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FILOGENIA MOLECULAR, EVOLUÇÃO E BIOGEOGRAFIA DO GÊNERO *CRYPTANTHUS* OTTO & DIETR. (BROMELIACEAE).

RECIFE
MARÇO/2013

GEYNER ALVES DOS SANTOS CRUZ

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Tese apresentada à coordenação do Programa de Pós Graduação em Ciências Biológicas como parte dos requisitos para obtenção do título de Doutor em Ciências Biológicas, na área de concentração Biotecnologia/Biologia Celular e Molecular.

Orientador(a): Prof[a] Dr[a] Ana Maria Benko Iseppon

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Aos meus pais

Cícero e Rosalva, à minha irmã Geizy

e ao meu amor Lene

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RESUMO

Cryptanthus Otto & Dietr. é endêmico do Brasil e composto por 67 espécies distribuídas em floresta Atlântica, restingas, campos rupestres e Caatinga. Apresenta espécies terrícolas endêmicas e em sua maioria ameaçadas de extinção, devido à perda do habitat natural. O presente trabalho teve como objetivo reconstituir a filogenia molecular do gênero, medir e associar o tamanho genômico com a história evolutiva do grupo e estabelecer marcadores microssatélites para estudos populacionais. No primeiro capítulo utilizando 104 espécimes de *Cryptanthus,* foi reconstruída a filogenia molecular para o grupo a partir de AFLP, e realizada análise do estado de caracter ancestral para flores andromonóicas e hermafroditas. Foi observado que os subgêneros *Cryptanthus* e *Hoplocryptanthus* Mez. não são monofiléticos. Além disso, os grupos morfológicos previamente propostos para o gênero, apresentaram caracteres homoplásicos, exceto larcedae. Em relação à biogeografia, a colonização da floresta Atlântica parece ter surgido dentro do grupo múltiplas vezes, sendo predominante no subgênero *Cryptanthus.* Em *Hoplocryptanthus,* as espécies de Campos Rupestres e de floresta Atlântica apresentam uma separação bem definida, consistindo em mais um indício da condição polifilética deste grupo. A análise de estado de caracter ancestral, mostrou a importância das flores andromonóicas na diversificação do gênero especialmente na floresta Atlântica. No segundo capítulo, o tamanho genômico de 47 espécies de *Cryptanthus* foi estimado e comparado com o estado de caracter ancestral de diferentes tipos de habitats em que o gênero ocorre, a partir de uma filogenia molecular pré-estabelecida. Foi observado diferenciação significativa entre os dois subgêneros em relação à variação do tamanho genômico e as relações filogenéticas. Adicionalmente, diferenças significativas entre tamanho genômico e preferência por diferentes habitas, também foram observadas. Contudo, as espécies que ocorrem em floresta Atlântica não se diferenciam em relação apenas a preferência por habitats, assim sugerindo que às relações filogenéticas provavelmente são os fatores mais determinantes na variação observada do tamanho genômico de *Cryptanthus*. O terceiro capítulo abordou a avaliação de 34 loci de microssatélite plastidial (cpSSR) da espécie *Dyckia marnier-lapostollei* L.B. Smith., permitindo o estabelecimento de 29 loci, dos quais sete foram genotipados em três populações da espécie *C. schwackeanus* Mez e três da espécie *C. warren-loosei* Leme. Seis loci apresentaram polimorfismo entre as populações, assim demonstrando que os cpSSR estabelecidos são uma boa ferramenta para estudos populacionais no gênero. No conjunto os dados representam os primeiros passos para o entendimento da evolução e das relações do grupo com ferramentas moleculares.

Palavras-chave: Bromelioideae, AFLP, *Hoplocryptanthus*, evolução do tamanho genômico, microssatélites, preferência de habitats, andromonoicismo.

ABSTRACT

Cryptanthus Otto & Dietr. is endemic to Brazil consisting 67 of species occurring in the Atlantic forest, *restingas*, *campos rupestres* and *caatinga*. The group presents endemic terrestrial species, mostly threatened due to loss of the natural habitat. The present study aimed to reconstruct the molecular phylogeny of the genus, to estimate and associate the genome size with the evolutionary history of the group and to establish microsatellite markers for population analysis. In the first chapter, using 104 specimens of *Cryptanthus* and AFLP markers, the molecular phylogeny was reconstructed, and the ancestral character state analysis was performed using staminate and hermaphrodite flowers. It was observed that the subgenera *Cryptanthus* and *Hoplocryptanthus* Mez. are not monophyletic. Moreover, the morphological groups previously proposed for the genus showed homoplasic characters, except for larcedae. Regarding it's biogeography, the colonization of the Atlantic forest appears to have arisen multiple times within the group, being more prevalent in the subgenus *Cryptanthus*. In *Hoplocryptanthus*, the *campos rupestres* and the Atlantic forest presented a well-defined separation pattern, raising a further indication of the polyphyletic condition of this group. The ancestral character analysis showed the importance of the staminate flowers on the diversification of the genus mainly in the Atlantic forest. In the second chapter, the genome size of 47 *Cryptanthus* species has been estimated and compared with the character state of different types of habitats where the genus occur. It was observed a significant difference between the two subgenera toward the genome size variation and the phylogenetic relationships. In addition, significant difference between the habitat preference and genome size was also observed. However, the species which occur in Altantic forest do not significantly differ from one another in terms of habitat preference, thus suggesting that the phylogenetic relationships are likely the most determinant factors for the observed genome size variation in *Cryptanthus*. The third chapter deal with the evaluation of 34 microsatellite plastidial loci (cpSSR) from the species *Dyckia marnier-lapostollei* L.B.Smith., allowing the establishment of 29 loci, for *Crypthanthus* seven loci were genotyped in three populations of *C. schwackeanus* Mez and three of *C. warren-loosei* Leme, with 10 individuals being used for each population. Six loci showed polymorphism among populations, thus demonstrating that the established cpSSRs are good tools for population analysis in the genus. Altogether, the data represent the first steps to understanding the evolution and relationships of the group with molecular tools.

Keywords: Bromelioideae, AFLP, *Hoplocryptanthus,* genome size evolution, microsatellite, habitat preference, andromonoecy.

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LISTA DE ABREVIATURAS

AFLP	***Amplified fragment length polymorphism*; Polimorfismo de tamanho do fragmento amplificado**
BS	***Bootstrap***
BSA	***Bovine serum albumin*; Albumina de soro bovino**
ca.	**Cerca de**
CAM	***Crassulacean acid metabolism*; Metabolismo do ácido das crassuláceas**
CAPES	**Coordenação de Aperfeiçoamento de Pessoal de Nível Superior**
CI	***Consistency indices*; Índice de consistência**
CpSSRs	***Plastid microsatellite marker*; Marcador de microssatélite plastidial**
CR	**Criticamente ameaçada**
CTAB	***Cetyl trimethylammonium bromide*; Brometo de cetil trimetil amônio**
CV	***Coefficient of variation;* Coeficiente de variação**
DAAD	***Deutscher Akademischer Austauschdienst*; Serviço Alemão de intercâmbio acadêmico**
DNA	***Desoxyribonucleic Acid*; Ácido Desoxirribonucleico**
dNTP	***Deoxynucleoside triphosphate;* Desoxirribonucleotídeo trifosfato**
EP	**Em perigo**
EUA	**Estados Unidos da América**
EXN	**Extinto**
FCM	***Flow cytometry*; Citometria de fluxo**
Ma.	**Milhões de anos**
$MgCl_2$	**Cloreto de Magnésio**
ML	***Maximum likelihood*; Máxima verossimilhança**
mM	**Milimolar**
MP	***Maximum parsimony*; Máxima parcimônia**
PCR	***Polymerase chain reaction*; Reação em cadeia da polimerase**

PNADB	**Programa de Apoio ao Desenvolvimento da Botânica**
PROBRAL	**Programa de apoio a projetos de pesquisa Brasil/Alemanha**
RAS	***Random addition sequence*; Sequência de adição aleatória**
RI	***Retention indice*; Índice de retenção**
RNA	***Ribonucleic Acid*; Ácido Ribonucleico**
SNS	***Single nucleotide sequence*; Sequência de nucleotídeo único**
spp.	**Espécies**
SSR	***Simple Sequence Repeat*; Repetição de Sequência Simples**
Taq	***Thermus aquaticus***
TBE	**Tris/Borate/EDTA**
TBR	**Tree bisection reconnection**
UFPE	**Universidade Federal de Pernambuco**
VU	**Vulnerável**

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INTRODUÇÃO

A família Neotropical Bromeliaceae Juss. exibe notável variabilidade em termos de morfologia, fisiologia, hábito, habitat e interação animal-planta, onde a seleção de fontes de captação de água e nutrientes, aparentemente, tem exercido um importante impulso para a evolução na família. Adicionalmente, a origem independente e evolução convergente de características associadas com a adaptação a extremas condições ambientais são importantes componentes da história evolutiva do grupo (Smith e Downs, 1974, 1977, 1979; Benzing, 2000). Devido à ampla variedade de características adaptativas, Bromeliaceae possui espécies distribuídas em grande parte dos ecossistemas americanos, principalmente nas florestas tropicais, campos rupestres e savanas, a exceção de uma única espécie que ocorre no continente Africano (Smith e Downs, 1974; Jaques-Felix, 2000). As bromélias também constituem uma importante fonte alimentar e de plantas ornamentais (Benzing, 2000).

Em vista da importância ecológica, econômica e cultural de Bromeliaceae, estudos filogenéticos recentes têm buscado entender os passos evolutivos que levaram à sua diversificação em toda América do Sul e Central (Crayn et al., 2000, 2004; Givnish et al., 2007, 2011; Horres et al., 2007; Shulte et al., 2005, 2009; Rex et al., 2009). Com base nesses estudos, vários gêneros de Bromeliaceae não apresentam clara delimitação, representando assim grupos não monofiléticos (Sass e Spech, 2010; Shulte et al., 2005, 2009). Contudo, muitos estudos de filogenia molecular abrangem apenas poucos representantes das linhagens que compõe a família, permanecendo em aberto muitas questões taxonômicas e evolutivas envolvendo os gêneros, principalmente no que diz respeito às relações infragenéricas. Adicionalmente, informações cariotípicas têm se tornado uma ferramenta útil na elucidação das relações filogenéticas e tendências evolutivas da família, embora ainda são poucas as espécies estudadas citogeneticamente (Gitaí et al., 2005).

O gênero *Cryptanthus* Otto & Dietr. é classificado dentro da subfamília Bromelioideae (Shulte e Zizka, 2008; Shulte et al., 2009; Givnish et al., 2011), a qual engloba 67 espécies de hábito terrícola endêmicas do Brasil e distribuídas em Floresta Atlântica, restingas, campos rupestres e Caatinga, desde a região Nordeste até o Sudeste (Luther, 2010). É considerado um grupo distinto dentro da família, por apresentar número cromossômico básico x=17, enquanto que para o restante da família x=25, apresenta características consideradas pleisiomórficas como, sistema radicular bem desenvolvido, e também derivadas, como é o caso da fotossíntese do tipo CAM (Ramirez-Morillo, 1996). O gênero é dividido em dois subgêneros *Cryptanthus* e *Hoplocryptanthus,* diferenciados por

características morfológicas, ecológicas e distribuição geográfica (Mez, 1896; Ramirez-Morillo,1998).

As espécies de *Cryptanthus*, em sua maioria, estão concentradas em Campos Rupestres e especialmente na Floresta Atlântica, apresentando um alto grau de endemismo. Estes ambientes são historicamente sujeitos a exploração antrópica, principalmente por desmatamentos, extrativismo e mineração, causando a diminuição do habitat natural (Silva e Andrade, 2005; Versieux et al., 2008). Neste cenário, estudos sobre a diversidade e padrões de endemismo da família Bromeliaceae nestes biomas apontam a presença de várias espécies do gênero em listas de espécies ameaçadas de extinção (Martinelli et al., 2008).

O processo de fragmentação e modificação dos habitats naturais causam a erosão genética, fato preocupante, pois a perda de variabilidade influencia diretamente na adaptação dos indivíduos e na viabilidade em curto prazo das populações remanescentes (Frankham, 2005), especialmente no caso das espécies de *Cryptanthus* que são micro-endêmicas em sua maioria. Portanto, atualmente a conservação dos processos evolutivos e conhecimento da diversidade filogenética são também reconhecidos como prioridades (Mace et al., 2003). Mesmo diante da importância ecológica e evolutiva de *Cryptanthus* e seu atual estado de conservação, ainda são poucos os trabalhos abordando a filogenia e evolução do gênero. Desta forma, a reunião de informações como filogenia molecular, reconstrução de estado de caráter ancestral, tamanho genômico e estudos populacionais, torna este estudo uma base que possibilitará prospecções sobre o manejo e a conservação de *Cryptanthus*.

Nesse contexto, os objetivos desse estudo foram:

- Reconstruir a filogenia molecular do gênero *Cryptanthus* a partir de AFLP no intuito de elucidar as relações infragenéricas.
- Medir e associar o tamanho genômico com a filogenia molecular e ocupação de diferentes habitats do gênero.
- Estabelecer marcadores microssatélites (cpSSR) para estudos de genética populacional.

1. Características Gerais da Família Bromeliaceae

A família Bromeliaceae Juss. é composta por cerca de 58 gêneros e 3248 espécies e está entre as mais características da região neotropical (Luther, 2010). Distribui-se em grande parte dos ecossistemas americanos, desde o sudeste dos EUA até regiões do Chile e Argentina na América do Sul. Apenas uma única espécie é encontrada no oeste do continente Africano [*Pitcairnia feliciana* (A.Chev.) Harms & Mildbr.], provavelmente fruto de um evento de dispersão em longa distância (Smith e Downs, 1974; Jaques-Felix, 2000; Givnish et al., 2004).

As espécies de Bromeliaceae são plantas herbáceas, em geral rizomatosas, com folhas simples lanceoladas em roseta, formando por vezes um recipiente central ou fitotelmo, que retém água e nutrientes. As inflorescências geralmente são racemosas situadas na posição central da roseta contendo flores com três pétalas e ovário variando de súpero a ínfero com placentação axial. Os frutos podem ser de dois tipos, cápsula ou baga, com sementes aladas, plumosas ou desprovidas de apêndices (Smith e Downs, 1974, 1977, 1979; Kubitzki, 1998; Benzing, 2000).

No que se refere aos aspectos ecológicos, a família apresenta distintas adaptações, ocorrentes nas formas de vida terrícola e epifítica incluindo hemiepifítica, o que possibilita a colonização de diferentes ambientes, bem como uma significativa diversificação dentro dos neotrópicos, tornando-se um caso notável de radiação adaptativa (Benzing, 2000; Givnish et al., 2004). O sucesso da colonização de diversos ambientes está intimamente ligado à presença de inovações-chave, tais como tricomas epidérmicos responsáveis pela absorção de água e o desenvolvimento de várias estratégias de adaptação ao estresse hídrico, como suculência, represamento foliar e fotossíntese do tipo CAM (Crayn et al., 2004; Shulte e Zizka, 2008). Em vista destas diversas estratégias, as bromélias destacam-se como importantes elementos ecológicos em muitas comunidades, contribuindo para a complexidade estrutural do ambiente, o que se reflete diretamente na riqueza e diversidade do grupo, incluindo associações de flora e fauna envolvendo seus representantes (Benzing, 2000).

