple sequence repeats (cpSSRs), also called chloroplast microsatellites, are among the most sensitive tools for evaluating plastid DNA variation (Ebert and Peakall, 2009). To achieve a better-resolved genus-wide plastid phylogeography of *Dyckia*, we developed a set of 12 polymorphic cpSSR markers based on de novo 454 sequencing.

METHODS AND RESULTS

Total genomic DNA was isolated from one individual plant of *Dyckia marnier-lapostollei* L. B. Sm. var. *estevesii* Rauh from Goiania, central Brazil (see Appendix 1), using a modified cetyltrimethylammonium bromide (CTAB) procedure (Tel-Zur et al., 1999). This species was chosen because its plastid haplotype takes a central position in a statistical parsimony network, suggesting an ancestral state within the genus (Krapp, unpublished results). Fragmentation of a 5-µg DNA aliquot by nebulization, preparation of barcoded libraries, and shotgun sequencing on a Roche 454 GS-FLX with the Titanium Sequencing Kit XLR70 and the Titanium PicoTiterPlate Kit (Roche Diagnostics, Penzberg, Germany) were performed as described previously (Wöhrmann et al., 2012a). Altogether, 59 624 reads were obtained from three independent runs. The proportion of a single 454 sequencing lane devoted to *D. marnier-lapostollei* was 4.2%, 2.1%, and 4.1% in the first, second, and third run, respectively. Sequences of plastid origin were identified using the BLAST function of the software package Geneious (Drummond et al., 2010). The fully sequenced plastome of *Typha latifolia* L. (Guisinger et al., 2010)

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Table 1. Characteristics of 12 chloroplast microsatellite primer pairs developed in *Dyckia marnier-lapostollei* var. *estevesii*.

Locus	Primer sequences (5′–3′)	Position	Repeat motif	Size (bp)	GenBank accession no.[a]
DSSR-L01	F: GTCAATTTTCAAGTTCAGCC	*atp*B-*rbc*L	$(T)_{13}C(A)_{10}$	75	JQ743912
	R: TCACGATTTCATCTACTTGC	intergenic			
DSSR-L04	F: AAAGGATGAGATCAATTCGG	*ndh*A	$(T)_9$*	94	JQ743913
	R: AAGATACATCGGAAAGTCCC	intronic			
DSSR-L06	F: ATTGATTGAATAAACCGGGG	*trn*K-UUU-*rps*16	$(T)_{13}$	77	JQ743914
	R: TAAATAAGAAATTGGAATGG	intergenic			
DSSR-N01	F: GTTCCCAGTAAGAACCAACC	*rpo*C1	$(T)_{14}$	102	JQ743915
	R: CTCAATAATTTCACATTTCC	intronic			
DSSR-N04	F: GAAATCAATGTGTCGATTCC	*clp*P	$(T)_{11}$	87	JQ743916
	R: TTTNAATAGAAAGAAGACCC	intronic			
DSSR-N05	F: TGAGATGAGTTTTGGCTCCC	*clp*P	$(A)_{12}$	85	JQ743917
	R: AACAATACATCAATGATAGG	intronic			
DSSR-N07	F: ATTATATACATCTGAAACCC	*trn*P-UGG-*psa*J	$(A)_{13}$	74	JQ743918
	R: CTTCCTCCTGAGCATTACGG	intergenic			
DSSR-N10	F: TNAATCAATATGGCGAAGGC	*clp*P	$(T)_{10}$	79	JQ743919
	R: ATTCCCTCACGCTTGGCGCC	intronic			
DSSR-N11	F: ATAGATAAAATTATCGGGCC	*ndh*G-*ndh*I	$(A)_{18}$	100	JQ743920
	R: AAATTTAAACTACATATTCC	intergenic			
DSSR-N15	F: CTTCCATTTATCCATATCCC	*rpl*16	$(T)_{11}$	64	JQ743921
	R: AAAATAAATCTGATTATGGG	intronic			
DSSR-N16	F: TTATACCAAATGATCAATCG	*rpl*16-*rps*3	$(T)_{13}$	90	JQ743922
	R: ACTCTTTCATTCTTTTTCCG	intergenic			
DSSR-N18	F: AAATAGGTAATCTATTCCCC	*psb*K-*psb*I	$(A)_{15}$	63	JQ743923
	R: GTAAGCATTACACAATCTCC	intergenic			

[a] GenBank accession numbers of the sequences on which the primers are based.

* The SSR motif at DSSR-L04 had only nine T residues in *D. marnier-lapostollei*, but had up to 14 T residues in other *Dyckia* species for which sequence data were available for primer design.

was taken as a reference. A total of 3826 plastid reads were assembled into 77 contigs and 12 singletons, which together represent 113 449 bases of the *D. marnier-lapostollei* plastome (counting the two inverted repeats only once). This corresponds to an overall coverage of ~84%, when compared to the *T. latifolia* plastome. A total of 36 mononucleotide repeats with ≥10 bases were detected (181 with ≥7 bases) using the FIND function of PhyDE (Müller et al., 2010). Besides two short dinucleotide repeats, each with an $(AT)_5$ motif, no other types of SSRs were observed. Flanking primer pairs were designed by eye for 34 loci, with a default length of 20 nucleotides and a GC clamp of up to three nucleotides at the 3′ end. For three loci (DSSR-L01, DSSR-L04, and DSSR-L06; Table 1), consensus primers were derived from alignments of the *D. marnier-lapostollei* sequence with sequence data previously generated by Sanger sequencing of the same loci in other *Dyckia* species (Krapp, unpublished data).

Primer functionality was initially tested on a single accession each of *D. marnier-lapostollei*, *D. dissitiflora* Schult. f., and *D. pernambucana* L. B. Sm. PCRs were carried out in 10-μL volumes using a Biometra T1-cycler or a Biometra T-Gradient cycler (Biometra GmbH, Göttingen, Germany), using

Table 2. Observed allele sizes at 12 chloroplast microsatellite loci in three populations of *D. dissitiflora* and *D. pernambucana* and one population of *D. limae*, allele numbers and size range in 19 different *Dyckia* species (one individual each), and cross-amplification in eight additional genera of Bromeliaceae (see Appendix 1).

	Allele sizes									Cross-amplification in other bromeliad genera[†]							
	D. dissitiflora			*D. pernambucana*			*D. limae*	19 *Dyckia* species									
Locus	Cachoeira* (*N* = 4)	Lajes* (*N* = 4)	Morrão* (*N* = 4)	Aldeia* (*N* = 4)	Brejo* (*N* = 4)	Papagaio* (*N* = 4)	Jerusalém* (*N* = 4)	No. of alleles	Size range (bp)	En	De	Fo	Pi	Pu	An	He	Ti
DSSR-L01	75	75, 76	75, 76	77	75, 76	77	76, 77	9	72–80	+	+	+	+	+	+	+	—
DSSR-L04	98	98	98	99	97	98	99	6	94–99	+	+	+	+	+	+	+	+
DSSR-L06	79	79, 80	79, 80	78	78	78	78	8	73–82	+	+	+	+	+	+	+	+
DSSR-N01	102	102	102	102	102	102	102	8	98–109	+	+	+	+	+	+	+	+
DSSR-N04	91	91	91	91	92	91	91	8	87–98	+	+	—	—	—	—	—	—
DSSR-N05	86	86	86	85, 86	85, 86	86	85, 86	4	84–87	+	+	+	+	+	+	+	+
DSSR-N07	74	74	74	74	74	74	74	5	71–75	+	+	—	+	+	+	+	+
DSSR-N10	81	79	79, 81	79	79	79	79	3	79–81	+	+	+	+	+	+	+	+
DSSR-N11	99	96 100	99	97	98	98	98	10	94–104	+	+	+	—	—	—	+	—
DSSR-N15	65	65	65	65	65	65	65	3	64–66	+	+	+	+	+	+	+	+
DSSR-N16	90	90	90	90	90	90	90	5	87–91	+	+	+	+	+	+	+	+
DSSR-N18	68	72, 73	68, 72	66	66	67	62, 66	9	62–73	+	+	+	+	+	+	+	+

Note: + = amplification; — = no amplification; An = *Ananas* (Bromelioideae); De = *Deuterocohnia*; En = *Encholirium*; Fo = *Fosterella*; Pi = *Pitcairnia* (all Pitcairnioideae); He = Hechtia (Hechtioideae); Pu = *Puya* (Puyoideae); Ti = *Tillandsia* (Tillandsoideae).

* Locality information for the populations is provided in Appendix 1.

[†] Single PCR product in the expected size range.

the indirect fluorescence labeling procedure described by Schuelke (2000). Each assay contained approximately 1 ng of template DNA, 1× Mango-*Taq* reaction buffer (Bioline, Taunton, Massachusetts, USA), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.04 μM forward primer carrying a 5′-M13 tail, 0.16 μM of M13 forward primer with fluorescent 5′-IRD700 modification, 0.16 μM unlabeled reverse primer, 0.5 μg/μL BSA, and 0.05 U Mango-*Taq* DNA polymerase (Bioline). All loci were amplified using a standard PCR program with an initial denaturation at 80°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 52°C for 1 min, and elongation at 65°C for 2 min. Final extension was performed at 65°C for 10 min. Samples were electrophoresed on denaturing 6% polyacrylamide gels in 1× TBE buffer, using an automated sequencer (Li-Cor 4200 IR^2; Li-Cor Biosciences, Bad Homburg, Germany). Fragment sizes were determined by visual examination with the help an external size standard, as outlined by Wöhrmann et al. (2012a). Allele sizes were validated by repeated PCRs of subsets of samples using either Mango-*Taq* polymerase or a set of proofreading polymerases (Long High Fidelity Enzyme Mix; Rovalab, Teltow, Germany), following the protocol supplied by the manufacturer.

The 12 most polymorphic cpSSR loci were used to genotype (1) population samples from *D. limae* L. B. Sm., *D. dissitiflora*, and *D. pernambucana*; (2) single accessions from 16 additional *Dyckia* species; and (3) one or two species each of eight bromeliad genera belonging to five subfamilies. *Dyckia dissitiflora* was chosen as an example of a *Dyckia* species with a relatively large distribution range across Brazil, whereas *D. limae* and *D. pernambucana* were taken as a typical example of two species that are not clearly distinguishable by morphological characters. Locus characteristics, primer sequences, and GenBank accession numbers of these 12 markers are summarized in Table 1, fragment sizes for all samples and loci are compiled in Table 2, and all plant materials used in this study are listed in Appendix 1.

Three, five, and six cpSSR loci were polymorphic among four individuals of *D. limae*, 12 individuals from three populations of *D. dissitiflora*, and 12 individuals from three populations of *D. pernambucana*, respectively (Table 2). Two to three alleles were observed per locus and species. All loci were highly polymorphic at the species level, with three to 10 alleles per locus across 19 *Dyckia* species (Table 2). Allele size distributions were generally compatible, with a variable number of mononucleotide repeats being the molecular basis for size variation. Overall, only six out of 540 individual PCRs performed with any *Dyckia* species failed. All loci produced single PCR fragments within the expected size range in the closely related genera *Encholirium* and *Deuterocohnia* Mez, and nine of the 12 primer pairs successfully cross-amplified in six other genera from five subfamilies of Bromeliaceae (Table 2).

CONCLUSIONS

The set of 12 novel cpSSR markers presented here provides a promising toolbox for reconstructing plastid genealogies and elucidating phylogeographical patterns within *Dyckia*. In conjunction with nuclear SSR markers that are currently being developed in our group (Wöhrmann et al., 2012b), the cpSSRs are also promising candidates for population genetic analyses in *D. dissitiflora*, *D. limae*, *D. pernambucana*, and probably many other *Dyckia* species. Primer binding sites appear to be well-conserved among Bromeliaceae, suggesting that the 12 cpSSR markers may be applicable for genetic studies throughout the family.