Apesar de amplamente distribuída nos neotrópicos, a família concentra o maior riqueza de espécies nas Florestas Tropicais, com destaque para a Floresta Atlântica brasileira (considerada um centro de diversidade de Bromeliaceae) com mais de 500 espécies e variedades morfológicas (Martinelli, 1994; Benzing, 2000). No entanto, a progressiva destruição da Floresta Atlântica tem causado a perda do habitat e consequente fragmentação deste bioma. Este cenário com alta influência antrópica tem levado à retirada de árvores, que são habitat natural das bromélias epífitas e dos

vertebrados dispersores de suas sementes (Benzing, 2000; Dimmit, 2000; Laurance et al., 2000; Chapman et al., 2003), além da invasão do habitat por plantas ruderais e da exposição da floresta a queimadas e coletas pela população humana local (Clark et al., 1995; Tabarelli et al., 2004). Estas circunstâncias, aliadas à distribuição geográfica restrita de várias espécies, têm levado a uma diminuição na variedade das espécies de bromélias ocorrentes neste bioma (Siqueira e Tabarelli, 2006).

A ampla variedade de adaptações, distribuição em diferentes ecossistemas e riqueza de espécies de Bromeliaceae não parecem estar diretamente relacionados aos números cromossômicos descritos para as espécies da família, uma vez que o número básico (x=25) e diploide (2n=50) são relativamente conservados (Marchant, 1967; Mcwillians, 1974; Brown e Gilmartin, 1983, 1989; Brown et al., 1984, 1997). No entanto, são observadas algumas variações onde o principal mecanismo evolutivo encontrado é a poliploidia, como por exemplo, em espécies dos gêneros *Bromelia* L., *Orthophytum* Beer, *Deuterocohnia* Mez e *Deinacanthon* Mez com números cromossômicos variando entre 2n=100, ca. 150 e ca. 160 (Cotias-de-Oliveira et al., 2000, 2004; Gitaí et al., 2005; Louzada et al., 2010).

O gênero *Cryptanthus* Otto & A. Dietr. representa, por sua vez, um caso único na família, diferindo do padrão citado anteriormente por apresentar 2n=34, 36, 42 e 54 e número básico x=17, além da presença de cromossomos B em *C. bahianus* L.B. Sm. A hipótese mais aceita para tal discrepância é disploidia descendente como mecanismo responsável pela variação dos números cromossômicos (Gitaí et al., 2005; Ceita et al., 2008). A estabilidade do número cromossômico para a família é em geral refletida também no tamanho genômico, com a maioria das espécies apresentando genomas pequenos e em média com 2*C*=1.16 pg (Favoreto et al., 2012).

Bromeliaceae é mundialmente reconhecida não só pelo importante papel ecológico, mas também devido ao seu significativo valor econômico como ornamental e para fins alimentícios, como é o caso do abacaxi [*Ananas comosus* (L.) Merr.]. Nas últimas duas décadas, a família tornou-se mais popular no Brasil devido ao seu valor ornamental em casas e jardins, porém esta tendência tem levado a um aumento da pressão antrópica nas populações naturais (Versieux e Wendt, 2007). As bromélias cultivadas para ornamentação são, em parte, exportadas para países como EUA, movimentando um comércio bastante lucrativo na ordem de milhões de dólares (Cathcart, 1995).

2. Filogenia e Evolução de Bromeliaceae

A família Bromeliaceae foi considerada como parte da ordem Bromeliales com base em características morfológicas (especialmente) e número cromossômico (Cronquist, 1981; Brown e Gilmartin, 1984; Dahlgren et al., 1985). No entanto, com o avanço dos estudos filogenéticos

utilizando dados morfológicos associados a dados moleculares, Bromeliaceae foi posicionada na ordem Poales formando um grupo monofilético considerado como irmão do restante das famílias desta ordem (Gilmartin e Brown, 1987; Crayn et al., 2004; Linder e Rudall, 2005; APG III, 2009; Givnish et al., 2007, 2010, 2011).

Smith e Downs (1974, 1977, 1979), analisando a morfologia de flores, frutos e sementes, dividiram a família em três subfamílias: Pitcairnioideae, Bromelioideae e Tillandsioideae, sendo a primeira um grupo polifilético (Terry et al. 1997; Horres et al. 2000; Crayn et al. 2004; Givnish et al. 2004, 2007). Vários estudos em Bromeliaceae, baseados especialmente em caracteres morfológicos e em alguns casos também em dados moleculares (Gilmartin e Brown, 1987; Givnish et al., 1992; Clark et al., 1993), foram realizados no intuito de elucidar as relações filogenéticas entre as subfamílias. Tais análises, porém, apresentaram progresso limitado principalmente devido ao alto índice de homoplasias morfológicas, bem como a baixa resolução obtida através das regiões genômicas plastidiais empregadas (Givnish et al., 2007).

A partir da utilização de novas regiões plastidiais, inclusão de sequências nucleares e/ou da combinação de ambas, a topologia e suporte estatístico dos estudos filogenéticos aumentou. Um dos primeiros trabalhos seguindo esta tendência foi o de Terry et al. (1997), os quais usaram uma nova região plastidial de rápida evolução (*ndh*F) trazendo maior resolução filogenética em Bromeliaceae. Neste estudo foi observado que Tillandsioideae é um grupo monofilético formando um ramo divergente, enquanto que as espécies de Pitcairnioideae formaram um grupo parafilético, sendo o gênero *Puya* irmão do grupo monofilético Bromelioideae. A partir deste trabalho, vários outros estudos foram conduzidos a partir de diferentes sequências genômicas plastidiais e nucleares (Tabela 1). Dentre os mais recentes, Givnish et al. (2007, 2011) baseados na combinação de regiões plastidiais, apontaram a divisão da família em oito subfamílias (Brocchinioideae, Bromelioideae, Hechtioideae, Lindmanioideae, Navioideae, Pitcairnioideae, Puyoideae e Tillandsioideae).

Além das sequências genômicas outros marcadores como o AFLP (Tabela 1), estão sendo usados geralmente direcionados a grupos específicos. De acordo com Després et al. (2003), em comparação com o sequenciamento de genes, o AFLP permite obter rapidamente muitos marcadores polimórficos amplamente distribuídos em todo o genoma das espécies estudadas, sem o conhecimento prévio sobre este genoma. Desta forma, em vez de gerar uma árvore de um gene em particular, que não reflete necessariamente a classificação filogenética das espécies (especialmente entre táxons intimamente relacionados e híbridos sujeitos a evolução reticulada), a análise simultânea de muitos loci representando todo o genoma tem o potencial de gerar uma árvore mais robusta. Portanto, o AFLP tem sido uma ferramenta útil em análises de filogenia molecular e diferenciação genética na família Bromeliaceae.

Tabela 1: Publicações referentes à filogenia molecular e parentesco genético em Bromeliaceae, ordenadas cronologicamente. Legenda para abreviações: Plastidial (P); Nuclear (N).

Autores	Sequências / Marcadores	Grupo taxonômico
Horres et al. (2000)	*trn*L (UUA) intron (P)	Bromeliaceae
Reinert et al. (2003)	*mat*K (P)	Pitcairnioideae
Crayn et al. (2004)	*mat*K, *rps*16 (P)	Pitcairnioideae
Givnish et al. (2004)	*ndh*F (P)	Bromeliaceae
Shulte et al. (2005)	*mat*K, *trn*K intron, *trn*L intron, *trn*K-*trn*F espaçador (P)	Bromeliaceae
Horres et al. (2007)	*trn*L intron, *trn*T-*trn*L, *trn*T-*trn*F espaçador	Bromelioideae
Sousa et al. (2007)	*mat*K, *psb*A-*trn*K, *trn*L-*trn*F (P)	*Lymania* R.W. Read
Rex et al. (2007)	Marcadores AFLP	*Fosterella* L.B. Smith
Shulte & Zizka (2008)	*atp*B-*rbc*L espaçador, *trn*L intron, *trn*L-*trn*F espaçador, *mat*K-*trn*K (P)	Bromelioideae
Rex et al. (2009)	*atp*B-*rbc*L espaçador, *psb*B-*psb*H espaçador, *mat*K, *rps*16 intron (P)	Pitcairnioideae
Shulte et al. (2009)	PRK (N); *atp*B-*rbc*L, *trn*L-*trn*F espaçador (P)	Bromelioideae
Sass & Spech (2010)	ETS, *rpb*2, g3*pdh* (N); *trn*L intron, *trn*F espaçador	*Aechmea* Ruiz & Pav.
Shulte et al. (2010)	Marcadores AFLP	*Puya* Molina
Jabaily & Sytsma (2010)	*trn*S-*trn*G, *mat*K, *rps*16 (P); PHYC (N)	*Puya* Molina
Jabaily & Sytsma (2012)	Marcadores AFLP	*Puya* Molina
Louzada (2012)	PHYC (N); *trn*L-F, *psb*A-*trn*H (P)	*Orthophytum* Beer
Versieux et al. (2012)	Marcadores microssatélite (nSSR); *trn*K-*rps*16, *trn*C-*pet*, FLO/LFY (N)	*Alcantarea* (E.Morren ex Mez) Harms
Wagner et al. (2012)	*atp*B-*rbc*L espaçador, *psb*B-*psb*H espaçador, *rps*16 intron, *mat*K, *rpl*32-*trn*L, *rps*16-*trn*K espaçador (P)	*Fosterella* L.B. Smith
Zhang et al. (2012)	Marcadores AFLP	*Aechmea* Ruiz & Pav.
Louzada et al. (*in prep.*)	Marcadores AFLP	*Orthophytum* Beer
Pinangé et al. (*in prep.*)	Marcadores AFLP	*Dyckia* Schult. & Schult.f.
Wagner et al. (*in prep.*)	Marcadores AFLP	*Fosterella* L.B. Smith

Apesar dos avanços alcançados no entendimento das relações filogenéticas, ainda são muitas as dificuldades na construção de filogenias moleculares com melhor resolução entre os táxons de Bromeliaceae. Desta forma, análises alternativas também são utilizadas, como por exemplo o "*DNA Barcoding*" que segue três princípios a padronização, o minimalismo e a escalabilidade (Hollingsworth et al., 2011). Contudo, em plantas é difícil obter uma sequência de DNA com estas características, de forma que geralmente são usados combinações de sequências (Hollingsworth et al., 2011). Em Bromeliaceae, 101 acessos abrangendo 46 espécies foram testados por Maia et al. (2012) usando a técnica de *DNA Barcoding* a partir de duas sequências plastidiais (*rbc*L/*mat*K). Quando comparada a outras angiospermas (Asteraceae e Orchidaceae) analisadas em estudos anteriores, a família apresentou o menor índice de sucesso para identificação de espécies. Segundo os autores, tais resultados podem ser explicados pela presença de diversos complexos taxonômicos, grande variedade ecológica e recente evento de divergência (19 Ma) entre suas linhagens.

No que diz respeito aos aspectos evolutivos, Bromeliaceae compreende grupos eco-morfológicos distintos. Espécies das primeiras linhagens divergentes apresentam sistema radicular bem desenvolvido (caso da maioria das espécies que apresentam hábito terrícola e litofítico), possibilitando a captação de água e nutrientes diretamente do solo com pouca ou nenhuma capacidade de armazenamento externo de água, onde cada folha forma um fitotelmo distinto. Por outro lado, o grupo composto de espécies consideradas derivadas (na sua maioria epífitas) apresenta como principais características tricomas capazes de reter água e nutrientes, além de possuir capacidade de armazenamento de água (o tanque que constitui o fitotelmo) na parte central da roseta (Smith e Downs, 1979; Shulte et al., 2009). As Tillandsioideae que dependem apenas do indumento foliar no armazenamento de água e nutrientes, têm sido propostas como o grupo mais derivado de toda família (Shulte et al., 2009).

Características morfológicas e ecológicas também vêm sendo utilizadas conjuntamente com caracteres moleculares em estudos filogenéticos a fim de avaliar e datar a evolução de caracteres dentro da família. Neste sentido, Givnish et al. (2004, 2007, 2011) utilizaram um relógio molecular baseado em análises anteriores com monocotiledôneas para a determinação da história biogeográfica de Bromeliaceae. Os autores sugerem que a família surgiu há 100 Ma (milhões de anos) na região do escudo das Guianas na costa oeste da América do Sul, entre o Brasil e a Venezuela, tendo se distribuído pelas Américas entre 16-13 Ma, com diversificação entre as linhagens modernas há cerca de 19 Ma. Por sua vez, a única espécie de bromélia africana (*P. feliciana*) foi datada em 9,3 Ma.

Outros estudos de filogenia molecular gerados para grupos de Bromeliaceae foram focados na análise evolutiva de caracteres de valor sistemático. Este tipo de abordagem foi utilizado, por exemplo, por Shulte e Zizka (2008), que em análises de filogenia molecular baseada em cinco loci do

genoma plastidial observaram que a presença de pétalas com ou sem apêndices é um caráter problemático na delimitação genérica da subfamília Bromelioideae, exibindo um alto índice de homoplasia. Diferentes estudos (Crayn et al., 2004; Shulte et al., 2005, 2009; Jabaily e Sytsma, 2010) mostram que outros caracteres de importância evolutiva como distribuição geográfica, simetria das pétalas, hábito tanque e padrão fotossintético também são informativos para a reconstrução de estados de caráter ancestrais em diferentes grupos de Bromeliaceae.

3. O gênero *Cryptanthus* Otto & A. Dietr.

3.1 Características gerais e distribuição geográfica

Endêmico do Brasil, o gênero é composto por 67 espécies (Luther, 2010) ocupando uma grande variedade de habitats como a Floresta Atlântica, os ambientes de restinga, os campos rupestres e a Caatinga. Todas as espécies conhecidas são terrícolas ou rupícolas, distribuindo-se geralmente em locais sombreados em sub-bosques, ou ainda – mais raramente – totalmente expostas em campos de altitude. As espécies ocorrem desde o estado do Rio Grande do Norte no Nordeste até Minas Gerais no Sudeste do país, tendo como centro de diversidade a Floresta Atlântica e os campos rupestres nos estados do Espírito Santo, Bahia e Minas Gerais (Ramirez-Morillo, 1996, Ramirez-Morillo e Brown, 2001).