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Appendix 1. Plant material used for this study.

Species	Collector (Herbarium)[a]	Location[b]	GPS coordinates
Dyckia dissitiflora Schult. f. Pop. Cachoeira (*N* = 4)	*A. M. Iseppon, Pinangé, D. & Cruz, G. 1605* (UFP)	Cachoeira "Ferro Doido," Bahia (BR)	−11.6279; −41.0005
Dyckia dissitiflora Schult. f. Pop. Lajes (*N* = 4)	*A. M. Iseppon, Pinangé, D. & Cruz, G. 1598* (UFP)	Lajes, Bahia (BR)	−11.6010; −41.1645
Dyckia dissitiflora Schult. f. Pop. Morrão (*N* = 4)	*A. M. Iseppon, Pinangé, D. & Cruz, G. 1562* (UFP)	Morrão, Bahia (BR)	−11.5901; −41.2072
Dyckia limae L. B. Sm. Pop. Jerusalém (*N* = 4)	*A. M. Wanderley s.n.* (UFP)	Serra de Jerusalém, Pernambuco (BR)	−8.5837; −37.2384
Dyckia pernambucana L. B. Sm. Pop. Aldeia (*N* = 4)	*D. Pinangé et al. DCKB/09.2009* (UFP)	Aldeia Couro d'Anta, Pernambuco (BR)	−8.3254; −36.7562
Dyckia pernambucana L. B. Sm. Pop. Brejo (*N* = 4)	*D. Pinangé et al. DKCA/09.2009* (UFP)	Brejo da Madre de Deus, Pernambuco (BR)	−8.1894; −36.3931
Dyckia pernambucana L. B. Sm. Pop. Papagaio (*N* = 4)	*A. M. Wanderley s.n.* (UFP)	Pico do Papagaio, Pernambuco (BR)	−7.8228; −38.0554
Dyckia aff. *brevifolia* Baker	*P. Braun 840* (HD)	Itacambira, Minas Gerais (BR)	−17.0667; −43.3000
Dyckia estevesii Rauh	*P. Braun s.n.* (HD)	BR	NA
Dyckia ferox Mez	*W. Rauh 64237* (HD)	Cerro Colorado, Cordoba (RA)	−30.1000; −63.9333
Dyckia goehringii E. Gross & Rauh	*W. Rauh 67622* (HD)	Diamantina, Minas Gerais (BR)	−18.2500; −43.6000
Dyckia granmogulensis Rauh	*W. Rauh 56484* (HD)	Grão Mogol, Minas Gerais (BR)	−16.5667; −42.9000
Dyckia aff. *ibiramensis* Reitz	*L. Horst 1287* (HD)	Diamantina, Minas Gerais (BR)	−18.2500; −43.6000
Dyckia leptostachya Baker	*H. Amerhauser s.n.* (WU)	Caacupé, Cordillera (PY)	−25.3833; −57.1500
Dyckia lindevaldae Rauh	*P. Braun BR 691* (HD)	Alto Paraiso, Goiás (BR)	−14.1167; −47.5167
Dyckia macedoi L. B. Sm.	*R. B. Louzada, Pinangé, D. & Medeiros, M. 151* (SP)	Santana do Riacho, Minas Gerais (BR)	−19.3539; −43.6237
Dyckia marnier-lapostollei var. *estevesii* Rauh	*L. Horst 5* (HD)	Goiania, Goiás (BR)	−16.6667; −49.2667
Dyckia marnier-lapostollei L. B. Sm.	*L. Horst 4* (HD)	Cristalina, Goiás (BR)	−16.7500; −47.6000
Dyckia microcalyx Baker	*W. Till 6020* (WU)	Cerros Acahay, Paraguari (PY)	−25.9167; −57.1500
Dyckia aff. *pumila* L. B. Sm.	*P. Braun BR 696* (HD)	Ponte Branca, Mato Grosso (BR)	−16.4500; −52.6667
Dyckia remotiflora var. indet. Otto & A. Dietr.	*L. Horst s.n.* (HD)	BR	NA
Dyckia tobatiensis Hassl.	*W. & S. Till 6050* (WU)	Tobati, Cordillera (PY)	−25.2500; −57.0667
Dyckia velascana Mez	*W. & S. Till 5012* (WU)	Ascochinga, Cordoba (RA)	−30.9500; −64.2667
Dyckia vestita Hassl.	*W. & S. Till 6018* (WU)	Paraguari, Paraguari (PY)	−25.6333; −57.1500
Encholirium horridum L. B. Sm.	*W. Schindhelm s.n.* (HD)	Pedra Azul, Minas Gerais (BR)	−15.9867; −41.4069
Encholirium magalhaesii L. B. Sm.	*s.n.* (BONN)	BR	NA
Deuterocohnia brevispicata Rauh & L. Hrom.	*N. Schütz 06/028* (FR)	Florida, Santa Cruz (BOL)	−18.0154; −64.1001
Deuterocohnia glandulosa E. Gross	*N. Schütz 06/019* (FR)	Ipati, Santa Cruz (BOL)	−19.7063; −63.6521
Fosterella villosula (Harms) L. B. Sm.	*J. Peters 06.0105* (HD)	Cochabamba, Cochabamba (BOL)	−17.0611; −65.6444
Fosterella weddelliana (Brongn. ex Baker) L. B. Sm.	*M. Miyagawa s.n.* (HD)	Solacana (BOL)	NA
Pitcairnia feliciana (A. Chev.) Harms & Mildbr.	*I. Ebert & D. Bangoura s.n. ex coll. P. Bak* (WU)	RG	NA
Pitcairnia heterophylla (Lindl.) Beer	*K. Senghas O-11230* (HD)	Cruz de Ocotte, Guerrero (MEX)	17.5500; 99.8833
Puya ferruginea (Ruiz & Pav.) L. B. Sm.	*W. Rauh s.n.* (HD)	Rio Marañon (PE)	NA
Puya herzogii Wittm.	*T. Krömer 6581* (HD)	Carrasco, Cochabamba (BOL)	−17.1933; −64.9731
Ananas ananassoides (Baker) L. B. Sm.	*P. Maas s.n.* (HD)	Est. Amazonas (BR)	NA
Hechtia caerulea (Matuda) L. B. Sm.	*W. Rauh s.n.* (HD)	Est. Mexico (MEX)	NA
Tillandsia usneoides (L.) L.	*W. Rauh s.n.* (HD)	Yungas Cachi (RA)	NA

Note: *N* = population size; NA = not available.

[a] Herbaria: BONN = University of Bonn; FR = Senckenberg Research Institute, Frankfurt; HD = Botanical Garden of Heidelberg; SP = Instituto de Botânica; São Paulo; UFP = Universidade Federal de Pernambuco; WU = University of Vienna.

[b] BOL = Bolivia; BR = Brazil; MEX = Mexico; PE = Peru; PY = Paraguay; RA = Argentina; RG = Guinea.

Capítulo III

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Development of 15 nuclear microsatellite markers in the genus *Dyckia* (Pticairnioideae; Bromeliaceae) using 454 pyrosequencing

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TECHNICAL NOTE

Development of 15 nuclear microsatellite markers in the genus *Dyckia* (Pitcairnioideae; Bromeliaceae) using 454 pyrosequencing

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Abstract The genus *Dyckia* (Bromeliaceae) comprises 147 species that are distributed in Brazil and adjacent countries. Many species are rare and narrow endemics. We used 454 pyrosequencing to isolate 1,587 microsatellite loci in *Dyckia marnier-lapostollei*. Of 50 loci that were selected for primer design, 15 markers proved to be polymorphic in five populations from three heterologous species, *Dyckia dissitiflora*, *Dyckia pernambucana* and *Dyckia limae*. Numbers of alleles per locus varied from 3 to 30, and expected and observed heterozygosities ranged from 0.21 to 0.53 and from 0.16 to 0.44, respectively, in the overall sample. The 15 new microsatellite markers are promising tools for studying population genetics in *Dyckia* species.

Keywords Bromeliaceae · Cross-species transferability · *Dyckia* · Microsatellites · 454 Pyrosequencing

The 147 species of *Dyckia* Schult. and Schult.f. (Bromeliaceae) form terrestrial or epilithic perennial rosettes that are characterized by a xeromorphic habit and showy flowers (Smith and Downs 1974; Luther 2008). *Dyckia* species are distributed in rocky habitats of Brazil and adjacent countries, with a centre of diversity in the Cerrado of Minas Gerais (Versieux and Wendt 2007). Many narrow endemics have been described, and almost one third of the species listed by Smith and Downs (1974) are known from the type collection only. It is therefore no surprise that the conservation status of many *Dyckia* species is considered as endangered (Versieux and Wendt 2007; Hmeljevski et al. 2011). Little is known about infrageneric relationships within *Dyckia*, the genetic structure and variation within its species, intra- and interspecific gene flow, and mechanisms of speciation. A long-recognized problem is the high degree of intraspecific morphological plasticity, which makes species delimitation in *Dyckia* notoriously difficult (Smith 1934). Nuclear microsatellite markers were shown to be sensitive tools for assessing population genetic parameters and for determining species limits in other bromeliad genera like *Alcantarea* (Barbará et al. 2007; Versieux et al. 2012) and *Pitcairnia* (Palma-Silva et al. 2011). We therefore initiated a 454 pyrosequencing project to isolate polymorphic microsatellite markers in *Dyckia*, and to test their cross-species transferability.

Genomic DNA was extracted from fresh leaves of one individual plant of *Dyckia marnier-lapostollei* L.B.Smith var. *estevesii* Rauh following Tel-Zur et al. (1999). We choose this species because its chloroplast haplotype takes a central position in a parsimony network, suggesting an ancestral state of the species (Krapp unpublished data). We assume that an ancestral rather than derived position within the genus would facilitate cross-species transferability of markers. This is an important aspect, because we aim to use the microsatellite markers for assessing species boundaries across the whole genus. Library preparation and shotgun-sequencing of a 5-µg DNA aliquot on a Roche 454 GS-FLX (Roche Diagnostics) were carried out as described

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Table 1 Characteristics of 15 microsatellite loci and primer pairs developed for *Dyckia marnier-lapostollei* var. *estevesii*

Locus	Primer sequences (5′–3′)	Repeat motif	Size (bp)	T_a (°C)	GenBank accession number
ngDy_1	F: AAAGAGGTGTCAATTGCTAAA	$(AAC)_8$	146	55	JX051855
	R: GCTAACTCTCTCCTCTCTTGG				
ngDy_3	F: GTCATCCGAAAACCCTACTAC	$(GAA)_7$	146	56	JX051856
	R: AGACCATCATCATCGGATATT				
ngDy_8	F: AGGTTCCAAGTCTCAATTTTT	$(TCT)_9$	148	55	JX051857
	R: TGACAAAGTTAAAGGAGCGTA				
ngDy_10	F: TGTCCATCCCTGAATTAAGTA	$(TTA)_7$	170	55	JX051858
	R: CATCCAACCACTCTTTTATTG				
ngDy_16	F: GTTAAGATTGAAGGGCAAAAA	$(AAT)_9$	154	55	JX051859
	R: ATCAGAAGATATTGGACGACA				
ngDy_17	F: GCGAGGCTTTTGTTTTATATT	$(AAT)_{10}$	142	55	JX051860
	R: AGCTCTAAGTCTGTTTGGTCA				
ngDy_22	F: TCGTCGATACGGTTTTTC	$(GTC)_9$	129	55	JX051861
	R: CCTAGAGCAAAGAGAAACGA				
ngDy_24	F: AATGGTTAGTAGTGCCGTTTA	$(TAC)_{10}$	166	54	JX051862
	R: CGCAGATCGAAATAAAGAATA				
ngDy_25	F: CTCTCCTCTCATTTGAAACCT	$(CCT)_6$	188	56	JX051863
	R: GACGGATCCTGACGAAAC				
ngDy_27	F: ATCAAATCGACCGAGAAAC	$(GCG)_6$	147	55	JX051864
	R: TACAAGTACATGGAGGAGGAG				
ngDy_30	F: CATAGATCGAAAAACCTACCC	$(AAGTTC)_6$	164	55	JX051865
	R: CATTAAGCGTTCTTCATCCTA				
ngDy_31	F: TTACATACTCGCCTCTTGAAA	$(CA)_{11}$	151	55	JX051866
	R: GCTAATATTTCATCGTTTTCTCT				
ngDy_32	F: AGATGATTCTCACCTGAGTTCT	$(CA)_{19}$	126	53	JX051867
	R: GGATAGGCTAGGTACATTTTT				
ngDy_45	F: CCATTTCTGCTGAGTTTATTT	$(ATTT)_8$	161	54	JX051868
	R: GTGGGGAACATGATCTTAAA				
ngDy_49	F: ATATTCCGCTATGTTCCAGAG	$(GCGAGG)_5$	199	56	JX051869
	R: TCTAAATCGAGCCATCAGATA				