3.2 Conservação

A Floresta Atlântica, apesar de ser uma das mais importantes do mundo devido à sua riqueza de espécies e grande número de endemismos, vem sofrendo uma drástica redução de suas áreas através de atividades antrópicas (Silva e Andrade, 2005). De acordo com Martinelli et al. (2008), grande parte do gênero (87%) ocorre no domínio da Floresta Atlântica [maiores concentrações nos estados do Espírito Santo (25 ssp.) e da Bahia (10 spp.)], *Cryptanthus* apresenta em torno de 25 espécies presentes em listas de extinção em diferentes categorias, sendo 20 como vulneráveis (VU), três em perigo (EP), uma criticamente ameaçada (CR) e uma extinta da natureza (EXN). Adicionalmente, a cadeia do Espinhaço, que se estende pelos estados de Minas Gerais e da Bahia, abriga os campos rupestres, onde *Cryptanthus* é um dos gêneros que apresenta maior grau de endemismo entre as bromélias com 73% das espécies endêmicas. Estas espécies, assim como as ocorrentes na Floresta Atlântica, também estão sob forte pressão devido à exploração antrópica deste habitat (Versieux et al., 2008).

3.3 Aspectos morfológicos e filogenia

Em geral, as espécies do gênero apresentam folhas formando uma roseta (sem fitotelmo), sistema radicular bem desenvolvido, com algumas podendo ser caulescentes curtas ou longas. Adicionalmente, *Cryptanthus* apresenta pétalas conadas de margens inteiras sem apêndices, além de estames antepétalos adnatos (Louzada e Versieux, 2010). A reprodução ocorre através da formação de brotos basais ou axiais, estolões ou por sementes (Ramirez-Morillo, 1996). De acordo com os principais estudos disponíveis para o grupo baseados em características morfológicas, ecológicas e distribuição geográfica (Mez, 1896; Smith e Downs 1979; Ramirez-Morillo, 1996), o gênero é dividido em dois subgêneros, *Cryptanthus* e *Hoplocryptanthus*. A partir da revisão do grupo feita por Ramirez-Morillo (1996), baseado em caracteres morfológicos, foi proposto a divisão dos subgêneros em seções. Contudo, como estas hipóteses não são validamente publicadas, iremos tratar aqui como "grupos morfológicos". Desta forma, o subgênero *Cryptanthus* é subdividido em cinco grupos morfológicos e apresenta como uma das principais características a tendência ao andromonoicismo (flores hermafroditas e masculinas no mesmo indivíduo). Adicionalmente, suas espécies possuem estigmas eretos, flores sem odor, pólen reticulado e cerca de oito sementes por fruto. Seus membros habitam ambientes de florestas, restingas e caatinga. Por sua vez, o subgênero *Hoplocryptanthus* é subdividido em quatro grupos morfológicos, sendo formado por espécies hermafroditas (como grande parte das espécies de Bromeliaceae) também com estigmas eretos, mas com flores que emitem odor, grãos de pólen com uma fina camada reticulada e várias sementes por fruto. Seus representantes ocorrem em florestas e campos rupestres (Ramirez-Morillo, 1996, 1998).

Em relação aos aspectos filogenéticos, *Cryptanthus* pertence à subfamília Bromelioideae, sendo classificado como parte do grupo situado no clado 'Eu-Bromelioids', tendo como grupos relacionados *Orthophytum* e *Lapanthus* Louzada & Versieux (Shulte e Zizka, 2008; Shulte et al., 2009; Louzada e Versieux, 2010; Givnish et al., 2011). Algumas características morfológicas indicam *Cryptanthus* como um gênero derivado em Bromelioideae, tais como ovário ínfero, fotossíntese do tipo CAM e tendência ao andromonoicismo. Outra característica que aponta para essa posição mais derivada, diz respeito ao número cromossômico básico menor (x=17) encontrado no gênero – enquanto os demais grupos apresentam uma predominância de x=25 – resultante do mecanismo de disploidia (Gitaí et al., 2005; Ceita et al., 2008). No sentido oposto, evidências como a morfologia das sementes, nectários e o sistema radicular desenvolvido posicionam o gênero como um grupo basal (Böihme, 1988; Gross, 1988). Em estudos recentes de filogenia molecular baseados em regiões nucleares e plastidiais (Shulte et al., 2005, 2009; Shulte e Zizka, 2008) *Cryptanthus* também vem sendo posicionado como grupo basal em Bromelioideae.

Mesmo após a revisão taxonômica do gênero por Ramirez-Morillo (1996), o relacionamento infragenérico e filogenético de *Cryptanthus* com os gêneros de Bromelioidae ainda apresentam lacunas, uma vez que o gênero tem sido representado por poucas espécies nas filogenias moleculares recentes (Shulte et al., 2005, 2009; Sousa et al., 2007; Shulte e Zizka, 2008; Givnish et al., 2011, Louzada, 2012).

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CAPÍTULO I
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Molecular Phylogeny, Character Evolution and Biogeography of the Genus *Cryptanthus* Otto & A. Dietr. (Bromeliaceae)

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Abstract

The genus *Cryptanthus* comprises about 67 endemic species in eastern Brazil occurring in Atlantic forest, *restinga*, *campos rupestres* and *Caatinga*. The majority of the species are threatened due to habitat loss. Here we present the first molecular phylogeny of *Cryptanthus* based on AFLP analysis of 48 species. In the generated phylogenetic tree, the subgenera *Cryptanthus* and *Hoplocryptanthu* appeared as non monophyletic. The proposed morphological groups of the genus presented homoplasic characters, with exception of the inconclusive lacerdae. Phylogenetic relationships between the species remained ambiguous due to low resolution. Further, some of the clades that received good support – mainly in the subgenus *Cryptanthus* – did not follow the species delimitation. Regarding the occurrence on different habitats, *Cryptanthus* seems to have occupied the Atlantic forest multiple times, with a tendency to the predominance of this habitat in the subgenus *Cryptanthus*. Atlantic forest and *campos rupestres* showed a defined pattern of occupation within subgenus *Hoplocryptanthus,* being also an evidence of the polyphyletic condition of this lineage at the habitat level. The phylogeny was also used to infer on the evolution of staminate and hermaphrodite flowers. The character reconstruction revealed the importance of the staminate flower morphology for the diversification of the subgenus *Cryptanthus* in the Atlantic forest domain.

Keywords: Bromelioideae, *Cryptanthus,* AFLPs, *Hoplocryptanthus,* Habitat.

1. Introduction

The monocot family Bromeliaceae Juss. (58 genera, ca. 3,248 species) is almost exclusively Neotropical, with a single disjunctive species [*Pitcairnia feliciana* (A. Chev.) Harms and Mildbraed] occurring in the African continent (Jaques-Félix, 2000; Luther, 2010). The bromeliad family constitutes a noteworthy case of adaptive radiation highlighted by their high ecological versatility, occupying a wide range of terrestrial, lithophytic and epiphytic habitats including arid coastal plains, humid forests and dry environments on rocky soils (Benzing, 2000; Rex et al. 2007; Shulte and Zizka, 2008). The great diversity and ecologic versatility seem to be linked to several key innovations, such as the unique leaf trichomes capable of water absorption, leaf succulence, tank habit, foliar impoundment and CAM photosynthesis (Crayn et al. 2004). Besides the ecological importance, Bromeliaceae are also important in an economic context, as ornamental plants and also for its agricultural value as in the case of *Ananas comosus* (L.) Merr.

Based on morphological characters (as flower, fruit and seed morphology), the family was initially classified into three subfamilies: Pitcairnioideae, Bromelioideae and Tillandsioideae (Smith and Downs, 1974, 1977, 1979). Molecular phylogeny, however, confirmed the monophyly of Bromelioideae and Tillandsioideae whereas Pitcairnioideae was recognized as a polyphyletic group (Horres et al. 2000; Crayn et al. 2004; Givnish et al. 2004, 2007; Terry et al. 1997). Considering those evidences, in the most recent molecular phylogenetic approaches, the family was divided into eight subfamilies (Brocchinioideae, Bromelioideae, Hecthioideae Lindmanioideae, Navioideae, Pitcairnioideae, Puyioideae and Tillandsioideae) with a well supported phylogeny based on plastid loci (Givnish et al. 2007, 2011).

The subfamily Bromelioideae comprises 33 genera and nearly 880 species (Luther, 2010) distributed throughout Central and South America with a centre of diversity in eastern Brazil (Smith and Downs, 1979). Molecular studies (Shulte et al. 2005, 2009; Shulte and Zizka, 2008) based on the combination of plastid and nuclear regions identified several basal lineages (i.e. *Bromelia* Juss., *Deinacanthon* Mez, *Fascicularia* Mez, *Greigia* Regel, *Ochagavia* Phil.). The remaining lineages of the subfamily are referred to as Eu-Bromelioideae, being subdivided in "Core Bromelioids" (advanced group) and a second basal group, where *Cryptanthus* Otto & Dietr is positioned (Givnish et al. 2011; Shulte et al. 2009; Shulte and Zizka, 2008).

The genus *Cryptanthus* comprises 67 terrestrial and rupicolous species (Luther, 2010) and is endemic to Brazil, occupying a wide variety of habitats, including the Atlantic forest and *restinga* (sandy coastal plain vegetation), but also ecologically dryer environments like the *campos rupestres* (rocky fields) and the *Caatinga* (semiarid) formation. The species occur from sea level up to 2,000 m of altitude, growing on shady places in lowland forests as well as on exposed habitats at high

altitudes. The centres of diversity are in the Atlantic forest and *campos rupestres* in the states of Espírito Santo and Minas Gerais (Ramirez-Morillo, 1996; Ramirez-Morillo and Brown 2001).

In general, the species have narrow geographic distributions. The majority of the species (52) occur within the Atlantic forest domain with 48 endemic to this biome. Due to the high extent of deforestation of the Atlantic forest 26 species of *Cryptanthus* are already included in the Brazilian list of threatened species (Martinelli et al. 2008). It is clear that the habitat loss and fragmentation is responsible for the reduction of the abundance of *Cryptanthus* species, once several species occupy narrow geographic ranges. Additionally, forest fragmentation leads to habitat dissection and invasion by invasive plants, and turn fragments prone to forest fires, also facilitating plant collecting by local human populations (Clark et al., 1995; Tabarelli et al., 2004). There are also endemic species of *Cryptanthus* occurring on *campos rupestres* of the states Minas Gerais and Bahia. These habitats are increasingly influenced by mining, the use as natural pastures, anthropogenic fires and the extraction of gallery forests, compromising the survival of the species (Versieux et al. 2008).

Vegetatively, the *Cryptanthus* species resemble species of other bromelioid genera such as *Orthophytum*, but can be basically separated considering the presence of bisexual and male flowers (Ramirez-Morillo, 1996). The genus is divided into two subgenera: (1) *Cryptanthus*, composed by andromonoecious plants and (2) *Hoplocryptanthus* Mez, with hermaphrodite flowers (Mez, 1896; Ramirez-Morillo, 1996, 1998). According to Ramirez-Morillo, 1996 and Ramirez-Morillo and Brown, 2001, *Cryptanthus* has been classified as a highly derived genus, mainly because of the tendency to andromonoecy, presence of fragrant flowers, CAM and by the uncommon chromosome numbers within Bromeliaceae (mainly 2n = 34, as compared to 2n = 50 for most remaining members of the family). The unique systematic study (Ramirez-Morillo, 1996) based on morphological characters, chromosome numbers and DNA amount recognize five sections for the subgenus *Cryptanthus* and four for *Hoplocryptanthus.*

In the most recent molecular phylogenetic studies including *Cryptanthus* (e.g. Schulte et al., 2005, 2009; Schulte and Zizka, 2008), the genus has usually been represented by only a few taxa and was placed as a sister group to *Orthophytum*. Due to the low sampling on the previous phylogenetic approaches, a more comprehensive molecular phylogenetic study is mandatory to clarify the infrageneric relationships of *Cryptanthus*.

Amplified fragment length polymorphism (AFLP; Vos et al., 1995) is applicable to all organisms without a priori sequence information. It combines high reproducibility with high variability and enables a reasonably genome-wide sampling. AFLPs have proven to be a powerful tool to assess species relationships in evolutionary complex groups, such as those undergoing hybridization, introgression and polyploidization (Jakob and Blattner, 2010; Koopman et al., 2008;

Perrie and Shepherd, 2009; Rebernig et al., 2010). AFLP technique is the method of choice for analyzing phylogenetic relationships between closely related taxa (Després et al., 2003; Hodkinson et al., 2000), applied also successfully at the inter- and intraspecific level within Bromeliaceae (Horres et al., 2007; Rex et al., 2009).

In the current study, we used AFLPs to reconstruct the first molecular phylogeny of the genus *Cryptanthus,* in order to find out whether species distinguished by morphological and ecological characters also represent evolutionary distinct units. Furthermore, this phylogenetic framework is used to analyse the evolution of the key morphological characters used here to help infrageneric delimitation, notably staminate central flowers.

2. Material and Methods

2.1 Taxon sampling

A data set of 109 samples was analysed (Table 1) including 48 *Cryptanthus* and five *Orthophytum* species chosen as outgroup based on the evidence provided by previous phylogenetic analysis (Schulte et al., 2005, 2009; Schulte and Zizka, 2008; Givnish et al., 2011).

The plant material was collected during field expeditions as well as from scientific living collections. Leaf material collected was transferred into a sodium chloride saturated aqueous solution of cetyl-trimethylammonium-bromide (20 g CTAB/L) according with Rogstad (1992), and stored at 7 °C until processing.

2.2 DNA Isolation

Total genomic DNA was isolated according to Doyle and Doyle (1987) with modifications described by Weising et al. (2005). DNA purification included precipitation of polysaccharides (Michaels et al., 1994) and RNAse (10 μg/mL) treatment during 2 hours at 37 ºC. DNA quality and quantity were verified by measuring its absorbance at 260 and 280 nm with a NanoDrop® 2000c (NanoDrop Technologies, Wilmington, DE).

2.3 AFLP assays

AFLP analyses were accomplished following Vos et al. (1995), with modifications introduced by Bänfer et al. (2004), Rex et al. (2007) and Carmen Jung (personal communication, May 15, 2011). The first step consisted of the genomic DNA digestion in a final volume of 25 μL at 37 °C with the restriction endonucleases *Hind*III and *Mse*I for 12 h and ligation to *Hind*III and *Mse*I adapters in the same reaction. Two consecutive PCR amplifications were performed as pre-selective

and selective amplification using an Eppendorf Mastercycler Pro thermal cycler using primers with one (+1), (+2) or (+3) selective nucleotides at their 3'ends. The reaction mix of the pre-selective PCR (total volume: 10 μl) contained 2 μl of the 1:10 diluted restriction–ligation product, 0.5 μM of unlabelled *Hind*III (+1) primer, 0.5 μM of unlabelled *Mse*I (+1) primer, 1 x PCR buffer (Peqlab blue), 2 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate (dNTP), and 0.025 U *Taq* DNA polymerase (Peqlab blue, Germany). The amplifications were subjected to an initial denaturation of 94 ºC for 2 min followed by 30 cycles of amplification, each consisting of 94 °C for 20 s, 56 °C for 30 s, and 72 °C for 2 min. Final extension was at 72 °C for 2 min, followed by 60 °C for 30 min.

An initial screening of selective primers using 12 primer combinations with three selective nucleotides each was performed on seven species of *Cryptanthus*. From this initial test nine primer combinations were chosen for the final analyses since they produced well scorable polymorphic patterns (Supplementary Table S1). The selective PCRs were carried out with 2.5 μl of the 1:20 diluted preselective PCR product and different combinations of the unlabelled *Mse*I (+3) primer (0.25 μM) (Carl Roth, Karlsruhe, Germany) and the fluorescence-labelled *Hind*III primer (NED, VIC and FAM, Sigma Aldrich, Munich, Germany) (0.05 μM) with three selective bases. Furthermore, the PCR reaction contained 1 x PCR buffer (Peqlab blue), 2 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate (dNTP), and 0.025 U *Taq* DNA polymerase (Peqlab blue, Germany). The protocol included an initial denaturation at 94 ºC for 2 min, followed by 35 cycles which the initial 15 cycles consisted of 94 °C for 20 s, 66 °C for 30 s and 72 °C for 2 min, reducing the annealing temperature by 0.7 °C at each step, followed by 20 cycles of 94 °C for 20 s, 56 °C for 30 s and 72 °C for 2 min. Final extension was at 60 °C for 30 min. Final products of the selective PCR were run on an automated sequencer (ABI Applied Biosystems) as a multiplex of three primer combinations labelled with distinct fluorescent dyes (NED, VIC and FAM, respectively) and an internal size standard (DNA Size Standard, ABI Applied Biosystems). The AFLP data set were analyzed and stored as electropherograms.

2.4 Data Analysis

The AFLP banding pattern was scored by manual procedure using the software GeneMarker, version 1.7 (SoftGenetics, State College, PA, USA) as presence or absence of a band at a particular position. A reproducibility test was carried out in order to increase the confidence of the AFLP findings. This approach aimed to compare a set of 16,5% of the whole sampling to check whether the amplified DNA fragments are reproducible with the nine used primer combinations. In the cases that the fragments were not present, the data was scored as missing.

2.5 Phylogenetic analyses

Phylogenetic reconstruction of the binary AFLP matrix was carried out using maximum parsimony (MP) in PAUP 4.0b (Swofford, 2002), maximum likelihood (ML) in RAxML (v. 7.2.8; Stamatakis, 2006) via the graphical front-end raxmlGUI (Silvestro and Michalak, 2012) and Bayesian analyses (BI) in MrBayes 3.2 (Ronquist et al., 2012). In parsimony analysis, a strict consensus tree was generated from heuristic searches with 10,000 random addition sequence (RAS) replicates, branch swapping via tree bisection reconnection (TBR), and the MULTREES option in effect. Statistical support of the tree topology was assessed with bootstrap (BS) analysis performing 1,000 pseudo-replicates, with 10 RAS replicates and TBR branch swapping. The extent of homoplasy was estimated using the consistency (CI) and retention indices (RI). For maximum likelihood ten independent ML searches were conducted under the Bingamma substitution model. The test for clade support were obtained from 1,000 bootstrap pseudo-replicates and reported on the tree with the highest likelihood value found over all runs.

A Bayesian phylogenetic reconstruction was obtained by Metropolis-coupled Markov Chain Monte Carlo (MCMC), as implemented in the program MrBayes v. 3.2 (Ronquist and Huelsenbeck, 2003). Two evolutionary models, with and without the gamma distributed across site rate heterogeneity, were tested and compared by the respective marginal likelihoods. These values were calculated using the stepping-stone algorithm (Xie et al., 2011) assuming 10 steps, each sampled for 1,000,000 generations after an initial burn-in.

The substitution model with rate heterogeneity (cf. RAxML's BINGAMMA) obtained a strong support (marginal likelihood = -20,016.76) over the simpler model without gamma variation (marginal likelihood = -20,755.86). Therefore, the Bayesian analysis was performed based on the gamma model with four independent MCMC runs and heated chains, for 10,000,000 generations, sampling trees every 1,000 interactions. The burn-in phase and the efficiency of the MCMC sampling were assessed by examining the log files with the program Tracer (Rambaut and Drummond, 2007). After excluding the burn-in fraction (i.e. the initial two million generations), the four independent runs were combined to generate a consensus tree with posterior probability values (PP) quantifying the statistical support to the nodes.

2.6 Biogeographical characterization

The habitat preference was studied to further explore and discuss the relationship found among the features and *Cryptanthus* clades. Thus, the habitat occurrence considered for each species were: Atlantic forest, *restinga* (sandy coastal plain vegetation), *campos rupestres* (rocky fields),

canga (iron rocky fields) and *caatinga* (semiarid environment). Coloured symbols mapped along the side of the phylogeny tree indicate the states used for each species.

2.7 Reconstructing Ancestral Character States

We explored the character transitions of staminate central flowers (andromonoecius and hermaphrodite - present or absent). The information about the staminate flowers was scored for each species represented in the phylogeny. We have traced the selected characters by overlying them onto the Bayesian tree by the maximum parsimony method using Mesquite 2.75 (Maddison and Maddison, 2011).

3. Results

3.1 AFLP data

Distinct AFLP profiles with nine primer sets (Supplementary Table S1) of 109 accessions produced 489 characters, including the outgroup. The scored characters per primer combination varied between 44 and 66 with the fragment sizes ranging from 90 to 480 base pairs. Of 489 scored characters, 98% were variable.

The pairwise Nei-Li distance values within the total data set, ranged from 0.05 to 0.48, where the lowest value was detected at the intraspecific level between *C. sergipensis*, whereas the highest occurred at interspecific level between *C. beuckeri* and *C. lavrasensis*. The range of infraspecific Nei-Li distances was smallest in *C. sergipensis* (with distances between 0.05 and 0.13) and highest in *C. beuckeri* (with distances between 0.14 and 0.42).

3.2 Phylogenetic relationships

The binary character matrix, subjected to a different phylogenetic evaluation, generated trees by maximum parsimony, maximum likelihood and Bayesian methods. In parsimony analysis, a total of 28 most parsimonious trees were retained with length of 5,793 steps. The consistency index (CI) for these trees was 0.08 and the retention index (RI) was 0.47. Altogether, from 489 characters found, 470 of which were parsimony-informative with 15 constant characters. In general the tree topologies resulting from maximum parsimony (Supplementary Figure S1), maximum likelihood and Bayesian inference, are highly similar with only few incongruences at lower taxonomic levels (Figure 1). The data sets were therefore subjected to Bayesian inference with also the BS supports from maximum likelihood subscribed on the tree.

The consensus tree (Figure 1), with *Orthophytum* species as outgroup, showed five clades that received various levels of BS and PP. The clades I and II shelter species of the subgenus

Cryptanthus, whereas the remaining clades comprised the species that belong to the subgenus *Hoplocryptanthus.*

The clade I depicted four subclades composed of 15 species and 35 accessions, where all specimens of *C. felixii* and *C. sergipensis* grouped together by species, further *C. pickelii*, plus the splitted species *C. lyman-smithii* (57E), *C. ubairensis* (13E) and an unclassified species *C.* sp. (37E) are sheltered in an unsupported subclade A with *C. ubairensis* (59E) and *C. lyman-smithii* (10E) forming a basal grade. Representatives of *C. beuckeri* (58E and 9E) and *C. bromelioides* (4E) comprised a moderately-supported (PP 0.90) subclade B. The subclade C comprised one representative of *C. beuckeri* (3E), in addition of the splitted species *C. sinuosus* (7E, 30E, 45E and 63E), plus *C. acaulis*, *C. dorothyae, C. ubairensis* (78E) and *C. lutherianus* (in the basal position) with the unclassified *C.* aff. *praetextus* completing the group. The weakly defined subclade D grouped the species *C. maritimus*, *C. correia-araujoi*, *C. zonatus*, other specimen of *C. beuckeri* (2E), and the unclassified *C.* aff. *bivittatus* and *C.* aff. *bromelioides*.

The clade II comprised a group of 18 species and 35 specimens. Some species present in the clade I were also found in this clade as *C. beuckeri* (54E and 16E), *C. bromelioides* (53E) and *C. sinuosus* (68E). Most of the specimens representing different species grouped together, as in *C. colnagoi* (32E and 76E), *C. dianae* (31E and 90E), *C. capitellatus* (23E and 26E) and the strongly well-supported (BS 100, PP 1) *C. bahianus*. The remaining species formed very mixed groups which, in most cases, were poorly supported, with few exceptions that show moderately or strongly well-supported nodes [*C. beuckeri* (54E) with *C. marginatus*; *C. beuckeri* (16E) with *C. teretifolius* and *C. reisii* with *C. sp.* (83E)]. The species *C. coriaceus* was nested as basal in the clade. With some exceptions, the relationships among the species within each clade as well as between them remain at low resolution.

Moderately to well-supported (BS 66, PP 0.98), the clade III united the species *C. leopoldo-horstii* and *C. micrus*. On the other hand, the clade IV depicted a monophyletic strongly well-supported group (BS 100, PP 1) that comprised the species *C. regius*, *C. caracensis*, *C. glazioui*, *C. lavrasensis*, *C. ferrarius*, *C. tiradentesensis* and *C. schwackeanus.* Most of those accessions, from the last three mentioned species, were grouped by moderately well-supported nodes. In turn, the species *C. caracensis, C. glazioui* and *C. lavrasensis* were placed in a basal position of the clade. Furthermore, the unclassified *C.* aff. *glazioui* (24E) and *C.* aff. *schwackeanus* formed a strongly well-supported group (BS 94, PP 1) with *C.* aff. *regius* (11E) and *C.* aff. *glazoui* (18E) also present in this clade. Regarding the weakly supported clade V (BS 50), it comprised the species *C. sanctaluciae* (forming a strongly well-supported group), *C. latifolius, C. pseudoglazioui*, *C. microglazioui* (group moderately well-supported), *C. odoratissimus* and *C. scaposus* with *C. whitmanii* forming a

moderately well-supported group. Additionally, the unclassified *C.* aff. *leuzingerae* is also present in this clade, with *C.* aff. *scaposus* and *C. pseudoscaposus* on the basal position. The clades III, IV and V comprised only species from the subgenus *Hoplocryptanthus*.

3.3 Biogeographical characterization

Regarding the habitat preference of *Cryptanthus*, most of the species occur in the Atlantic forest (Figure 1). Members of the subgenus *Cryptanthus* appeared in more derived clades, with exception of *C. arelli* that occurs in *campos rupestres*, while *C. acaulis*, *C. dorothye, C. sinuosus* and *C.* aff. *burle-marxii* are found in Atlantic forest and *restinga*, and *C. bahianus* is found in *Caatinga*. Based on these results, 80% of the whole sampling for this subgenus grows in the Atlantic forest. Considering the species of the subgenus *Hoplocryptanthus* – found in a basal position of the phylogeny – there was a division between the species from *campos rupestres*, associated with *canga*, and in Atlantic forest, where the species occurrence is about 33%, 11% and 44% respectively.

4. Discussion

Cryptanthus is a very distinctive genus compared with the rest of the family Bromeliaceae. Particular features include the tendency to andromonoecy (subgenus *Cryptanthus*) and the wide occurrence in different habitats (Ramirez-Morillo, 1996, 1998). Furthermore, the genus presents a unique chromosome number (2n = 34) which Brown and Gilmartin (1989b) suggested as a synapomorphic character that could separate the genus in an own subfamily. Therefore, as a very peculiar group within the family, *Cryptanthus* has been the subject of some approaches such as the taxonomic revision by Ramirez-Morillo (1996). However, the species boundaries of certain group of species of the genus still remain difficult to define (e.g. *C. acaulis*, *C. bromelioides*, *C. beuckeri*). The current AFLP study is the first molecular phylogeny of the genus based on a significant sampling, as an attempt to clarify the infrageneric relationships of *Cryptanthus*.

4.1 Phylogenetic relationships

The phylogenetic reconstruction does not support the subgenus concept that divide the genus in two subgenera, *Cryptanthus* and *Hoplocryptanthus* (Figure 1), based also on morphological characters, ecological features and geographic distribution. The subgenus *Cryptanthus* is characterized by species with odorless flowers that are andromonoecious, petals are nearly always sublinear-lanceolate and pollen with reticulate surface (Ramirez-Morillo, 1996; Leme et al., 2010), while in *Hoplocryptanthus* the species have perfumed hermaphroditic flowers, petals broadly spathulate or obovate (Leme et al., 2010) and pollen with a smooth or finely reticulate surface

(Ramirez-Morillo, 1996). Additionally, subgenus *Cryptanthus* presents fewer seeds per fruit and occurs from the State of Rio de Janeiro, over Minas Gerais and Espírito Santo to the State of Rio Grande do Norte, from sea level to ca. 700 m elevation. On the other hand, the subgenus *Hoplocryptanthus* presents species with higher number of seeds per fruit and is distributed in the mountain Atlantic forest of Espírito Santo and the mountains of the Espinhaço range in Minas Gerais, mainly in wet sites at elevations over 600 m (Leme et al. 2010; Leme and Siqueira-Filho, 2006; Ramirez-Morillo, 1996).

In a taxonomic review of the genus *Cryptanthus*, Ramirez-Morillo (1996) proposed the division of the two subgenera into some sections. However, such an approach is not officially released, since it was not officially released, being available only as an unpublished thesis. Despite of that, this work is still very important and provide huge data evaluation for comparisons within the genus and should be considered at least to discuss the value of the characters considered. Therefore, in this work we will refer to these hypotetical sections as morphological groups.

4.1.1 The subgenus Cryptanthus

Subgenus *Cryptanthus* is a monophyletic group, consisting of the clade I which comprises species of the morphological groups bahianae, beuckeri and cryptanthus and the clade II that unites all groups proposed for the subgenus, corresponding to the previously mentioned, plus lacerdae and zonatae. In the clade I, the species which form a complex living north of São Francisco River (Siqueira et al., 2007) were grouped in different clades, which are represented by *C. pickelii*, *C. alagoanus* and *C. felixii* forming a well supported monophyletic group, and *C. zonatus* in an unsupported subclade D (Figure 1). Moreover, other representatives as *C. dianae* and *C.* aff. *burle-marxii* grouped separately in the clade II, suggesting no geographic correlation to the phylogenetic relationships for this complex of species. Additionally, *C. burle-marxii* and *C. zonatus* which are splitted in the clades I and II, belong to the group *Zonatae* which bears a unique pattern of transversal bands formed by peltate trichomes on the foliar blades (Ramirez-Morillo, 1996). Therefore, we consider this morphological character questionable in a phylogenetic context.

Even though *C. sergipensis* and *C. bahianus* belong the group *Bahianae* and present some morphological affinities, like narrow triangular leaves, serrate leave border, glabrous adaxially and lepidote abaxially surfaces (Ramirez-Morillo, 1996), these species were found in very well supported subclades A and F within the subgenus *Cryptanthus* suggesting that bahianae was established by morphological homoplasic characters, regarding an artificial group (Figure 1).