(Wöhrmann et al. 2012), resulting in a total of 59,624 sequence reads. BatchPrimer3 software (You et al. 2008) was used to search for perfect microsatellites and for primer design. Accepting minimum thresholds of 15 repeat units for mononucleotide repeats, seven for di-, six for tri-, five for tetra- and four units for penta- and hexanucleotide repeats, a total of 1,587 perfect SSRs were found. This corresponds to one SSR per 5.6 kb.

Primer pairs were designed for 50 loci. Of these, 15 yielded distinct PCR products on agarose gels, and were used to genotype 59 individual plants from five populations of three heterologous species, *Dyckia dissitiflora* Schult.F., *Dyckia pernambucana* L.B.Smith and *Dyckia limae* L.B.Smith. *Dyckia pernambucana* and *D. limae* are found in the "Brejos de Altitudes" of the state of Pernambuco, an interzone including Caatinga, Cerrado and Atlantic Rainforest elements (Siqueira-Filho et al. 2006). Morphologically, the two species are closely related, but differ in some minor characters of the inflorescence, leaves, and spines of the leaf blades. A presumably close relative is *D. dissitiflora* Schult.F. that occurs in rupestrian fields of the Chapada Diamantina (Bahia) and in the Caatinga biome of the Brazilian northeast (Conceição and Pirani 2007).

For genotyping, total DNA was extracted from silica-dried leaves as above, and PCRs were carried out in 12.5 µL volumes using a Biometra T-Gradient cycler. Each assay contained approximately 10 ng of DNA in 1xPCR Mango-buffer (Bioline), 5 µg BSA, 1.5 mmol/L $MgCl_2$,

Table 2 Population genetic parameters determined in five populations of *D. dissitiflora* and *D. pernambucana* (two populations each) and *D. limae* (one population)

	D. dissitiflora							*D. limae*			*D. pernambucana*							Overall ($N = 59$)
	"Cachoeira" ($N = 20$)			"Lajes" ($N = 10$)				"Jerusalém" ($N = 10$)			"Brejo" ($N = 9$)			"Aldeia" ($N = 10$)				
Locus	N_a	H_o	H_e	N_a	H_o	H_e	N_{amd}	N_a	H_o	H_e	N_a	H_o	H_e	N_a	H_o	H_e	N_{amp}	N_{mean}
ngDy_1	4	**0.25**	0.54	3	**0.00**	0.36	5	2	0.20	0.19	2	0.33	0.42	2	0.30	0.27	3	8
ngDy_3	5	0.55	0.52	3	0.60	0.62	5	2	0.20	0.19	3	0.56	0.65	4	0.60	0.62	4	9
ngDy_8	2	0.05	0.05	1	0.00	0.00	2	2	0.10	0.10	1	0.00	0.00	1	0.00	0.00	1	3
ngDy_10	3	0.70	0.62	3	0.90	0.67	3	1	0.00	0.00	1	0.00	0.00	2	0.10	0.10	2	4
ngDy_16	6	0.70	0.70	7	0.60	0.83	9	3	**0.20**	0.57	1	0.00	0.00	2	0.30	0.27	2	11
ngDy_17	1	0.00	0.00	3	0.20	0.19	3	3	0.60	0.54	1	0.00	0.00	1	0.00	0.00	2	4
ngDy_22	4	**0.30**	0.64	5	0.40	0.69	7	5	0.80	0.69	2	**0.11**	0.50	1	0.00	0.00	2	8
ngDy_24	10	0.85	0.87	14	1.00	0.97	19	12	1.00	0.94	4	0.56	0.71	7	0.70	0.85	9	30
ngDy_25	2	0.50	0.38	2	0.30	0.27	2	1	0.00	0.00	2	0.11	0.11	1	0.00	0.00	2	3
ngDy_27	1	0.00	0.00	2	0.10	0.10	2	5	0.50	0.66	1	0.00	0.00	3	0.20	0.19	3	6
ngDy_30	5	0.50	0.51	4	0.50	0.54	7	1	0.00	0.00	1	0.00	0.00	2	0.20	0.19	2	7
ngDy_31	7	**0.50**	0.84	5	0.50	0.73	8	2	0.30	0.27	1	0.00	0.00	1	0.00	0.00	1	9
ngDy_32	8	0.70	0.83	9	**0.30**	0.89	13	6	0.90	0.76	2	0.22	0.21	4	**0.20**	0.50	6	17
ngDy_45	7	0.65	0.68	9	0.90	0.86	11	4	0.60	0.66	1	0.00	0.00	3	**0.30**	0.62	3	13
ngDy_49	1	0.00	0.00	3	0.30	0.28	3	3	0.30	0.28	2	0.56	0.50	3	0.90	0.61	4	8
Mean	4.4	0.41	0.48	4.87	0.44	0.53	6.60	3.47	0.38	0.39	1.67	0.16	0.21	2.47	0.25	0.28	3.07	9.33

Significant deviations from Hardy–Weinberg equilibrium ($P < 0.05$) are written in bold

H_e expected heterozygosity, H_o observed heterozygosity, N sample size, N_a number of alleles, N_{amd} mean number of alleles across all *D. dissitiflora* samples, N_{amp} mean number of alleles across all *D. pernambucana* samples, N_{mean} mean number of alleles across all 59 *Dyckia* samples

0.1 mmol/L of each dNTP, 10 pmol of each primer fluorescently labelled with IRDye700 or IRDye800, and 0.1 U of *Taq* DNA polymerase (Mango-*Taq*, Bioline). Cycling conditions for touchdown PCR were 94 °C for 6 min followed by 12 cycles of 94 °C for 45 s, 64° for 30 s with a reduction of 1 °C per cycle, 72 °C for 45 s, then 19 cycles of 94 °C for 45 s, 52 °C for 30 s, 72 °C for 45 s, and a final extension at 72 °C for 20 min. PCR fragments were electrophoresed on denaturing 6 % polyacrylamide gels in 1× TBE buffer, using an automated sequencer (Li-Cor 4200 IR^2, Li-Cor Biosciences). Fragment sizes were scored as described (Wöhrmann et al. 2012). Locus characteristics, primer sequences and GenBank accession numbers of the 15 microsatellite loci are summarized in Table 1, and population genetic parameters determined with ARLEQUIN (Excoffier et al. 2005) and GENEPOP (Raymond and Rousset 1995) are compiled in Table 2.

All 15 loci were polymorphic in *D. dissitiflora* with 2–19 alleles, whereas two and three loci were monomorphic in *D. pernambucana* and *D. limae*, respectively. Three to 30 alleles per locus were found across the overall sample, whereas expected and observed heterozygosities ranged from 0.21 to 0.53, and from 0.16 to 0.44, respectively (Table 2). Deviations from Hardy–Weinberg equilibrium were observed in a few cases only. Preliminary cluster analyses clearly separated *D. limae/D. pernambucana* from *D. dissitiflora* populations (not shown). The 15 new microsatellite markers are thus promising tools for studying population genetics of *Dyckia* species.

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Capítulo IV

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Population genetics of closely related species *Dyckia pernambucana, D. limae* and *D. dissitiflora* (Bromeliaceae) based on microsatellite and AFLP markers

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Abstract

In the Atlantic Rainforest located north of the São Francisco River, the humids enclaves (or continental islands) called "*Brejos de Altitude"* play a significant role in the dynamics of diversity of local Flora and Fauna, due to their exclusive conditions of humidity and microclimates. The endangered species *Dyckia pernambucana* L.B.Smith and *D. limae* L.B.Smith (Pticairnioideae, Bromeliaceae) are characterized by their narrow endemic occurrence in such "*Brejos de Altitudes*" of the Borborema Plateau (Pernambuco, Brazil). Further, according to previous morphological inferences, the species status of these species remains unclear. In order to understand the patterns of gene flow and genetic variability among populations, both microsatellite loci (nuclear and plastid) and AFLP marks were employed in 50 individuals, distributed in five populations of Pernambuco Group (*D. limae* and *D. pernambucana*), as well as 37 individuals of a the closest species (*D. dissitiflora*), with 87 individuals in total. The levels of diversity found in the present work were high, despite the possible influence of genetic drift and selfing rates. In accordance with this, significant values of inbreeding coefficients were observed with the three markers used, indicating that the populations are notably structured. *D. limae* could not be undoubtedly distinguished from the remaining populations of Pernambuco. However, the associations found here were consistent with the historical patterns of colonization and fragmentation of the Atlantic Rainforest located north of São Francisco River.

Keywords: Pticairnioideae, genetic diversity, gene flow, molecular markers, "*Brejos de Altitude*"

1 Introduction

Among several biogeographical units that comprise the Atlantic Forest domain, the Northeastern Atlantic Forest is considered as one of the most relevant centers of endemism due to its historical association with other Brazilian vegetation types, such as the Amazon Forest and semi arid Caatinga (Prance, 1982; Tabarelli & Santos, 2004). Within this part of Atlantic Forest, located at the north of the São Francisco River, the humid enclaves called "*brejos de altitude"* (also continental islands or inselbergs) play an important role in the diversity of the local flora and fauna composition, because of their unique conditions. Thus, this vegetation type found in the states of Pernambuco, Paraíba, Alagoas, Rio Grande do Norte and Ceará, is thought as "exception areas" in the semi-arid region. Therefore, the "*brejos de altitude*" are characterized as interzones, having Atlantic Forest and Caatinga elements with peculiar vegetation, humidity and microclimates, being also recognized as refuges of biodiversity (Lins, 1989; Tabarelli & Santos, 2004).

The genus *Dyckia* Schult & Schult.f. (Bromeliaceae), one of the largest groups of the subfamily Pticairnioideae with currently 150 described species, is distributed exclusively across eastern South America, and approximately 80% of the species are endemic to the Brazilian territory. *Dyckia* species generally inhabit xerophytic, terrestrial or rupicolous habitats where their specimens thrive essentially in formations of "*campos rupestres*" of Cerrado and Caatinga, as well as in the Atlantic Forest domain (Smith & Downs, 1974). Besides, *Dyckia* is also known for its high degree of intraspecific morphological plasticity, which makes species delimitation extremely difficult (Leme *et al*., 2012; Florian, 2013a)

The endangered species *Dyckia pernambucana* L.B.Smith and *D. limae* L.B.Smith are characterized by their narrow endemic occurrence in the "*brejos de altitudes*" of Pernambuco (Siqueira-Filho, 2004). Further, according to Siqueira-Filho and Leme (2006) these species are closely related, regarding their morphological characters, with small differences in the

inflorescence (shorter in *D. limae*), leaves (arcuate vs. unilaterally and recurved) and spines in leaf blades (longer and partially retrorse in *D. limae*), which arise some questions about the correct assignment of populations and/or species. Regarding the pollination syndromes, the ornithophily has been reported for both species, with records for hummingbirds (*Chlorostilbon aureoventris* and *Chrysolampis mosquitos*) as the main floral visitors (Machado & Porto, 2004).