The species *C. beuckeri* only known to the states of Bahia in the southern and Espírito Santo in the northern (Thomas et al., 2003), is the unique taxon of the morphological group beuckeriae

which has petiolate leaves as synapomorphic feature, with no further close relative recognizable within the genus (Ramirez-Morillo, 1996). Nevertheless, this species presents an interesting phylogenetic pattern, once grouped with different species within the subgenus *Cryptanthus*. It is present in subclades B with *C. bromelioides*; which the closely related species is *C. ubairensis*, in unsupported subclade D, in subclade C with *C. acaulis*; vegetatively similar to the *C. minarum*, in subclade E with *C. marginatus*; probably closest to *C. acaulis*, and in subclade H with *C. teretifolius* (Figure 1). According to Ramirez-Morillo (1996), the morphological variation found for *C. beuckeri* and the facility to hybridize with other *Cryptanthus* species have caused confusion. However, natural hybridization was never demonstrated or documented, and there is no record of sympatry of other *Cryptanthus* species with *C. beuckeri*. Therefore, the occurrence of hybridization in nature between *C. beuckeri* and other *Cryptanthus* species seems to be unlikely. Possibly, the reported confusion is caused by the unusual petiolate leaves only attributed to *C. beuckeri*, which may contribute to a tendency to identify potentially distinct species with petiolate leaves under this binomial. Nevertheless, the different populations identified as *C. beuckeri* need to be better investigated by genetic analysis to confirm the species boundaries.

The group lacerdae includes a single species (*C. lacerdae*), which presents leaves with three longitudinal silvery lines formed by peltate trichomes, greenish flowers and fossulate pollen (Ramirez-Morillo, 1996). Although *C. lacerdae* presents unique features that could influence its phylogenetic relationship, it does not show a clearer phylogenetic position, hence occupying an unsupported clade within the subgenus *Cryptanthus* (Figure 1). Future improvement of the phylogenetic signal will allow us to obtain a more conclusive answer to this phylogenetic question.

Based on the phylogenetic reconstruction, the group Cryptanthus is also an artificial group, once the species *C. acaulis, C. bromelioides, C. colnagoi, C. coriaceus, C. correia-araujoi, C. dianae, C. lutherianus, C. marginatus, C. maritimus* and *C. ubairensis* are splitted between the sister clades of the subgenus *Cryptanthus* (Figure 1). This group is described by a combination of characters, of which some stand out as: acaulescent or rarely caulescent plants, reproduction by axilar or basal offsets, leaves adaxially glabrous or sparsely lepidote and petals with or without a pair of calli at the base of the antepetalous stamens (Ramirez-Morillo, 1996). This range of morphological traits might be homoplasic, then hampering the delimitation of the group.

4.1.2 The subgenus Hoplocryptanthus Mez

Hoplocryptanthus forms a polyphyletic group represented by the clade III, which sheltered the morphological group *Xerophyticae*, clade IV, consisting of the morphological group already cited plus *Schwackeanae, Hoplocryptanthus* and *Mesophyticae*, and clade V, covering the two last

mentioned groups. The moderately well-supported clade III formed a group in full accordance with the species designation, once the recently described species *C. micrus* seems to be morphologically closest to the *C. leopoldo-horstii* (Versieux et al., 2010). Nevertheless, the latter species belongs to the group *Xerophyticae* that appears to be an artificial group since the other representative (*C. caracensis*) was present in the clade IV.

The clade IV is strongly supported (Figure 1), where all species shared a suite of morphologic similarities with *C. shwackeanus,* but also with synapomorphies that distinguish them, as seen in the presented phylogeny (Leme, 2007; Leme et al., 2009; Ramirez-Morillo, 1996). *C. shwackeanus* and *C. ferrarius* occur in iron-rich rocky soils (*canga*), a region target of a large number of iron-mining activities, which may be contributing to the genetic differentiation of the studied specimens that grouped separately in the clade. In turn, *C. regius* formed a moderatally to well supported group with *C. tiradentesensis* which shares just some morphologic features (e.g. stemless, propagating by short basal rhizomes, inflorescence bipinnate). Moreover, it was reported that *C. tiradentesensis* is close related with *C. caracensis* (Leme, 2007), which form a moderately supported group with *C. glazioui* that, according to Ramirez-Morillo (1996), is close related to *C. pseudoglazioui* found in the clade V.

The group *Hoplocryptanthus* is just defined by a long-caulescent habit (Ramirez-Morillo, 1996), being here represented by *C. glazioui* in the clade IV, *C. microglazioui* (in a moderately supported subclade) and *C. pseudoglazioui* in the clade V, suggesting that the character used to circumscribe this group is also homoplasic (Figure 1). Furthermore, the group *Mesophyticae* characterized by the leave morphology (adaxially glabrous, abaxially densely white lepidote or white with brown transversal bands; Ramirez-Morillo, 1996) was represented by the species *C. latifolius*, *C. odoratisssimus, C. pseudoscaposus, C. scaposus* and *C. whitmanii*, all in the clade V. Therefore, also presenting homoplasic characters. Moreover, the latter two mentioned species are, according to Ramirez-Morillo (1996), related in accordance with the phylogeny (Figure 1). Additionally, the group *Schwackeanae* characterized by stigmas with three straight lobes whose margins smooth and compound by the species *C. shwackeanus* (Ramirez-Morillo, 1996), as already observed for other groups, are also based on homoplasic characters once the representatives are splitted in the clade IV.

4.2 Biogeographical notes

The Atlantic forest domain is generally known by three different formations which are the coastal plain vegetation, the forests slope and the altitude forests (Joly et al., 1991). Considering the phytogeographic aspects, it is divided in two regions: the South and Southeast, comprising the forests slope, and the Northeast constituted by lowland forests. Additionally, the Espírito Santo state presents an intermediate formation (Rizzini, 1979; Siqueira, 1994). Despite all the different

formations of the Atlantic forest, in our analysis of habitat, the occupation by the species of the monophyletic subgenus *Cryptanthus* present no established pattern, suggesting a phytogeographical connectivity between these regions (Figure 1). Nevertheless, the clade II indicates also a tendency to occupation of drier habitats (e.g. *C. arelli,* and *C. bahianus*), more evident than in the clade I, suggesting in general the tendency of the most derived species of the genus to occupy wetter habitats.

The *campos rupestres* are one of the floristically richest regions in the world, and are also a refuge for many endemic species of Bromeliaceae as for some *Hoplocryptanthus* species (Giulietti et al., 1997; Versieux et al., 2008). Unlike the subgenus *Cryptanthus,* a clearer pattern of habitat occupation was evident for *Hoplocryptanthus,* where the species occurrence is divided in the *campos rupestres* with the associated environment *canga* and Atlantic forest (Figure 1). These findings reinforce the polyphyletic condition of the subgenus *Hoplocryptanthus* that appears divided into different groups also in regard to the habitat.

Within the genus *Cryptanthus*, the occupation of the Atlantic forest has occurred twice, with the dry environments like *campos rupestres* and *caatinga* virtually absent and with a predominance of the Atlantic forest on the most derived clades of the phylogenetic reconstruction (Figure 1).

4.3 Evolution of the staminate flowers

Andromonoecy is considered a derived character in Bromeliaceae, taking into account that almost all members of the family are essentially hermaphrodite. Only *Hechtia* Klotszch (Hechtioideae) and *Catopsis* Griseb. (Tillandsioideae) present some dioecious members (Smith and Downs, 1977; Brown and Gilmartin, 1989a). Therefore, the andromonoecious tendency of the subgenus *Cryptanthus* is one of the most interesting morphological traits, when compared with the rest of the family. In the andromonoecious species, the staminate flowers open first and are located mainly in the mid to apical sector of the inflorescence, while the perfect flowers are concentrated in the basal racemes (Leme et al., 2010). Furthermore, staminate flowers are smaller than hermaphrodite in the same inflorescence (Ramirez-Morillo, 1996).

The reconstruction of the evolution of staminate flowers within the genus *Cryptanthus* showed a clear pattern (Figure 2). The hermaphrodite sexual system is inferred as ancestral within the genus. The molecular phylogeny implies in a single origin of the andromonoecy sexual system within the group, where all species of the subgenus *Cryptanthus* are characterized by the occurrence of the andromonoecious flowers. Therefore, this trait can be regarded as a synapomorphy for the subgenus, which is pointed by the character reconstruction to be highly conserved.

When gaining this key innovation, the group may have favored by some advantages such as resource reallocation, in which the production of staminate flowers reduces the resource investment

in functionally male flowers, thus enabling the resources saved to be re-allocated to other fitness-enhancing traits (Bertin, 1982; Solomon, 1985; Spalik, 1991; Emms, 1993). The sex allocation is an example of this process, where the resource-depleting factors such as shading or water stress (Solomon, 1985), florivory (Krupnick and Weis, 1998) and fruiting success of earlier flowers (Diggle, 1994; Gibs et al., 1999) can alter the production of perfect and staminate flowers. Furthermore, the staminate flowers are more efficient as a donor of pollen and pollinator attractor for many reasons, such as higher production of pollen, which can also be larger (observed for the subgenus *Cryptanthus* – data not shown) and reduced pollen-pistil interference either within flowers or among flowers on the same plant (Podolsky, 1992, 1993; Harder and Barrett, 1996; Elle and Meagher, 2000; Barrett, 2003). These hypotheses have been tested in different andromonoecious species. For instance, Zhang and Tan (2009) studying the shrub *Capparis spinosa* L., observed that male flowers save resources for female function and they primarily serve to attract pollinators as pollen donors. The scenario indicated by the molecular data enables the species of the subgenus *Cryptanthus* to present better strategies towards the colonization of diverse habitats, mainly in the Atlantic forest domain.

5. Conclusions

The AFLP findings presented here provided the first insights on the molecular phylogenetic relationships of the genus *Cryptanthus*. Our phylogenetic reconstruction indicated that both subgenus *Cryptanthus* and H*oplocryptanthus* forms a polyphyletic group. In the phylogeny several nodes within the genus *Cryptanthus* remained unresolved maybe because they regard recent evolution trends within the Bromeliaceae. Thus, we cannot have sufficient information to reliably assess the phylogenetic relationship among all species studied. The infrageneric classification of *Cryptanthus* deserves further investigations, once the proposed morphological groups were defined based on homoplasies. Moreover, the data at hand do not showed a clear phylogenetic position of the group *Lacerdae* and it is necessary to improve the phylogeny resolution to get a more conclusive answer to this question.

Apparently, the occupation of the Atlantic forest by the genus occurred several times, where in the subgenus *Hoplocryptanthus* is well defined in the basal clade, whereas the *campos rupestres* and *canga* are associated with the remaining species of the subgenus. Moreover, this pattern brings further evidence in favour to a polyphyletic condition of the subgenus. The species of the subgenus *Cryptanthus* did not present a stabilished pattern, but a tendency to the species of the most derived clades occypie wetter environments on the Atlantic forest domain.

The character state reconstruction reveals the great importance of the evolution of staminate flowers, which possibly contribute for the species of the subgenus *Cryptanthus* to strive different habitats on the Atlantic forest domain.

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Table 1. Studied material. Abbreviations: ASE, Herbarium of Universidade Federal de Sergipe; BHCB, Herbarium of Universidade Federal de Minas Gerais; HB, Herbarium Bradeanum; IBt, Instituto de Botânica; LC, Living collection, RB, Herbarium of Jardim Botânico do Rio de Janeiro; RG, Refúgio dos Gravatás in Teresópolis, Rio de Janeiro; SP, Herbarium of Instituto de Botânica; UFPE, Universidade Federal de Pernambuco.

Species	Voucher	DNA No.	Locality
Cryptanthus acaulis (Lindl.) Beer	RG, 3359 (LC)	17E	RJ, Rio de Janeiro
Cryptanthus alagoanus Leme & J.A.Siqueira	RG, 6186 (LC)	62E	PE, Ipojuca
	RG, 6188 (LC)	77E	PE, Ipojuca
	RG, 5193 (LC)	81E	PE, Ipojuca
Cryptanthus arelii H.Luther	RG, 2830 (LC)	14E	BA, Chapada Diamantina
Cryptanthus argyrophyllus Leme	RG, 5143 (LC)	33E	Bahia
Cryptanthus bahianus L.B.Sm.	RG, 4223 (LC)	65E	BA, Santa Terezinha
	UFPE (LC)	GRV4	PE, Gravatá
	UFPE (LC)	GRV6	PE, Gravatá
Cryptanthus beuckeri E.Morren	RG, 3891 (LC)	16E	BA, Arraial da Ajuda
	RG, 6786 (LC)	2E	ES, Linhares
	RG, 4020 (LC)	3E	Bahia
	RG, 7341 (LC)	54E	BA, Camacan
	RG, 6994 (LC)	9E	BA, Itapebi
	RG, 5151 (LC)	58E	BA, Potiraguá
Cryptanthus bromelioides Otto & Dietrich.	RG, 2229 (LC)	4E	RJ, Rio de Janeiro
	RG, 3595 (LC)	53E	RJ, Rio de Janeiro
Cryptanthus capitellatus Leme & L.Kollmann	RG, 7988 (LC)	23E	ES, Santa Teresa
	RG, 6921 (LC)	26E	ES, Várzea Alegre
Cryptanthus caracensis Leme & E.Gross	RG, 1853 (LC)	89E	MG, Santa Bárbara
Cryptanthus colnagoi Rauh & Leme	RG, 7898 (LC)	32E	MG, Salto da Divisa
	RG, 1021 (LC)	66E	Bahia
	RG, 5313 (LC)	76E	BA, Itapetinga
Cryptanthus coriaceus Leme	Leme et al. 1114 (HB)	91E	ES, Serra
Cryptanthus correia-araujoi Leme	Leme et al. 2704 (HB)	39E	ES, Domingos Martins
Cryptanthus delicatus Leme	RG, 2236 (LC)	72E	RJ, Campos
Cryptanthus diamantinensis Leme	Leme et al. 3812 (HB)	64E	BA, Caeté-Açu
Cryptanthus dianae Leme	RG, 5038 (LC)	31E	PE, Jaqueira
	RG, 5037 (LC)	84E	PE, Jaqueira
	RG, 6814 (LC)	90E	PE, Jaqueira
Cryptanthus dorothyae Leme	RG, 2379 (LC)	19E	ES, Presidente Kennedy
Cryptanthus felixii J.A.Siqueira & Leme	Leme et al. 6100 (HB)	44E	PE, Bonito
	Leme et al. 5540 (HB)	47E	AL, Serra Lisa
Cryptanthus ferrarius Leme & C.C.Paula	RG, 6890 (LC)	27E	MG, Catas Altas
	RG, 6540 (LC)	60E	MG, Mariana
	RG, 6541 (LC)	67E	MG, Mariana
Cryptanthus giganteus Leme & A.P.Fontana	RG, 7988 (LC)	20E	MG, Salto da Divisa
Cryptanthus glazioui Mez	RG, 1856 (LC)	5E	MG, Santa Bárbara
Cryptanthus lacerdae Antoine	RG, 3400 (LC)	38E	BA, Camaçã
Cryptanthus latifolius Leme	RG, 5220 (LC)	12E	ES, Guarapari
Cryptanthus lavrasensis Leme	RG, 7620 (LC)	8E	MG, Santa Bárbara
Cryptanthus leopoldo-horstii Rauh	RG, 5657 (LC)	41E	MG, São Gonçalo R. Pratas
	RG, 5565 (LC)	82E	MG, Diamantina
	Louzada et al. 384520 (SP)	R20	Minas Gerais
Cryptanthus lutherianus I.Ramirez	RG, 3083 (LC)	22E	ES, Ibiraçu
Cryptanthus lyman-smithii Leme	RG, 4344 (LC)	10E	BA, Jaguaripe
	Leme et al. 4342 (HB)	57E	BA, Jaguaripe
Cryptanthus marginatus L.B.Sm.	RG, 0290 (LC)	80E	ES, Domingos Martins
Cryptanthus maritimus L.B.Sm.	Leme et al. 1582 (HB)	92E	ES, Vila Velha
Cryptanthus microglazioui I.Ramirez	RG, 0152 (LC)	15E	ES, Domingos Martins
	Louzada et al. 396794 (SP)	R13	Espírito Santo