The understanding of the mechanisms that have shaped evolutionary and biogeographic histories of natural populations in such particular environments can be achieved through the studies of genetic variability. Hence, molecular markers can provide a relatively unbiased method of quantifying genetic diversity levels within and between species (Hamrick, 1994; Hamrick and Godt, 1996; Frankham *et al*., 2010). In plants, the differentiation dynamics among populations is a result of the intrinsic effects of pollen and seed dispersal, which is always related to the history of colonization of both populations and species (Chapman *et al*., 2000). Genetic structure analyses among populations have been reported for some bromeliads (Cavallari *et al*., 2006; Barbará *et al*., 2007a, 2008, 2009; Palma-Silva *et al*., 2009, 2011; Hmeljevski *et al*., 2011) providing significant insights about connectivity patterns of species with varying breeding systems and life histories.

Despite the existence of some previous population analyses in the family, little is known about infrageneric relationships, genetic structure, gene flow patterns and mechanisms of speciation within *Dyckia.* Thereby, the data provided by Hmeljevski *et al.* (2011) appears as the only record of population genetic analysis in this genus. In this work, levels of genetic structure and the occurrence of genetic drift were evaluated with allozyme markers in 30 individuals from populations of the temperate rare micro endemic species *D. ibiramensis* Reitz from southern Brazil.

Here we provide a population genetic survey regarding three closely related *Dyckia* species by analyzing patterns of structure and genetic diversity and comparing indices from multilocus DNA fingerprinting and nuclear and plastid co-dominant markers, focusing on the "Pernambuco Group" (PG), which is composed by *D. pernambucana* and *D. limae*. We also compare the usefulness of each marker system in providing insights regarding taxa delimitation as well as their life histories.

2 Materials and methods

2.1 Population sampling and DNA extraction

Populations of the three morphologically related saxicolous *Dyckia* species that are endemic to outcrops (*D. dissitiflora*, *D. limae* and *D. pernambucana*) in northeastern Brazil were sampled (Table 1; Figure 1). Individuals of PG populations (*D. limae* and *D. pernambucana*) were randomly sampled in five inselbergs rocky outcrops, which comprised 50 samples in total (one and four populations of *D. limae* and *D. pernambucana*, respectively), with 10 individuals per population (Table 1). *Dyckia limae* is restricted to a sedimentary rock formation in the Catimbau National Park (Caatinga domain), in the São Francisco River basin (Geise *et al*., 2010). On the other hand, *D. pernambucana* has somewhat a larger distribution range in the granitic rock outcrops of the remaining inselbergs in Pernambuco (Table 1; Figure 1).

Dyckia dissitiflora was included in the analysis due to its taxonomic association with the species from the PG populations, being considered to be the closest to this group according to M.G. Wanderley (personal communication, September, 2011). Therefore, the samples of *D. dissitiflora* comprising 37 individuals collected from three sites (Table 1) were included for a better comparison. Thus, considering the entire sampling, young leaves of 87 individuals sampled were stored in sodium chloride satured aqueous solution of cetyl trimethylammonium bromide (20 g CTAB/L) following Zel-Tur *et al*. (1999), until DNA

extraction. The total genomic DNA was isolated, using the CTAB procedure of Doyle & Doyle (1987) adjusted as described by Weising *et al*. (2005).

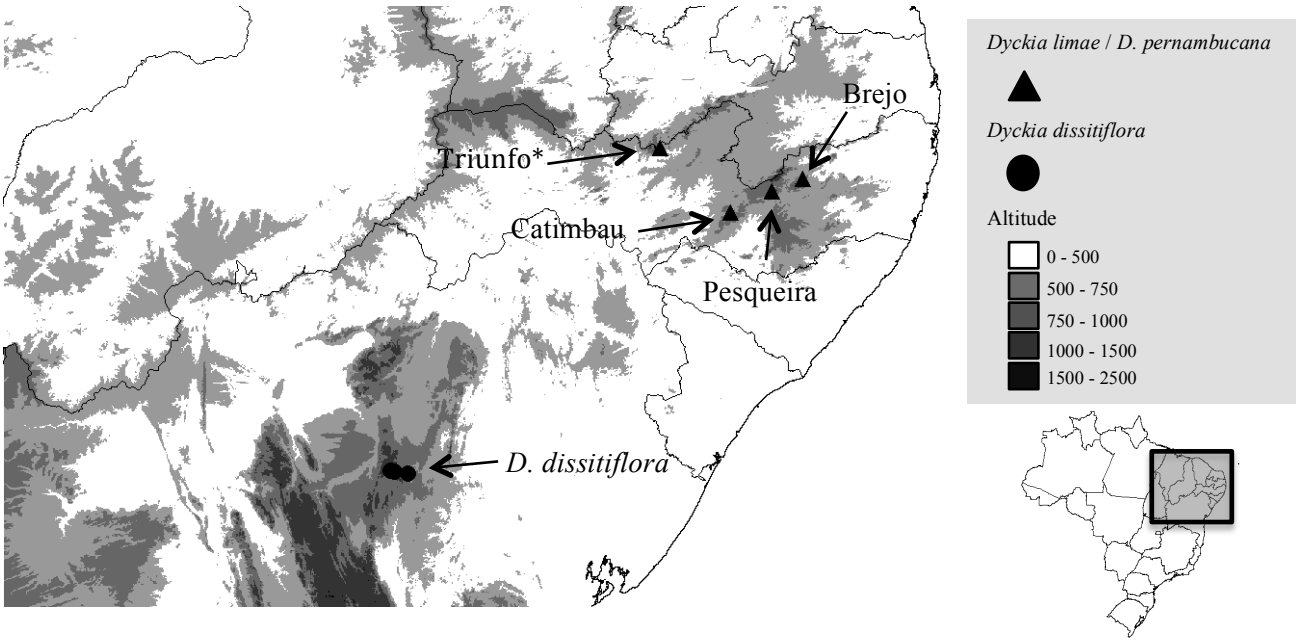

Figure 1. Altitudinal map showing the collections sites of *Dyckia dissitiflora* populations, located in the rocky outcrops of *Chapada Diamantina* and the representatives of PG populations: *D. pernambucana and D. limae* from the Borborema Plateau, Pernambuco, Brazil. (*) "*Pico*" and "*Srita*" populations located in Triunfo city.

2.2 Marker amplification and scoring

2.2.1 AFLP assays

The AFLP fingerprinting was performed according to the original protocol of Vos *et al.* (1995) and Debenet & Mattiesh (1999) with minor changes. In brief, 30 ng of genomic DNA were digested in a final volume of 25 µL at 37 °C with simultaneous restriction using endonucleases *Hind*III and *Mse*I, as well as adapters, for 12 h. The pre-selective and selective amplifications were carried out using primers with one (+1), two (+2) or three (+3) selective nucleotides at their 3' ends. The pre-selective PCR reactions contained 2 µL of 1:10 diluted restriction-ligation product, 0.5 µM of the unlabeled MseI +1 primers, 1 µL of the PCR buffer

10× (Peqlab blue), 25 mM $MgCl_2$, 0.2 mM of each deoxynuleoside triphosphate (dNTP) and 0.025 U of Taq polymerase (SawadyTaq, Peqlab, Germany). The mix reactions were subjected to 20 cycles of PCR amplification, each consisting of 94 °C for 20 s, 56 °C for 30 s and 72 °C for 2 min. Finally, the final extension was at 72 °C followed by 60 °C for 30 min.

A total of six primer combinations were used in the selective PCR (ACA/ACC, AAC/ACA, AAC/ATC, AGC/ATC, AAC/CAG, AGC/CTA) as follows: 2.5 µL of the 1:20 diluted pre-amplification product and different combinations of the *Mse*I (+3) primer (Carl Roth, Karlsruhe, Germany) (0.25 µM) and the fluorescence-labeled *Hind*III (+3) primer (WellRED- D2, -D3, -D4, Sigma Aldrich, Munich, Germany) (0.05 µM), were performed (Table 2). Final products of the selective PCR were run on an automated sequencer (CEQ8800, Beckman Coulter, Krefeld, Germany) as a multiplex of three primer combinations labeled and an internal size standard (GenomeLab DNA Size Standard Kit 600, Beckman Coulter, Krefeld, Germany).

The AFLP banding patterns were scored semi-automatically as presence or absence through the software Genemarker 1.9 (SoftGenetics, State College, PA, USA). The reproducibility test was also performed with approximately 15% of the total data set that had been taken randomly for this purpose. The intensity of each individual peak was normalized on the basis of a fixed threshold, with cutoff in less than 10% of the second highest signal intensity.

2.2.2 Nuclear and plastid microsatellite markers

In relation to the nuclear microsatellite markers, a total of 15 loci (Table 2), previously characterized for *D. manier-lapostollei* L.B.Sm (Wörhmann *et al.*, 2012b), were selected for the population analysis, using either unlabeled or fluorescent primer (forward or reverse), carrying a IRDye700 or IRDye800 label as detailed by Wörhman *et al.* (2012a).

Table 1. Population names, localities, geographical coordinates and sample sizes of *Dyckia* species from the inselbergs of Diamantina Plateau (Bahia) and "*brejos de altitudes*" (Pernambuco), northeastern Brazil.

Population Species code	City – State	GPS position	Altitude	Sample Size
"*Morrão*" *D. dissitiflora*	Morro do Chapéu – Bahia	S 11°35'24.5" W 41°12'26"	1276 m	9
"*Cachoeira*" *D. dissitiflora*	Morro do Chapéu – Bahia	S 11°37'40.5" W 41°00'01.8"	900 m	19
"*Lajes*" *D. dissitiflora*	Morro do Chapéu – Bahia	S 11°36'3.5" W 41°09'52.1"	1110 m	9
"*Catimbau*" *D. limae*	Buíque – Pernambuco	S 08°35'01.4" W 37°14'18.1"	898 m	10
"*Brejo*" *D. pernambucana*	Brejo da Madre de Deus – Pernambuco	S 08°12'11.56" W 36°23'53.34"	1040 m	10
Pesqueira ("*Pesq*") *D. pernabucana*	Pesqueira – Pernambuco	S8°20'33.64" 36°42'28.20"W	1060 m	10
Pico do Papagaio ("*Pico*") *D. pernambucana*	Triunfo – Pernambuco	S 07°49'21.9" W 38°03'19.3"	1183 m	10
Lajedo Santa Rita ("*Srita*") *D. pernambucana*	Triunfo – Pernambuco	S 07°49'23.4" W 38°03'52.9"	1172 m	10

In order to analyse maternal inheritance, all individuals were screened for variation at eight cpSSR markers (Supplementary Table S1), which were previously developed for *D. manier-lapostollei* (Krapp *et al*., 2012). The PCR amplifications were carried out using an indirect labeling fluorescence procedure, with the labeled forward primer IRDye700-M13 (Schuelke, 2000), as also described by Krapp *et al.* (2012). The PCR products were visualized in an automated sequencer (Li-Cor, 4200 IR^2, Li-Cor Biosciences, Bad Homburg, Germany), as detailed in Wörhmann *et al*. (2012a).