Species	Voucher	DNA No.	Locality
Cryptanthus micrus Louzada, Wand. & Versieux	Louzada et al. 1474 (BHCB)	R116	Minas Gerais
Cryptanthus odoratissimus Leme	RG, 5207 (LC)	34E	ES, Santa Leopoldina
	RG, 5216 (LC)	21E	ES, Domingos Martins
Cryptanthus pickelii L.B.Sm.	IBt (LC)	R9	Pernambuco
Cryptanthus pseudoglazioui Leme	Leme et al. 1560 (HB)	42E	ES, Santa Leopoldina
	Leme et al. 1556 (HB)	73E	ES, Santa Leopoldina
Cryptanthus pseudoscaposus L.B.Sm.	RG, 5218 (LC)	74E	ES, Domingos Martins
Cryptanthus regius Leme	Leme et al. 6372 (HB)	49E	MG, Tiradentes
Cryptanthus reisii Leme	RG, 5019 (LC)	85E	BA, Itapetinga
Cryptanthus sanctaluciae Leme & L. Kollmann	Louzada 162 IBt (LC)	STL1	ES, Santa Teresa
	Louzada 162 IBt (LC)	STL2	ES, Santa Teresa
Cryptanthus scaposus E.Pereira	RG, 5213 (LC)	36E	ES, Domingos Martins
Cryptanthus schwackeanus Mez	RG, 6981 (LC)	86E	MG, Ouro Preto
	Louzada et al. 441744 (SP)	SERB	MG, Ouro Preto
	Louzada et al. 441744 (SP)	SERP	MG, Caeté
Cryptanthus sergipensis I.Ramirez	Melo et al. 17279 (ASE)	G1	SE, Poço Redondo
	Melo et al. 17279 (ASE)	G2	SE, Poço Redondo
	Melo et al. 17279 (ASE)	G3	SE, Poço Redondo
	Melo et al. 17279 (ASE)	G4	SE, Poço Redondo
Cryptanthus sinuosus L.B.Sm.	RG, 2868 (LC)	68E	RJ, Arraial do Cabo
	RG, 5229 (LC)	30E	RJ, Búzios
	RG, 4184 (LC)	7E	RJ, São Pedro D'Aldeia
	RG, 5406 (LC)	45E	RJ, São Pedro D'Aldeia
	RG, 5084 (LC)	63E	RJ, São Pedro D'Aldeia
Cryptanthus teretifolius Leme	Leme et al. 3073 (HB)	51E	ES, Vitória
Cryptanthus tiradentesensis Leme	RG, 5240 (LC)	61E	MG, Tiradentes
	RG, 5825 (LC)	69E	MG, Tiradentes
Cryptanthus ubairensis I.Ramirez	RG, 5225 (LC)	13E	BA, Ubaira
	Leme et al. 7788 (RB)	59E	BA, Guaratinga
	RG, 5228 (LC)	78E	BA, Ubaira
Cryptanthus venecianus Leme & L.Kollmann	RG, 7743 (LC)	48E	ES, Nova Venecia
Cryptanthus viridipetalus Leme	RG, 8016 (LC)	46E	ES, Boa Esperança
Cryptanthus whitmanii Leme	RG, 5208 (LC)	71E	ES, Domingos Martins
Cryptanthus zonatus (Visiani) Beer	RG, 6518 (LC)	55E	PE, Jaqueira
Cryptanthus aff. *bivittatus*	RG, 3762 (LC)	25E	ES, Domingos Martins
	RG, 3080 (LC)	79E	ES, Domingos Martins
Cryptanthus aff. *bromelioides*	RG, 4244 (LC)	40E	RJ, Rio de Janeiro
Cryptanthus aff. *burle-marxii*	RG, 3860 (LC)	1E	RN, Natal
	RG, 3878 (LC)	28E	RN, Natal
	RG, 6260 (LC)	56E	Pernambuco
Cryptanthus aff. *diamantinensis*	RG, 6325 (LC)	93E	BA, Saude
Cryptanthus aff. *glazioui*	RG, 6877 (LC)	18E	MG, Barão de Cocais
	RG, 6872 (LC)	24E	MG, Barão de Cocais
Cryptanthus aff. *leuzingerae*	RG, 1144 (LC)	29E	ES, Santa Leopoldina
Cryptanthus aff. *praetextus*	RG, 5490 (LC)	52E	Espírito Santo
Cryptanthus aff. *regius*	RG, 6888 (LC)	11E	MG, Catas Altas
Cryptanthus aff. *scaposus*	RG, 2725 (LC)	70E	ES, Santa Rita
Cryptanthus aff. *schwackanus*	RG, 6266 (LC)	43E	MG, Sabará
Cryptanthus aff. *sinuosus*	RG, 8089 (LC)	50E	RJ, São Fidélis
Cryptanthus sp. nov.	RG, 6588 (LC)	88E	BA, Rio do Meio
Cryptanthus sp.	RG, 6479 (LC)	37E	BA, Vitória da Conquista
Cryptanthus sp.	RG, 6979 (LC)	6E	ES, Serra
Cryptanthus sp.	RG, 6477 (LC)	83E	BA, Bandeira do Colônia
Orthophytum amoenum (Ule) L.B.Sm.	Louzada, 7106 IBt (LC)	R23	BA, Chapada Diamantina
Orthophytum hatschbachii Leme	Louzada, 104 IBt (LC)	R19	BA, Rio de Contas
Orthophytum heleniceae Leme	Louzada et al. 2544 (SP)	R38	Bahia
Ortophytum ophiuroides Louzada & Wand.	Louzada, 88 IBt (LC)	R5	BA, Chapada Diamantina
Orthophytum ulei Louzada & Wand.	Louzada, 91 IBt (LC)	R43	BA, Chapada Diamantina

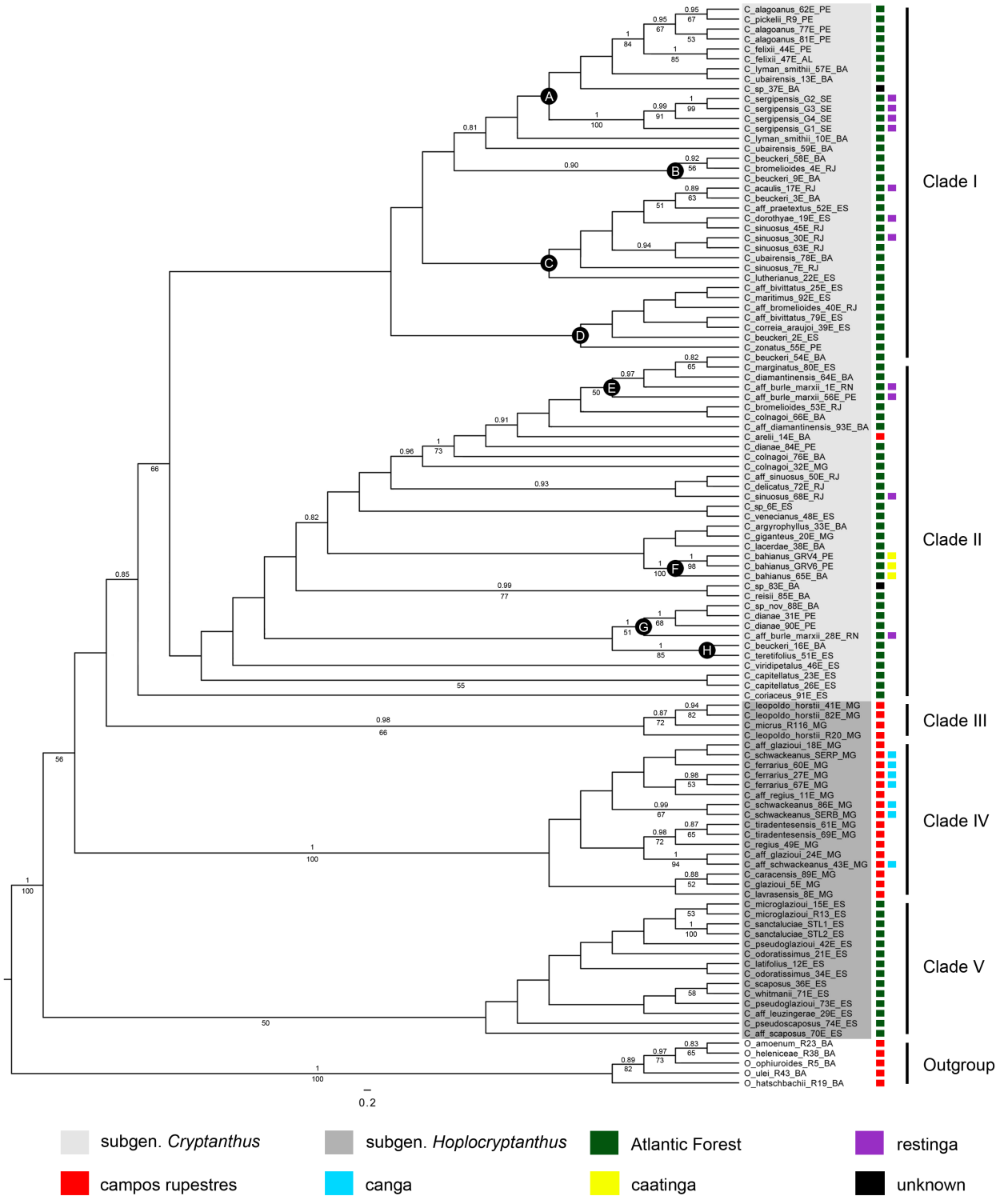

Figure 1: Bayesian inference tree of 104 *Cryptanthus* accessions based on 489 characters obtained with nine AFLP primer pair combinations and five *Orthophytum* as outgroup. Posterior probabilities (PP) > 80 are given above the branches and boostrap values (BS) > 50 below. Clades I to V and Subclades A to H are referred to in the text. The squares indicate the type of the subgenus and habitat, the references are indicated above in the figure.

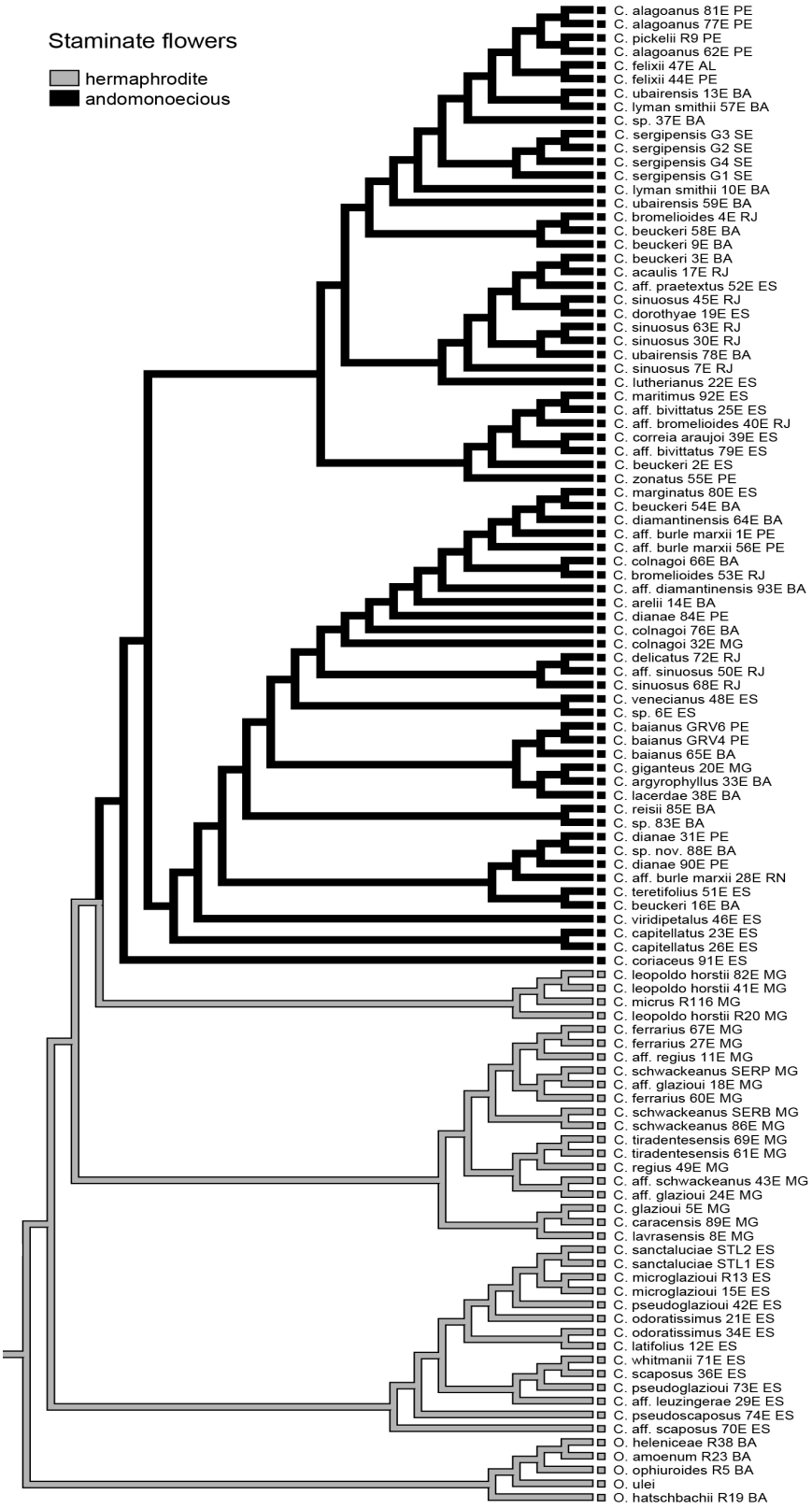

Figure 2: Most parsimonious reconstruction of the evolution of staminate flowers in *Cryptanthus*, based on relationships revealed by bayesian inference of nine AFLP primer pair combinations.