The scoring procedure was determined by visual examination of the fragment sizes, using an external size standard from sequences of the AT-rich *psbL-trnS* region of *Macaranga indistincta* that provides only the T reaction from the sequencing procedure (for details, see Guicking *et al.*, 2008). Thus, the identification and assignment of alleles were achieved from the resulting sets of T fragments with known sizes that were run in every sixth or seventh lane of the gel, as described by Wörhmann *et al*. (2012a).

2.3 Data analysis

2.3.1 Genetic diversity and F-statistics

For the AFLP assays, the percentage of polymorphic loci, unbiased estimates of genetic diversity (*Hj,* analogous to H_E) and the significance of genetic differentiation statistics (F_{ST}) among species/populations, were calculated using the software AFLP-SURV 1.0 (Vekemans *et al*., 2002) and obtained by means of 5,000 random permutations. Therefore, the allelic frequencies of AFLP fragments were evaluated using the Bayesian approach for diploid species proposed by Zhivotosky *et al*. (1999). This program was designed to use the approach of Lynch & Milligan (1994) to calculate population genetic parameters on the basis of the expected heterozygosity of dominant marker loci. The analysis of molecular variance (AMOVA) was performed, using the program Arlequin 3.5 (Excoffier *et al*., 2005), to

estimate variance components, partitioning of the variation of each species/individuals among populations with significance tests of 10.000 permutations.

Regarding the nuclear SSR loci, the number of polymorphic loci (P), number of alleles, heterozygosity measurements (H_O and H_E) and the variance in allele size (Var) were measured by using the software Microsatellite Analyser (MSA; Dieringer & Schlötterer, 2003). The software GENEPOP (Raymond & Rousset, 1995; Rousset, 2008) was used to estimate the inbreeding coefficient F_{IS} (Weir & Cockerham, 1984) and the deviation from the Hardy-Weinberg equilibrium (HWE). The global and pairwise index of genetic differentiation (F_{ST}) was estimated to infer the degree of population subdivision by using the MSA software, and resampling with 10,000 permutations to test its significance. The software Arlequin 3.5 was also employed in the nSSR data set in order to evaluate the molecular variance.

In the matter of the plastid DNA analysis, the identification of genetic variants of all plastid markers (haplotypes), the effective number (Ne), number of different haplotypes (A) and the number of private haplotypes (P_h) were estimated. Additionally, the haplotypic richness (H_R) was also estimated using the rarefaction method (El Mousadik & Petit, 1996) and the unbiased Nei's index of gene diversity (H_E) (Nei, 1973). Furthermore, the average genetic distance among individuals within datasets was estimated using the value of D_{sh}^2 (Goldestein *et al*., 1995) based on the averaged square sum of all length differences at microsatellites. All these indices were calculated by using the program Haplotype Analysis (Eliades & Eliades, 2009). In addition, the AMOVA was used to assess patterns of nuclear and plastid DNA differentiation in hierarchical models. Isolation by distance (Wright, 1965) was verified by calculating the correlation between geographical and genetic differentiation matrices, with application of Mantel test (Sokal & Rohlf, 1995), using 10.000 randomizations to determine significance.

2.3.2 Genetic distances

AFLP and microsatellite (from nucleus and chloroplast) data matrices were used to estimate genetic distances among individuals and populations. The Jaccard's dissimilarity coefficient was calculated for the binary data whereas the SSRs markers the simple matching method was employed by using the software DARwin 5.0 (Perrier & Jacquemoud-Collet, 2006). Dissimiliraty coefficients were used for clustering analysis based on weighted neighbor joining (WNJ) method.

2.3.3 Bayesian admixture analysis

A Bayesian clustering analysis was employed with the two nuclear marker systems (AFLP and nSSR) to assign individuals to genetic clusters (K) and to estimate admixture proportions (Q) for each individual, without considering sampling locations, using the software STRUCTURE 2.2 (Pritchard *et al*., 2000; Falusch *et al*., 2003a). The analyses were performed under the admixture model assuming correlated allelic frequencies and using a burn-in period of 50.000, run lengths of 300.000 and 10 iteractions per K. In order to determine the most likely number of clusters (K), we used the method proposed by Evanno *et al*. (2005), with the online program Structure Harvester to visualize the STRUCTURE 2.2 output.

3 Results

3.1 Variability across loci

The 15 nuclear microsatellite loci were significantly variable, varying from 3 (ngDy_8) to 36 (ngDy_27) alleles per locus (12 on average), and a total of 181 alleles identified among the 87 individuals from the populations of the three species (Table 2). The average for observed and expected heterozygosities ranged from 0.032 to 0.669 and from

0.032 to 0.834, respectively (Table 2). In relation to the F-statistics values, most of the nuclear SSR loci departed from the HWE in the intra-population level (F_{IS} estimates) for the PG populations (*D. limae* and *D. pernambucana*).

Regarding the cpSSRs, the eight loci generated two to six alleles per locus, as well as the identification of 25 individual haplotypes, 23 of them exclusive to one population (Supp. Table S1). On the other hand, bearing in mind only the PG populations, the combined genetic information yielded 12 distinguishable haplotypes, each one exclusive to a single population. The populations from "*Pico*" and "*Catimbau*" revealed the highest numbers of haplotypes: four and three, respectively (data not shown).

3.2 Genetic Diversity

The fraction of polymorphic nuclear SSR loci among species ranged from 66% to 93%, being slightly higher among populations of *D. dissitiflora* (Table 3). Likewise, the allelic richness (Rs) ranged from 2.183 ("*Brejo*") to 5.333 ("*Morrão*"), whereas expected heterozygosities ranged from 0.127 to 0.481 and 0.260 and 0.653, respectively, also with the *D. dissitiflora* populations exhibiting the highest values (Table 3). Conversely, the highest variation in allele size (Var) was obtained for a population from the PG ("*Pico*") with 206.547, and the smallest value (35.098) was also observed for a population of this group ("*Catimbau*"). Finally, most loci deviated from the HWE through the observation of their F_{IS} estimation (Table 3).

A total of 28 different cpSSR alleles within the whole sampling were found in the present work (ranging from 62 to 100 bp). Briefly, the cpDNA markers showed that all populations displayed polymorphic sites in their plastid microsatellite markers, except for the "*Srita*" population that showed a single fixed haplotype, whereas the remaining populations beared two to six haplotypes (Table 3; Supplementary Table S2). The populations "*Morrão*"

and "*Lajes*" exhibited the highest haplotypic richness (5.000 in each population) and values of genetic diversity (0.833 and 0.917, respectively; Table 3).

Table 2. Levels of genetic diversity and indices of F-statistics for 15 microsatellite loci (Wörhmann et al, 2012b) in *D. limae, D. pernambucana* and *D. dissitiflora* populations

Locus	*A*	H_O	H_E	F_{IT}*	F_{ST}*	F_{IS}*
ngDy_1	09	0.294	0.401	0.156	0.142	0.016
ngDy_3	09	0.331	0.385	0.252	0.247	0.006
ngDy_8	03	0.032	0.032	-0.013	-0.013	0.000
ngDy_10	05	0.317	0.269	-0.017	-0.017	0.000
ngDy_16	11	0.387	0.485	0.564	0.387	0.289
ngDy_17	14	0.298	0.520	0.628	0.184	0.544
ngDy_22	20	0.417	0.730	0.568	0.325	0.359
ngDy_24	13	0.243	0.375	0.591	0.374	0.346
ngDy_25	11	0.299	0.588	0.693	0.363	0.519
ngDy_27	36	0.669	0.834	0.378	0.147	0.271
ngDy_30	06	0.1 59	0.357	0.905	0.532	0.797
ngDy_31	11	0.185	0.370	0.732	0.168	0.679
ngDy_32	12	0.259	0.421	0.814	0.471	0.648
ngDy_45	13	0.508	0.520	0.397	0.319	0.114
ngDy_49	08	0.296	0.245	0.080	0.270	-0.259
Mean	**12.067**	**0.313**	**0.435**	**0.449**	**0.260**	**0.289**

Abbreviations: *A*, number of alleles per locus; *Ho*, observed heterozigosity; H_E, expected heterozigosity; F_{IT}, overall inbreeding coefficient; F_{ST}, fixation index; F_{IS}, inbreeding coefficient.
* Values calculated only for the focused populations of *Dyckia pernambucana* and *D. limae*.

The six AFLP primers combinations resulted in 347 non-monomorphic markers, from which 307 markers were present in the PG populations and 323 in the *D. dissitiflora* populations. The proportion of polymorphic loci ranged from 69.7% ("*Brejo*") to 84.5% ("*Morrão*") with the populations of *D. dissitiflora* exhibiting higher average values of polymorphic loci than the PG populations (Table 4).

Similarly, the Hj value within Pernambuco populations ranged from 0.192 ("*Brejo*") to 0.233 (*"Pico"*), with the populations of *D. dissitiflora* also displaying higher diversity values (Table 5). The global values of diversity were also observed for *D. dissitiflora* populations, where the diversity within populations (H_w) was higher than in the PG, whereas this latter revealed higher values of diversity between populations (H_b) (Table 4).

Table 3. Indices of genetic diversity in populations of *Dyckia pernambucana, D. limae* and *D. dissitiflora* yielded by the microsatellite markers

Population ID	Nuclear Microsatellites						Plastid DNA				
	P	*Rs*	*Var*	*Ho*	*He*	F_{IS} (W&C)	*N_e*	*Hpr*	*R_h*	*He*	D_{sh}^2
"*Catimbau*"[a]	73	3.350	35.098	0.380	0.391	0.010	1.852	3	1.900	0.511	1.114
"*Brejo*"[b]	93	2.183	68.790	0.127	0.260	0.367*	1.724	2	1.000	0.467	0.058
"*Pesq*"[b]	66	2.412	74.723	0.253	0.281	0.033*	1.724	2	1.000	0.467	0.058
"*Pico*"[b]	93	2.832	206.547	0.253	0.481	0.372*	2.941	4	2.800	0.733	0.167
"*Srita*"[b]	66	2.798	193.826	0.180	0.403	0.338*	1.000	1	0.000	0.000	0.000
PG/Mean	**78.200**	**2.715**	**115.797**	**0.239**	**0.363**	**0.010**	**2.060**	**2.750**	**1.675**	**0.545**	**0.349**
"*Morrão*"[c]	93	5.333	200.044	0.481	0.653	0.091*	3.857	5	5.000	0.833	3.174
"*Cachoeira*"[c]	80	3.788	51.540	0.460	0.482	0.142*	1.695	1	1.420	0.433	3.000
"*Lajes*"[c]	93	4.467	46.964	0.415	0.525	0.253*	5.400	5	5.000	0.917	2.569
Overall/mean	**82.125**	**3.395**	**109.692**	**0.319**	**0.4345**	**0.010**	**2.524**	**2.875**	**2.265**	**0.545**	**1.268**

Abbreviations: *P*, percentage of polimorphic loci; *Rs*, allelic richness; *Var,* variance in allele size; H_O, observed heterozigosity; H_E, expected heterozigosity; F_{IS}, inbreeding coefficient based on Weir and Cockerham (1984); *N_e*, effective number of haplotypes; *Hpr*, number of private haplotypes; *R_h*, haplotypic richness; H_E, genetic diversity; D_{sh}^2, mean genetic distance between individuals.

[a] *Dyckia limae;* [b] *D. pernambucana*; [c] *D. dissitiflora*

PG, Pernambuco Group

Departures of intra-population inbreeding coefficients from Hardy-Weinberg Equilibrium (HWE) are denoted by asterisk.

3.3 Patterns of genetic differentiation

The analyses of nSSR loci revealed notable values of divergence among PG populations, across all loci with a global F_{ST} value of 0.260 (Table 2). The AFLP markers also showed substantial variation among populations of the PG, however, lower than SSR analysis, with global F_{ST} of 0.196 (Table 4).