Appendix A. Supplementary material

Table S1. Primer combinations used for the selective amplification of *Cryptanthus* representatives.

Primer combination	Total bands	Polymorphic bands (%)
MseI + AGC/*Hind*III + AGC	55	100
*Mse*I + ATC/*Hind*III + ACA	66	100
*Mse*I + AGA/*Hind*III + AAC	44	98
*Mse*I + AGA/*Hind*III + AGC	48	98
*Mse*I + CAA/*Hind*III + ACA	51	100
*Mse*I + CAG/*Hind*III + AAC	63	96
*Mse*I + CTG/*Hind*III + AAC	57	98
*Mse*I + CTG/*Hind*III + AGC	52	98
*Mse*I + CAT/*Hind*III + ACA	53	100
Total	489	98

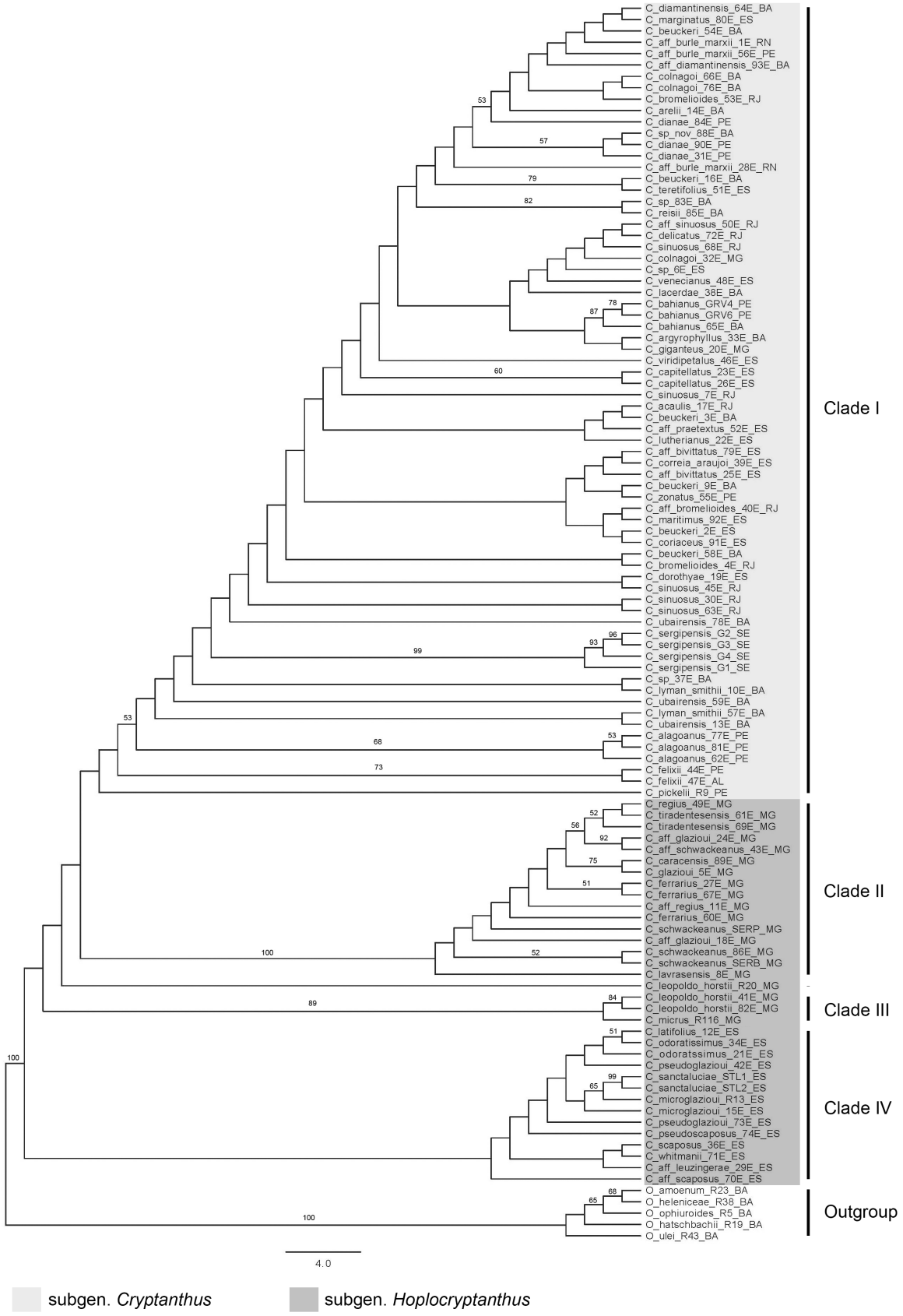

Figure S1: Strict consensus tree of a parsimony based on 489 characters obtained with nine AFLP primer pair combinations and *Orthophytum* as outgroup. The analysis yielded 28 most parsimonious trees of 5793 steps length (consistency index CI = 0.08, retention index RI = 0.47). The squares indicate the type of the subgenus, the references are indicated above in the figure.

CAPÍTULO II

**(Manuscrito preparação –
a ser submetido à revista *Annals of Botany*)**

Genome size diversity in the Genus *Cryptanthus* Otto & A. Dietr. (Bromeliaceae), consequences and evolution

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- *Background and Aims* The genus *Cryptanthus* is a distinct group within the family Bromeliaceae and occurs in a wide range of habitats. Due to high endemism, narrow geographic distribution, and loss of natural habitats, the genus presents many endangered species. The aim of the present study was to analyze nuclear genome size in a phylogenetic framework and to assess relationships between genome size and habitat preferences.
- *Methods* Genome sizes of 47 species of *Cryptanthus* and five of *Orthophytum* were estimated by flow cytometry and compared with the reconstruction of the character state of habitat type onto a given phylogenetic tree.
- *Key Results* The 1C DNA values differed up to 2.13-fold among species. The analyses of the genome size distribution on the *Cryptanthus* phylogenetic tree matched the two defined major lineages. Therefore, there was a significant difference in DNA content between the subgenera *Cryptanthus* and *Hoplocryptanthus*. Moreover, significant difference in genome sizes and habitats preference was observed, further the reconstruction of the evolution also showed this positive association. On the other hand, species from Atlantic forest do not significantly differ from one another in terms of habitat preferences.
- *Conclusions* this study demonstrates that various evolutionary forces and processes have shaped the observed genome size variation. Nevertheless, the phylogenetic relationships were the most determinant factors of the observed divergence in genome size at the basal nodes.

Keywords: Genome size evolution, *Cryptanthus*, nuclear DNA content, flow cytometry, habitat preferences, phylogeny.

Introduction

Genome size evolution has become a widely studied phenomenon, since the genomes are being regarded as a distinct level of biological organization, presenting a unique evolutionary history and diverse inherent properties (Gregory, 2005). Therefore, the interest has increased in plant evolutionary biology towards the significance of the genome size variation among angiosperms (Leitch and Bennett, 2007). The variation in genome size is mainly caused by polyploidy and the amount of non-coding repetitive DNA (e.g. satellite DNA, transposable elements, pseudogenes), which can be changed by mechanisms like chromosome accumulation, genomic duplications and amplification of transposable elements (Bennet and Leitch, 2005), among others. Alterations at the chromosome-level usually cause sudden and marked changes in genome size, while molecular mechanisms are more gradual, producing only slight modifications (Dušková *et al*., 2010). The differences in genome size and thus the actual amount of nuclear DNA may limit some plant traits, such as the occurrence in certain types of niches (Knight *et al.*, 2005; Francis *et al.*, 2008).

The taxonomic and evolutionary significance of the variation in genome size have been used as a supportive character at various taxonomic ranks, being a useful tool as an indicator of taxonomic heterogeneity or complex evolutionary history (Obermayer and Greilhuber 2005; Leong-Škorničková *et al.*, 2007; Suda *et al.*, 2007; Slovák *et al.*, 2009). In addition, the genome size has considerable biological effects on organisms independent of the encoded genetic information (the so-called nucleotypic theory; Bennett, 1972). Therefore, variation in genome size has been interpreted toward various phenotypic and/or life-history traits, sometimes correlating with significant consequences at the ecological level, influencing the range of the environmental conditions that a plant can tolerate (the so-called large genome constraint hypothesis; Knight *et al.*, 2005).

The biological and the evolutionary meaning of the genome size evolution are better understood by analyzing phylogenetic relationships, which are the most important factors to explain the genome size variation, outweighing any correlation with ecogeographic variables (Loureiro *et al*.,

2010). Moreover, whether phylogeny is not taken into account, the role of other factors (e.g. environmental conditions) in shaping the genome size may be overemphasized. Therefore, when searching for the causes of variation in genome size, both adaptive and non-adaptive components need to be considered (Dušková *et al*., 2010). Accordingly, comparative analyses between DNA sequences and genome size data sets can help in elucidating how genome size variation is linked to species evolution (Albach and Greilhuber, 2004; Grotkopp *et al.*, 2004; Jakob *et al.*, 2004; Weiss-Schneeweiss *et al.*, 2006; Chrtek *et al.*, 2009; Dušková *et al.*, 2010), mainly in challenging plant groups as the family Bromeliaceae.

Flow cytometry (FCM) is a rapid, accurate, and reliable technique which has been extensively used for estimating the nuclear DNA content of plants (Loureiro *et al.*, 2010). However, there are few studies that reported DNA C-values for Bromeliaceae (Arumuganathan and Earle 1991; Ebert and Till 1997; Ramírez-Morillo and Brown 2001; Sgorbati *et al.*, 2004; Zonneveld *et al.*, 2005; Leitch *et al*., 2010; Favoreto *et al*., 2012), which is one of the most important neotropical plant family, considering ecological and economic aspects (Benzing, 2000; Crayn *et al.*, 2004; Rex *et al.*, 2007; Shulte and Zizka, 2008; Versieux *et al.*, 2008).

The genus *Cryptanthus* (Bromeliaceae) with estimated 67 species (Luther, 2010) is endemic to Brazil. Their species present a wide distribution in the Atlantic forest, *restingas* (sandy coastal plain vegetation), *campos rupestres* (rocky fields) and *caatinga* (semiarid vegetation). The geographic distribution ranges from Rio Grande do Norte to Rio de Janeiro states. The greatest diversity of species is found in the states of Minas Gerais, Espírito Santo and Rio de Janeiro (Ramirez-Morillo, 1996; Ramirez-Morillo and Brown 2001). Within Bromeliaceae, the genus is a conspicuous group, presenting distinct autapomorphic features such as the tendency to andromonoecy (staminate and hermaphrodite central flowers at the same individual) and prevalence of the unique basic chromosome number $x = 17$ instead $x = 25$ for the remaining lineages of the family (Brown and Gilmartin, 1989; Ramirez-Morillo and Brown, 2001).

Most of the *Cryptanthus* species present a restricted geographic distribution, thus forming micro-endemic groups. The majority of the species (more than 60%) occur in the Atlantic forest, a biome widely recognized for its high levels of endemism (Martinelli *et al*., 2008). Moreover, there are also endemic species of the genus that occur in drier environments as *campos rupestres* (Versieux *et al*., 2008). Several factors as the restricted occurrence of many species, habitat degradation, and continued use for ornamentation, have contributed to the actual endangered status for many species (Martinelli *et al*., 2008; Versieux *et al*., 2008). However, just a few studies with this genus have been carried out so far.

Given the ecological importance and threatened condition of the genus, we employed FCM in a representative sampling of *Cryptanthus* and correlated this data to the phylogenetic reconstruction of the genus (Cruz *et al*., in prep.) to address the following questions: (i) what is the variation of the genome size (DNA C-values) within the genus? (ii) How does the observed genome size variation correlate with the molecular phylogeny of the group and available chromosome counts? (iii) Is there a correlation between the observed genome size and the habitat preferences?

Material and Methods

Taxon sampling

A data set of 156 samples was analysed (Table 1) including 47 *Cryptanthus* and five *Orthophytum* species. The plant material was collected during field expeditions and grown at the Jardim Botânico de São Paulo as well as other scientific living collection. Voucher specimens have been deposited at Herbarium Instituto de Botânica de São Paulo and Bradeanum.

Flow cytometry analysis

To estimate the DNA C-values, approximately 20-30 mg of fresh leaves from 47 *Cryptanthus* species, five *Orthophytum* species and from an internal reference standard (*Solanum lycopersicum*

cv. Stupicke polni tyckove) were chopped on ice with 1mL of LB01 and $MgSO_4$ buffers to release the nuclei, according to Dolezel *et al.* (1989). The chopped tissue was aspirated through two layers of cheesecloth using a plastic pipette, filtered through a 50 μm nylon filter, and collected into a polystyrene tube. Nuclei suspension was stained with 25 μL of 1% propidium iodide (w/v), and 5 μL of 20 mg L^{-1} RNase was added to each sample. The samples were stored at 4 °C in the dark and analyzed after 1-2 h. At least 5,000 nuclei were analyzed for each sample using a FACSCalibur cytometer (Becton-Dickinson). Each output flow cytometry histogram from Cell Quest software was analyzed using WinMDI 2.8 software. The DNA 2C-values of each sample were calculated by the relative fluorescence intensity of the sample and the internal reference standard (1.96 pg for *S. lycopersicum*). At least three plants and three samples per plant for each species were analyzed to obtain the mean DNA C value.

Reconstructing Ancestral Character States

For genome size evolution analysis, all available DNA C-values [including the previously obtained for *C. bahianus* (1C = 0.38 pg) by Ramirez-Morillo and Brown (2001)] were taken into account. Then, in order to simplify the data input for the reconstruction of ancestral character states, the range of DNA C-values was converted into a categorical data set, according to the Sturges's rule ($k = 1+\log_2 N$) for determining the number of classes, thus resulting in seven different groups. Habitat types of the sampled species were also recorded for the reconstruction of ancestral character states within the genus *Cryptanthus*. A total of five different patterns of distribution were observed: 1) Atlantic forest; 2) Atlantic forest and *restinga*; 3) Atlantic forest and *caatinga*; 4) *campos rupestres*; and 5) *canga*. The Bayesian phylogenetic tree of *Cryptanthus* based on AFLP markers (Cruz *et al*., in prep.) was used as the basis for reconstruction ancestral states for both genome size and habitat type by the maximum parsimony method with the software Mesquite 2.75 (Maddison and Maddison, 2011).