In general, pairwise population analysis revealed the differentiation among all pairs of populations with both SSR and AFLP markers, except for two near located populations ("*Pico*" and "*Srita*"- Table 5). Furthermore, a significant Mantel correlation was observed between genetic differentiation (F_{ST}) and geographical distance in both performed analysis (r = 0.473, p = 0.006 for nSSRs and r = 0.766, p = 0.020 for AFLP markers). Thus, the smallest geographic distance indicated lower differentiation among populations of *D. dissitiflora*, as well as the two populations mentioned above, which exhibited the shorter geographic distance (≈ 1 km) (Table 5).

The AMOVA results among the PG populations were highly congruent between the AFLPs and SSR data sets. Thus, the variation was higher within populations than among populations (69.19% and 68.63% respectively; Table 5). Conversely, the cpDNA data revealed that the variance relies on the variation among populations, instead of within populations (83.83% and 16.17%, respectively; Table 6).

3.4 Genetic distance-based analysis

The dissimilarity analysis performed (WNJ method) using the AFLP data resulted in a clear separation among populations of *D. dissitiflora* from the PG populations (Figure 2). While the populations of the first species did not show a clear separation, the populations of *D. pernambucana* and *D. limae* showed two major defined clades, except for Triunfo ("*Pico*" and "*Srita*"), indicating probably gene flow between those localities. Besides, *D. limae* was more closely related with Triunfo group, with significant statistical support (Figure 2).

Table 4. Comparison of genetic diversity and global differentiation among populations from Pernambuco group (*D. limae* and *D. pernambucana*) based on 347 AFLP markers

Populations	*N*	*TNL*	*PL*	*PPL*	H_J	S.E.(H_J)	H_T	H_W	H_b	*Global* F_{ST}
"*Catimbau*"	09	307	227	73.9	0.22091	0.00938				
"*Brejo*"	08	307	214	69.7	0.19118	0.00939				
"*Pesq*"	10	307	218	71.0	0.23320	0.00930	0.277	0.228	0.054	0.196
"*Pico*"	10	307	239	77.9	0.23921	0.01083				
"*Srita*"	10	307	237	77.2	0.22887	0.00977				
"*Morrão*"	09	323	273	84.5	0.30315	0.00915				
"*Cachoeira*"	17	323	236	73.1	0.27105	0.00987	0.304	0.285	0.018	0.060
"*Lajes*"	09	323	264	81.7	0.28217	0.00950				

N, number of individuals per population; PL, polymorphic loci; TLN, Total number of Loci; PPL, percentage of polymorphic loci; H_J, analogous expected heterozygosity (Zhivotvski, 1999); H_T, total diversity; H_W, average diversity within-population; H_b, average diversity between populations, F_{ST}, global fixation index.

Table 5. Pairwise F_{ST}-values (Weir and Cockerham, 1984) based on 15 SSRs loci (below the diagonal and 347 AFLPs loci (above the diagonal).

	Catimbau	Brejo	Pesqueira	Pico	Srita	Morrão	Cachoeira	Lajes
"Catimbau"	-	**0.245**	**0.152**	**0.127**	**0.125**	0.3002	0.3090	0.3028
"Brejo"	**0.345**	-	**0.179**	**0.312**	**0.296**	0.3581	0.3846	0.3719
"Pesqueira"	**0.305**	**0.491**	-	**0.225**	**0.222**	0.2889	0.3274	0.3169
"Pico"	**0.201**	**0.343**	**0.368**	-	**0.020**	0.2941	0.3270	0.3067
"Srita"	**0.237**	**0.367**	**0.381**	**0.034**	-	0.3089	0.3333	0.3101
"Morrão"	0.301	0.356	0.355	0.232	0.269	-	0.0697	0.0380
"Cachoeira"	0.391	0.434	0.422	0.353	0.380	0.117	-	0.0778
"Lajes"	0.370	0.451	0.423	0.322	0.366	0.041	0.078	-

Pairwise genetic differentiation within Pernambuco group (*D. limae* and *D. pernambucana*) in bold.

Table 6. Analysis of molecular variance (AMOVA) in the PG populations based on 307 AFLP, 15 nuclear SSRs and eight-chloroplast DNA loci.

Source of variation	*d.f.*	*Variance components*	*Percentage of variation*	*P-value*
AFLP markers				
Among populations	4	11.020 Va	30.81	P<0.001
Within populations	45	24.744 Va	69.19	P<0.001
Nuclear microsatellite				
Among populations	4	1.249 Va	31.64	P<0.001
Within populations	95	2.699 Va	68.63	P<0.001
*cp*DNA				
Among populations	4	1.314 Va	83.83	P<0.001
Within populations	45	0.253 Va	16.17	P<0.001

d.f., degrees of freedom

Furthermore, the tree based on the nSSRs yielded a different arrangement among populations and species, as well as lower bootstrap support in comparison with the AFLP data (Supplementary Figure S1). Five major groups could be identified, where all populations of *D. dissitiflora* comprised one group, except for three individuals of "*Morrão*", which grouped with "*Brejo*" individuals. The populations of "*Catimbau*" (*D. limae*) were more closely related with the two populations of Triunfo, which exhibited no clear separation among

individuals from different localities, as demonstrated by the AFLP analysis. Additionally, unlike the AFLP data, the populations from Triunfo yielded two different groups in the SSR analysis (Supplementary Figure S1).

3.5 Bayesian Clustering

The results of STRUCTURE analyses for both markers suggested the existence of three clusters, although the $K = 2$ also presented a high probability value in the AFLP analysis (Figures 3 and 4). Thus, the identification of the genetic clusters pinpointed one single group considering *D. dissitiflora* populations, in congruency with the dissimilarity analysis. Two groups were recognized for the PG populations. For the AFLP data set, one cluster comprised "*Brejo*" and "*Pesq*" populations and one cluster which included "*Catimbau*" and Triunfo.

For the SSR Bayesian STRUCTURE analysis, one single genetic cluster was also found for the populations of *D. dissitiflora* but, unlike the AFLP data, the two groups recognized for PG populations comprised the "*Catimbau*", "*Pesq*" and "*Brejo*" populations in one cluster, and some Triunfo individuals in another group (Figure 4). In both markers, the ΔK distribution supported the choice for $K = 3$, however, also $K = 2$ was applicable for AFLP data. The admixture proportion (Q) for each individual is shown in Figure 4.

4 Discussion

4.1 Populations genetic diversity

Dyckia species are configured as interesting models for population analysis due to their intrinsic biological features, such as the rupicolous/saxicolous nature, a wide range of morphological variability, adaptive radiation and isolated populations, exhibiting a particular colonization history (Forzza, 2001; Forzza *et al*., 2004; Hmeljevski *et al*., 2011; Leme *et al*., 2012) Thus, the population parameters provided here from different molecular markers (multilocus, nuclear and plastid) allow us to interpret some historical population patterns, in a

fine intra-specific scale.

Relationships among population may be affected by different factors, such as sample size, inbreeding, genetic drift, and fitness. In general, loss or maintenance of genetic diversity may depend on different characteristics of the plant species (mating system, pollination syndromes), mostly indicating a positive correlation between heterozygosity and populations fitness (Lovless & Hamrick, 1984; Hamrick and Godt, 1996; Kageyama *et al.*, 2003; Leimu *et al.*, 2006; Frankham *et al.*, 2010). In this regard, the overall genetic variation in the focused PG populations (*D. limae* and *D. pernambucana*) was high (nSSR, H_E = 0.343; cpSSR, H_E = 0.545 and AFLP, H_E = 0.277), when compared with other bromeliads previously studied (Izquierdo & Piñero, 2000 Sarthou *et al*., 2001; Sgobarti *et al.*, 2004) and also *Dyckia* species (*D. ibiramensis* with H_E = 0.219; Hmeljevski et al., 2011). On the other hand, the values of genetic diversity found in the inselberg bromeliad *Alcantarea imperialis* (Carrière) Harms and *A. geniculate* (Wawra) J.R.Grant (Barbará et al., 2007), as well as for populations of *Vriesea gigantea* Gaudich. and *Pticairnia* species (*P. albiflos*, *P. flammea* and *P. corcovandensis*; Palma-Silva *et al.*, 2009; 2011), were higher than the values observed here for *Dyckia* species.

The genetic diversity observed in the present analysis using dominant markers (AFLP fragments) was compatible with the data provided in the populations of three species of the sister genus *Encholirium* (Cavallari *et al.*, 2006). Despite the known occurrence of clonal habit in *Encholirium* species (also registered in some *Dyckia*), the dominant markers profiles indicated the maintenance of genetic variation. Additionally, the AFLP patterns shown for the *Dyckia* populations here were compatible with the genetic analysis among *Aechmea* species (Zhang *et al.*, 2012), revealing also great variability and genetic diversity.

Polymorphisms in chloroplast microsatellites have also been used to identify plant genetic diversity under various hierarchical levels (Provan *et al*., 2001; Cubas *et al.*, 2005; Terrab *et al.*, 2006; Pardo *et al.*, 2008). The cpSSRs markers employed in the present work

indicated that the focused PG populations seem to uphold a high level of genetic variation (Table 3), as observed in some populations of bromeliad with similar patterns of diversity (Palma-Silva *et al.*, 2009; 2011). This was also seen in the haplotypic richness values in the sampled populations, except for the "*Srita*" population, which exhibited a single haplotype and, therefore, no evidence of genetic variation (Table 3; Supplementary Table S2). These data suggest that despite the evidence of population bottleneck or recent re-colonization in the populations analysed in the present work, this in particular ("*Srita*") seemed to exhibit more clearly such phenomena. .

Furthermore, in accordance with the results of Barbará et al. (2007), the genetic diversity estimated at population level in both groups (PG and *D. dissitiflora* populations) showed higher values for *D. dissitiflora* populations in almost all parameters (allelic and haplotypic richness, observed and expected heterozygosities) with the three markers used. In the present case, it may also reflects the different geographical distribution ranges of the two groups (narrow endemic vs. endemic), given that narrow endemics or rares species are believed to have reduced genetic variability, which is essentially attributed to the influence of evolutionary forces, such as genetic drift or selection (Hamrick & Godt, 1996; Cole, 2003).

4.2 Genetic differentiation in fragmented populations of the Borborema Plateau

The evidence of moderated/high genetic divergence among PG populations was congruent between AFLP (F_{ST} = 0.196) and SSR (F_{ST} = 0.260) loci. This scenario of clear structuring also could be seen in the pairwise analysis (Table 5) among populations of the inselbergs in the Borborema Plateau. Similar patterns of differentiation have been found in other bromeliad species, especially those which exhibit comparable ecological features and life histories, such as populations of *Alcantarea* (Barbará *et al.*, 2007; 2008a), which inhabit rocky inselbergs in the Brazilian Atlantic Forest, or *V. gigantea* (Palma-Silva *et al*. 2009), *Pitcairnia* spp. (Palma-Silva *et al.*, 2011) and also, high differentiation among subpopulations

of the rare riparian *D. ibiramensis* (Hmeljevski *et al*., 2011). In this perspective, this isolated populations pattern suggest that, possibly, the low gene flow among populations allowed the influence of genetic drift (Wright, 1931).

According to Loveless & Hamrick (1984), the mating system is one of the factors that can play an important role in the spatial distribution of genetic variation within and among populations. In general, the genus *Dyckia* shows a mixed mating system (Hmeljevski *et al.*, 2011; Leme *et al.*, 2012). Thus, the existence of selfing or even mating between related individuals are thought to occur in the analyzed populations, possibly indicating an increase in inbreeding rates. Given that, the values of inbreeding coefficient (F_{IS}) were high in PG populations and significantly departed from de HWE, as expected for species that can present consistent selfing rates. Similar results of high inbreeding coefficients of populations (F_{IT}) were observed, implying, therefore, in the occurrence of the Wahlund effect (Hartl & Clark, 1997), which can be explained mainly by reduction of heterozygosity due to genetic subdivision.

The cpSSR haplotypes revealed stronger genetic structure when compared to nSSR and AFLP data, as also reported for *V. gigantea* (Palma-Silva *et al*., 2009). Nevertheless, in the present work there was no evidence of shared haplotype in the PG populations (Supplementary Table S2). The comparison of the partitioning of the genetic diversity (AMOVA) between nuclear and plastid markers revealed the distinct roles of pollen and seed dispersal. The congruent multilocus and nuclear data show that most of the variation lies within populations, in contrast to the results found for cpSSRs, which showed higher variation rates among populations (Table 6). Thereby, this remarkable trend can be positively associated with the hummingbird-driven pollination (Siqueira-Filho & Leme, 2006) and the wind-based seed dispersal in the *Dyckia* populations of the inselbergs, indicating higher efficiency in gene flow through pollen than seed dispersal in those populations, as recently

shown for other bromeliads (Barbará *et al*., 2008; Palma-Silva *et al*., 2009; Paggi *et al*., 2010; Palma-Silva *et al*., 2011).

Studies on the genetic diversity of bromeliad species of natural "terrestrial islands" with similar adaptive histories presented congruent results. Inselberg analyses in Bromeliaceae (Barbará *et al*., 2007; 2008a; 2009; Palma-Silva *et al.*, 2011), in comparison with the present work, revealed that the overview of the evolutionary processes in such populations were ruled essentially by biparental inbreeding, selfing, clonal growth and restricted seed dispersal as main determinants of the historical genetic structure in naturally fragmented populations. As suggested for *Vriesea* and *Alcantarea* populations, the capacity of *Dyckia* populations to colonize isolated rocky outcrops probably stems from their flexible mating systems and ability to endure inbreeding. In addition, the only population work available for *Dyckia* species (Hmeljevski *et al.*, 2011) also support this mentioned capacity to maintain genetic diversity and adaptive success (in this case, in a rheophytic environment) with significant inbreeding rates, in which the offspring was not composed exclusively by half-sibs, but a mixture of half-sibs, full-sibs and selfing-sibs.

4.3 Population subdivisions and historical fragmentation in the Borborema Plateau

The already mentioned isolation of the PG populations, in agreement with the inbreeding coefficients and isolation by distance (Mantel correlation test), was also supported by the WNJ distance analysis in the present work (Figure 2). The AFLP-based dendrogram confirmed *D. dissitiflora* as a relevant outgroup in the clustering analysis (Figure 2). On the other hand, *D. limae* ("*Catimbau*") could not be segregated from the populations of *D. pernambucana*. The distance-based analysis suggested the existence of two major groups in the "*brejos de altitude*": the "*Brejo*" and "*Pesqueira*" populations in one side and the Triunfo populations with *D. limae* in the other side, with significant statistical support (100% of

bootstrap; Figure 2). When compared with the Jaccard's coefficients, commonly used in binary distance matrices, the simple matching coefficient of the SSR dendrogram yielded a weaker resolution (Supplementary Figure S1), suggesting that in some cases, for co-dominant analysis the neighbor joining band-based approach may be not adequate (Bonin *et al.*, 2007).

The Bayesian cluster analysis also showed (and supported) these patterns and provided the first insights in the genetic structure of populations of *Dyckia* from Pernambuco. The AFLP clustering analysis yielded, as most-likely the existence of two clusters (K = 2), in accordance with the first node separation between the two major groups, while K = 3 also supported the existence of two genetic clusters within the PG populations, with a close relation between Triunfo and *D. limae* (Figures S2 A and 4A). The SSR clustering analysis also displayed a similar result, whith *D. limae* not distinguished as an isolated genetic cluster (Figures S2B and 4B). Based on the combined data from the markers used here, we believe that the three genetic differentiated groups seems to be the most likely scenario for the populations sampled in the inselbergs of the Borborema Plateau.

In this framework, one possible explanation relies on the biogeographical history of the Atlantic Forest at the north of the São Francisco River. According to the hypothesis proposed by Cavalcanti (2003), a sequence of divergence events would have defined a historical relation between the Atlantic Forest at the north of the São Francisco River and the southeastern portion of the Atlantic Forest. Briefly, it is proposed that the first event isolated the lowland forests from the large continuous forest, whereas the second event divided the forest corridor coming from south and southeast, but still with remaining connections with the inselbergs from "*Pesqueira*". The third big event probably separated the inselbergs in the city of Brejo da Madre de Deus ("*Brejo*") from the others: Triunfo, Buíque ("*Catimbau*") and Floresta. Consequently, the historical fragmentation of Atlantic Forest located at the north of the São Francisco River seems to be a possible explanation of the relationships, found in the

present work, between the populations of "*Catimbau*" and Triunfo ("*Pico*" and "*Srita*").

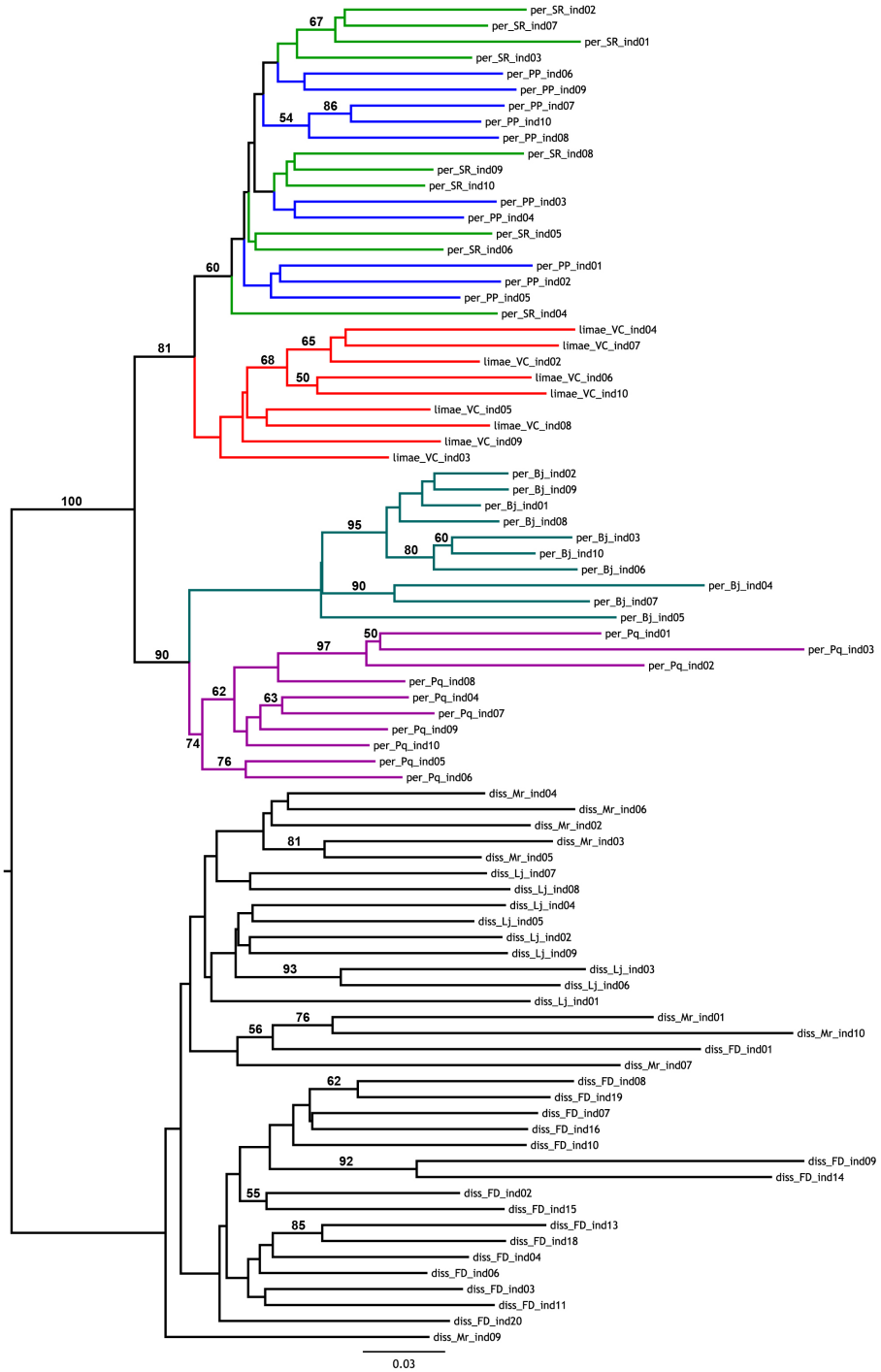

Figure 2. Dendrogram based on genetic distance of 347 AFLP bands using the Weighted Neighbour Joining method in all populations sampled. Black and colored branches indicate individuals of *D. dissitiflora* (black), *D. limae* (red) and populations of *D. pernambucana* (purple – "*Pesq*"; green – "*Srita*" and blue – "*Pico*"). Numbers above branches are bootstrap support values. Scale bar represents 3% divergence.

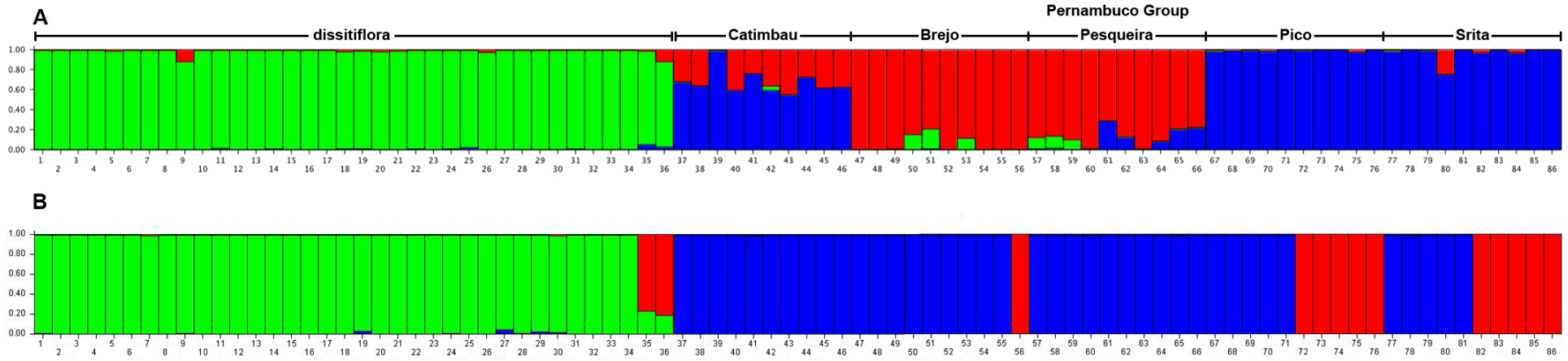

Figure 3. Bayesian admixture STRUCTURE proportions of individuals plants of *Dyckia* populations analyzed, for K=3 model. The results are based on 347 AFLP fragments (A) and 15 nuclear SSRs loci (B) and the identified three genetic clusters are indicated in different colors. The Pernambuco Group is formed by *D. limae* ("*Catimbau*") and (*"Brejo", "Pesqueira", "Pico"* and *"Srita"* populations)

5 Concluding Remarks

The combined results of genetic variation from different markers allowed us to identify hotspots of diversity in bromeliads from two important and exclusive types of vegetation: the "*Chapada Diamantina*" and the "*brejos de altitude*". The data suggested that despite the possible influence of genetic drift and selfing rates, the populations showed interesting patterns of diversity in the "continental islands-type" as high as seen in other bromeliad studies.

Considering the results discussed in this study, the application of AFLP and two types of SSR markers (nuclear and chloroplastidial), with particular evolutionary histories in the genome, has proved to be significantly powerful in evaluated *Dyckia* populations. Taking into account the morphological characteristics, as well as the molecular data achieved here, *D. limae* could not be confirmed and established as a separate taxonomic unit from the remaining populations sampled in Pernambuco. The PARNA Catimbau, the National Park located in the city of Buíque is the only sampling location in the Borborema Plateau with a sedimentary formation (instead of the remaining granitic formations), what could possibly explain the existing adaptive distinctions.

Studies concerning the fine-scale structure, phenology, paternity correlation and population dynamics should be addressed in the populations from Triunfo and Catimbau National Park to deepen the understanding of their relationship and also the real species status in the populations of *Dyckia* from Borborema Plateau, Pernambuco.

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Supplementary data

Table S1. Distribuition and frequencies of the haplotypes (cpDNA) in all *Dyckia* populations sampled

Haplotype Code	*Haplotype composition**	*Population*	*Frequencies*
Haplo-1	97 92 76 85 78 66 79 98	Brejo	0.70
Haplo-2	97 92 76 86 78 66 79 98	Brejo	0.30
Haplo-3	98 91 75 86 79 68 79 99	Mr	0.11
Haplo-4	98 91 75 86 79 68 81 99	Ca/Mr	0.74 / 0.44
Haplo-5	98 91 75 86 79 72 80 99	Lajes	0.11
Haplo-6	98 91 75 86 79 73 79 96	Lajes	0.22
Haplo-7	98 91 75 86 80 72 79 100	Ca/Lajes	0.21 / 0.22
Haplo-8	98 91 75 86 80 72 81 100	Lajes	0.05
Haplo-9	98 91 75 86 81 73 79 99	Lajes	0.11
Haplo-10	98 91 76 85 78 67 79 98	Pico	0.10
Haplo-11	98 91 76 86 79 72 79 99	Mr	0.11
Haplo-12	98 91 76 86 79 72 81 99	Mr	0.11
Haplo-13	98 91 76 86 79 73 79 96	Lajes	0.22
Haplo-14	98 91 76 86 79 73 79 99	Lajes	0.11
Haplo-15	98 91 76 86 80 72 79 99	Mr	0.11
Haplo-16	98 91 76 86 80 72 81 99	Mr	0.11
Haplo-17	98 91 77 85 78 67 79 98	Pico	0.40
Haplo-18	98 91 77 86 78 67 79 98	Pico	0.40
Haplo-19	98 92 77 85 78 67 79 98	Pico	0.10
Haplo-20	99 91 76 85 78 62 79 98	Cat	0.20
Haplo-21	99 91 76 85 78 62 80 98	Cat	0.10
Haplo-22	99 91 76 85 78 66 79 98	Cat	0.70
Haplo-23	99 91 76 85 78 67 79 98	Srita	1.00
Haplo-24	99 91 77 85 78 66 79 97	Pesq	0.70
Haplo-25	99 91 77 86 78 66 79 97	Pesq	0.30

* The haplotypes are characterized by their fragments sizes, in base pairs, across the eight plastid loci

Ca, "*Cachoeira*" population; Mr, "*Morrão*" population; Cat, "*Catimbau*" population

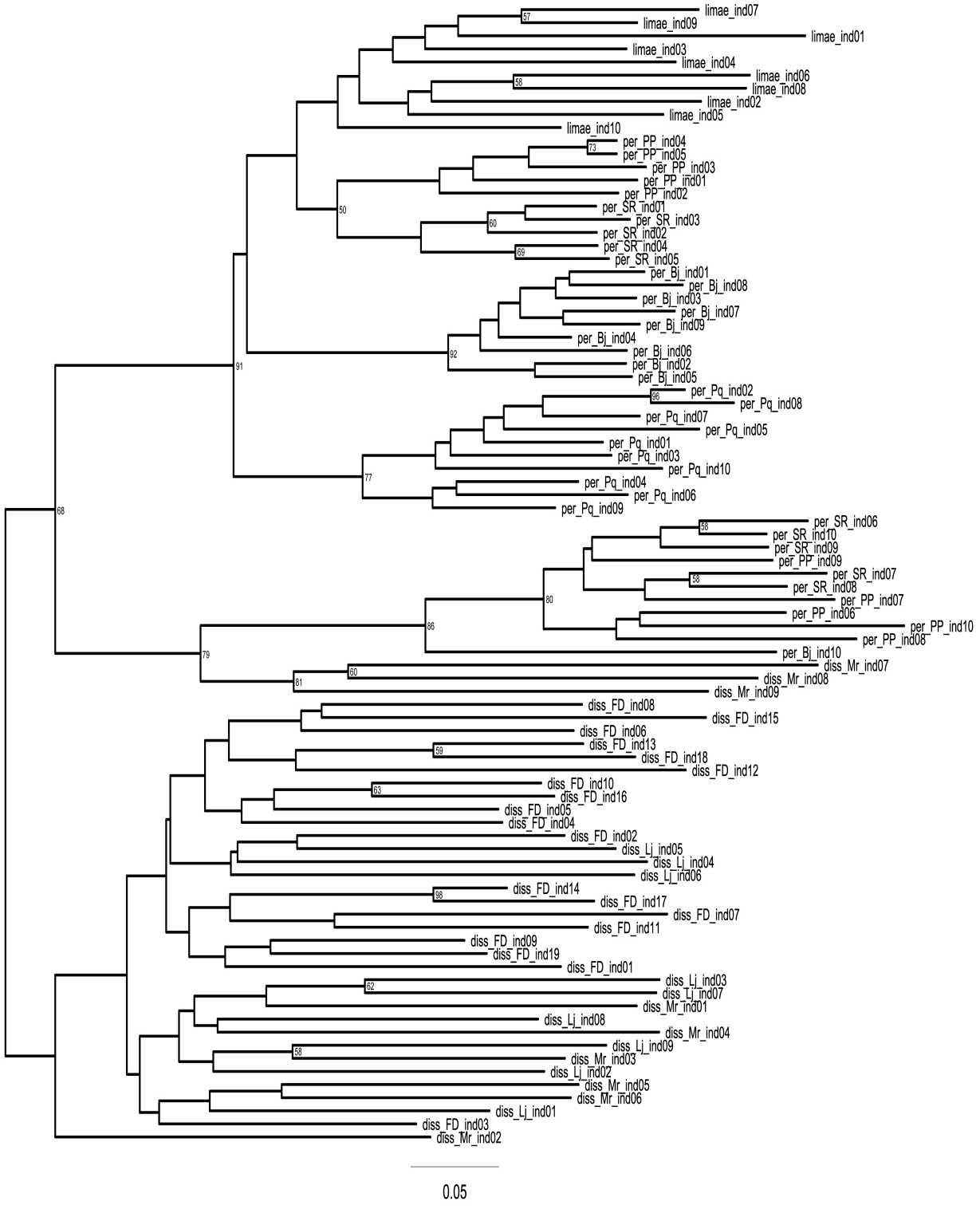

Figure S1. Weighted Neighbour joining dendrogram based on genetic distance of 15 nuclear SSR loci in populations of the three *Dyckia* species. Numbers are bootstrap support values.

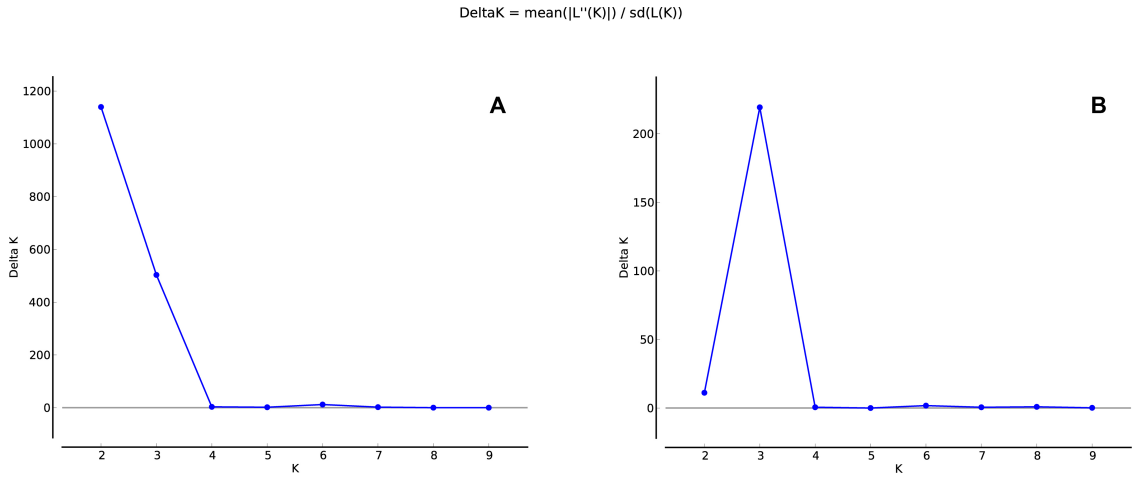

Figure S2. Indication of the most likely number of clusters after Evanno et al. (2005) in the STRUCTURE analisys. Results from 10 replicates for each $1 \leq \Delta K \leq 10$ values with both AFLP (A) and nSSRs (B).

Conclusões Gerais

- Os resultados, quanto à reconstrução filogenética aqui apresentados, forneceram os primeiros *insights* em nível infra-genérico no gênero *Dyckia*, reconhecido pela sua extrema diversidade morfológica e diversificação recente. Além disso, mesmo apresentando-se como um grupo claramente monofilético, o baixo suporte nos clados basais indica que, possivelmente, as barreiras reprodutivas inter-específicas ainda não estão inteiramente definidas. Desta forma, eventos de hibridizações e introgressões parecem ser comum em *Dyckia*, principal causa, portanto, do baixo sinal filogenético encontrado nas reconstruções realizadas.
- O conjunto de *primers* desenvolvidos (nucleares e plastidiais) mostraram-se promissores candidatos nas análises populacionais tanto dentro do gênero *Dyckia* como em outros grupos dentro da família Bromeliaceae, tendo em vista os elevados indices de amplificação heterólogas observados.
- Considerando os resultados populacionais apresentados, mediante a análise agregada de marcadores dominantes (AFLP) e co-dominantes (microssatélites), com particular histórias evolutivas dentro do genoma, notificou-se a significativa eficácia de tais marcadores em revelar padrões de conectividade e diversidade em populações de *Dyckia*
- Os dados moleculares em conjunto com as características morfológicas apresentadas pelas populações de *Dyckia,* ocorrentes nos inselbergs de Pernambuco, não puderam confirmar *D. limae* como uma unidade taxonômica definida, em relação as demais populações de *D. pernambucana*. Por ser micro-endêmica do PARNA do Catimbau, localizado na cidade de Buíque, e dada a natureza intrínseca do ambiente (única formação sedimentar do Planalto da Borborema), as distinções morfológicas encontradas possivelmente podem ser explicadas pelas particularidades adaptativas a tal formação rochosa elevados indices de diferenciação genetica.

Anexos

Instruções para autores:

American Journal of Botany

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Use conventions adopted by the scientific community for genetic symbols and nomenclature.

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Dow MM, Cheverud JM, Rhoads J, Friedlaender J. 1987b. Statistical comparison of biological and cultural/history variation. In: Friedlaender J, Howells WW, Rhoads J, eds. *Solomon Islands project: health, human biology, and cultural change*. New York: Oxford University Press, 265-281.
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