Phylogenetically corrected t-Tests

A *t*-test ($p < 0.05$) was performed in the software STATISTICA 8 (Statsoft, Inc.) to test for nonrandom differences in genome sizes among major well-supported clades of *Cryptanthus* according to Cruz *et al*. (*in prep*.): 1) between *Cryptanthus* subgen. *Cryptanthus* and *C*. subgen. *Hoplocryptanthus*; 2) between clades I and II (*C*. subgen. *Cryptanthus*); and 3) between clades IV and V (*C*. subgen. *Hoplocryptanthus*). Additionally, differences in genome sizes were also tested among species from the two main habitat types (Atlantic forest and *campos rupestres*).

Results

Flow cytometry analysis

This paper reports the DNA C-values for 47 *Cryptanthus* species from which, 43 (91,5 %) are first reports and five new values for *Orthophytum* species (Table 1), selected as sister group. The coefficient of variation (CV) of the G0/G1 peak varied from 1.99 to 7.11 (Figure 1). In the vast majority of the estimations (84.6%), the CV value was lower than 3 %, and only 1.92% of them were higher than 4 %. In all cases, the DNA C-values presented no meaningful differences among different individuals of the same species.

Genome size variation

The analysis revealed a pronounced variation in nuclear DNA content within the genus *Cryptanthus* (Table 1), spanning a 2.13-fold ranging from 1C = 0.38 pg (*C. bahianus*) to 1C = 0.83 pg (*C. correia-araujoi*). A continuous variation of the DNA C-values was sorted according to the prefered habitat, which was much clearer for the subgenus *Hoplocryptanthus*. The species that occur in *campos rupestres* of *Hoplocryptanthus* presented the lowest DNA contents (1C = 0.35-0.62 pg), followed by the species from the Atlantic forest of the same subgenus (1C = 0.42-056 pg). On the other hand, the subgenus *Cryptanthus* (1C = 0.76-0.83; Figure 2).

Genome size and molecular phylogeny

Based on the previously cited AFLP phylogenetic analysis (Cruz *et al*., in prep; Figure 2), the clade I (15 species and 35 accessions) showed DNA C-values ranging from 0.35 pg (*C. alagoanus*) to 0.83 pg in *C. correia-araujoi*. In turn, the clade II (19 species and 35 accessions) revealed a variation from 0.38 pg in *C. bahianus* to 0.76 pg in *C. bromelioides*. Regarding the basal lineages (subg. *Hoplocryptanthus*) in clade III (with just one species mensuared) no variation was observed, while in clade IV (seven species and 16 accessions) the DNA C-values were quite stable, given that they ranged from 0.39 pg in *C. caracensis* and *C. regius* to 0.41 pg in *C. ferrarius* and *C. schwackeanus*. Finally, for the clade V (eight species and 14 accessions) the DNA C-values ranged from 0.47 pg in *C. microglazioui* to 0.55 pg in *C. odoratissimus.*

Considering the mean size of each major clade within the genus *Cryptanthus*, it is clear that the subgenera *Cryptanthus* and *Hoplocryptanthus* differ markedly in DNA content variation (Figure 2). The phylogenetically corrected *t*-test showed significant differences between these two groups (p = 0.00214). Furthermore, the same was also observed between the clades IV and V within *Hoplocryptanthus* (p = 0.00565), where the species are split in *campos rupestres* and Atlantic forest respectively. The clades I and II within the subgenus *Cryptanthus* did not show significant differences. Relationships between DNA content and habitat preferences within the genus *Cryptanthus*, where the species occupy different habitats like Atlantic forest and *campos rupestres*, also differed significantly in their DNA C-values (p = 0.00102).

4. Discussion

This study aimed to investigate the role of different evolutionary forces in shaping the genome sizes within a diverse plant group of the family Bromeliaceae. We have estimated the DNA C-values in a significantly representative sample set of the genus *Cryptanthus* (47 species), further interpreted the results in the light of the phylogenetic relationships and habitat preferences.

Genome size diversity in Cryptanthus

The chromosome numbers determined by previous studies for *Cryptanthus*, except for the contested number for *C. beuckeri* (Supplementary Table S1), indicate that the genus is essentially diploid. Despite of this conservation in ploidy level with few variation, as reported in previous studies, genome size is a highly dynamic and bidirectional process bringing in some cases significant variation also among different homoploid species (Bennett and Leitch, 2005; Loureiro *et al.*, 2010). On the other hand, it tends to be very stable within the same evolutionary unit (Bennett *et al.*, 2000; Greilhuber, 2005; Murray, 2005). Therefore, the 2.13-fold genome size observed difference was interpreted as a genuine variation. For example, the species with the second largest genome (*C. bromelioides*; 1C = 0.76 pg), presents the same chromosome number (2n=34) as the species with the smallest genome here observed (*C. bahianus*; 1C = 0.38 pg). Amplification and deletion of satellite repeats as well as amplification and insertion of transposable genetic elements may have lead to this variation (Bennetzen *et al.*, 2005; Lim *et al.*, 2006; Vitte and Bennetzen, 2006).

Genome size evolution and molecular phylogeny

The analyses of the genome size distribution on the *Cryptanthus* phylogenetic tree (Cruz *et al.*, in prep) matches the two defined major lineages, the subgenera *Cryptanthus* and *Hoplocryptanthus*, suggesting that this difereciation could have evolved due to selective processes. Thus, the genome size evolution of the genus demonstrates the dynamic evolutionary processes of the extant species, which can be inferred from the strong association of nuclear DNA content with the evolutionary divergence of the two subgenera.

The significant genome size variation that was found between the clades IV and V may also be associated with the different habitat types where they occur, thus the genome variation found, as well as the portrayed phylogeny (Cruz *et al.*, in prep), suggest that the morphology of this group

(Ramirez-Morillo, 1996; Leme, 2007; Leme and de Paula, 2009) could be interpreted as a local adaptation to the *campos rupestres* (clade IV) and the Atlantic forest (clade V).

Within the subgenus *Cryptanthus*, there is no significant difference in genome sizes between the clades I and II. On the other hand, the tracing of character transitions onto the phylogenetic tree (Figure 2) indicates that there were numerous significant shifts within the subgenus *Cryptanthus*, in which we find an increase in genome sizes. The variation of the DNA content involve almost all the range observed for the genus. The molecular phylogeny of the genus (Cruz *et al.*, in prep) is sufficiently representative, once it covers the whole range of estimated genome sizes. However, given the high diversification of genome sizes observed in this subgenus, as well as the low phylogenetic resolution reported among some closely related species (Cruz *et al*., in prep), it still is very difficult to make further considerations on the ancestral states and possible directions of genome size evolution.

Genome size and habitat preferences

There are many studies that have been focusing on investigating associations between genome size and various environmental parameters (such as habitat, temperature, precipitation and altitude). For example, Dušková *et al.* (2010) found in the genus *Lasiocephalus* Willd. ex Schltdl. (Asteraceae) a strong correlation between several environmental factors (e.g. altitude, habitat and growth form) and the genome size. Similarly, Veseley *et al.* (2012) observed that genome size evolution in geophytes is closely related to their ecology (e.g. growth in humid conditions) and phenology features. In summary, the ecology of plants is determined by sets of traits, many of which are constrained by the genome size. Therefore, the amount of nuclear DNA determines the range of conditions in which a plant can evolve, while its genetic composition defines its actual phenotypic expression (Loureiro *et al*., 2010). The association between genome sizes and habitats preferences (*campos rupestres* and Atlantic forest, in this case) is reasonable, given that the existing variables of

the environments have shaped the historical biogeography and, therefore, the genetic diversity of the groups of plants (Carnaval *et al*., 2009; Martins, 2011).

The statistical t-test showed that there is a significant difference in genome sizes when comparing species from the *campos rupestres* and species from the Atlantic forest. In addition, the reconstruction of the evolution of genome sizes and habitat preferences (Figure 2) also presented a positive association between these two characters. In general, species from drier environments such as *campos rupestres* and *cangas* (clade IV plus *C. arelli* and the species from *caatinga C. bahianus*, both sheltered in the clade II) presented the smallest genomes, in average, while their counterparts from Atlantic forest had the largest genomes.

The rocky outcrop areas (*campos rupestres*) shelter a vegetation type that is considered peculiar (Porembski and Barthlott, 2000). Many of these species have a number of features that allow their survival in such environments with poor and narrow soils and high insolation levels, where such conditions have been key constraints to the selection of species that strive in those habitats (Giulietti *et al*., 1997; Porembski *et al*., 1998). In turn, the Atlantic forest is characterized by a strong seasonality, sharp environmental gradients and orographic driven rainfall, forming a diverse landscape that includes open, mixed, and closed evergreen, semi-deciduous and deciduous forests (Tabarelli *et al.*, 2010). Taking into account these environmental characteristics, it seems to be reasonable that the habitat preferences may have contributed somehow in shaping genome sizes within the genus, as observed in the high diversity among species from the Atlantic forest, in opposition to the more stable pattern that was found for the species from *campos rupestres*. Furthermore, according to the theory of the "large genome constraint" by Knight *et al*. (2005), species with large genomes are under-represented in environments with extreme conditions as *campos rupestres*. Based on our results, the species of the subgenus *Hoplocryptanthus* plus *C. bahianus* and *C. arelli* from this habitat are in accordance with this cited hypothesis, since they present the smallest genome sizes within the genus.

Despite the importance of the environmental factors in shaping the genome size, the statistical test showed that the clades I, II and V do not significantly differ from one another in terms of habitat preferences. The variation was structured according to the molecular phylogeny of the group, as also reflected by the positive association between genome sizes and phylogenetic position of the main clades. Therefore, the phylogenetic relationships were the most determinant factors of the observed divergence in genome size at the major lineages.

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Table 1. List of *Cryptanthus* species included in the study. Abbreviations: AF, Atlantic forest; CA, *caatinga*; CG, *canga*; CR, *campos rupestres*; HB, Herbarium Bradeanum; IBt, Instituto de Botânica; LC, Living collection; RE, Restinga; RG, Refúgio dos Gravatás in Teresópolis, Rio de Janeiro; SP, Herbarium of Instituto de Botânica. [K]Kew Gardens database (Ramirez-Morillo and Brown, 2001).

Species	Voucher	1C Value (pg)	Habitat type
Cryptanthus acaulis (Lindl.) Beer	RG, 3359 (LC)	0.69	AF
Cryptanthus alagoanus Leme & J.A.Siqueira	RG, 5380 (LC)	0.39	AF
Cryptanthus arelii H.Luther	RG, 2830 (LC)	0.49	CR
Cryptanthus bahianus L.B.Sm.	SEL 87-382	0.38[K]	AF, CA
Cryptanthus beuckeri E.Morren	RG, 7341 (LC)	0.73	AF
Cryptanthus bibarrensis Leme	RG, 5016 (LC)	0.71	AF
Cryptanthus brevifolious Leme	RG, 3841 (LC)	0.41	AF
Cryptanthus bromelioides Otto & Dietrich.	RG, 2229 (LC)	0.76	AF
Cryptanthus burle-marxii Leme	RG, 6195 (LC)	0.66	AF, RE
Cryptanthus capitatus Leme	RG, 1117 (LC)	0.68	AF
Cryptanthus capitellatus Leme & L.Kollmann	RG, 7988 (LC)	0.68	AF
Cryptanthus caracensis Leme & E.Gross	RG, 1853 (LC)	0.39	CR
Cryptanthus colnagoi Rauh & Leme	RG, 5144 (LC)	0.67	AF
Cryptanthus coriaceus Leme	Leme et al. 1114 (HB)	0.42	AF
Cryptanthus correia-araujoi Leme	Leme et al. 2704 (HB)	0.83	AF
Cryptanthus delicatus Leme	RG, 2236 (LC)	0.73	AF
Cryptanthus dianae Leme	RG, 3872 (LC)	0.68	AF
Cryptanthus ferrarius Leme & C.C.Paula	RG, 6540 (LC)	0.41	CR, CG
Cryptanthus giganteus Leme & A.P.Fontana	RG, 6913 (LC)	0.69	AF
Cryptanthus glazioui Mez	RG, 1856 (LC)	0.40	CR
Cryptanthus latifolius Leme	RG, 5220 (LC)	0.55	AF
Cryptanthus leopoldo-horstii Rauh	RG, 8414 (LC)	0.57	CR
Cryptanthus leuzingereae	RG, 1144 (LC)	0.49	AF
Cryptanthus lutherianus I.Ramirez	RG, 6956 (LC)	0.45	AF
Cryptanthus lyman-smithii Leme	RG, 4344 (LC)	0.69	AF
Cryptanthus marginatus L.B.Sm.	RG, 0290 (LC)	0.70	AF
Cryptanthus maritimus L.B.Sm.	Leme et al. 1582 (HB)	0.74	AF
Cryptanthus microglazioui I.Ramirez	RG, 0152 (LC)	0.47	AF
Cryptanthus odoratissimus Leme	RG, 5207 (LC)	0.55	AF
Cryptanthus pickelii L.B.Sm.	RG, 7516 (LC)	0.64	AF
Cryptanthus pseudoglazioui Leme	Leme et al. 1560 (HB)	0.50	AF
Cryptanthus pseudoscaposus L.B.Sm.	RG, 5211 (LC)	0.52	AF
Cryptanthus regius Leme	Leme et al. 6372 (HB)	0.39	CR
Cryptanthus reisii Leme	RG, 5015 (LC)	0.44	AF
Cryptanthus sanctaluciae Leme & L. Kollmann	RG, 6699 (LC)	0.50	AF
Cryptanthus scaposus E.Pereira	RG, 5214 (LC)	0.52	AF
Cryptanthus schwackeanus Mez	RG, 7205 (LC)	0.41	CR, CG
Cryptanthus sinuosus L.B.Sm.	RG, 2868 (LC)	0.52	AF, RE
Cryptanthus teretifolius Leme	Leme et al. 3073 (HB)	0.70	AF
Cryptanthus tiradentesensis Leme	RG, 7266 (LC)	0.40	CR
Cryptanthus ubairensis I.Ramirez	RG, 7788 (LC)	0.50	AF
Cryptanthus venecianus Leme & L.Kollmann	RG, 7743 (LC)	0.66	AF
Cryptanthus viridipetalus Leme	RG, 8016 (LC)	0.67	AF
Cryptanthus viridovinosus Leme	RG, 7674 (LC)	0.64	AF
Cryptanthus warren-loosei Leme	RG, 0481 (LC)	0.64	CR
Cryptanthus whitmanii Leme	RG, 5209 (LC)	0.53	AF
Cryptanthus zonatus (Visiani) Beer	RG, 6559 (LC)	0.52	AF
Orthophytum amoenum (Ule) L.B.Sm.	Louzada, 7106 IBt (LC)	0.52	CR
Orthophytum hatschbachii Leme	Louzada, 104 IBt (LC)	0.52	CR
Orthophytum heleniceae Leme	Louzada et al. 2544 (SP)	0.54	CR
Ortophytum ophiuroides Louzada & Wand.	Louzada, 88 IBt (LC)	0.51	CR
Orthophytum ulei Louzada & Wand.	Louzada, 91 IBt (LC)	0.75	CR

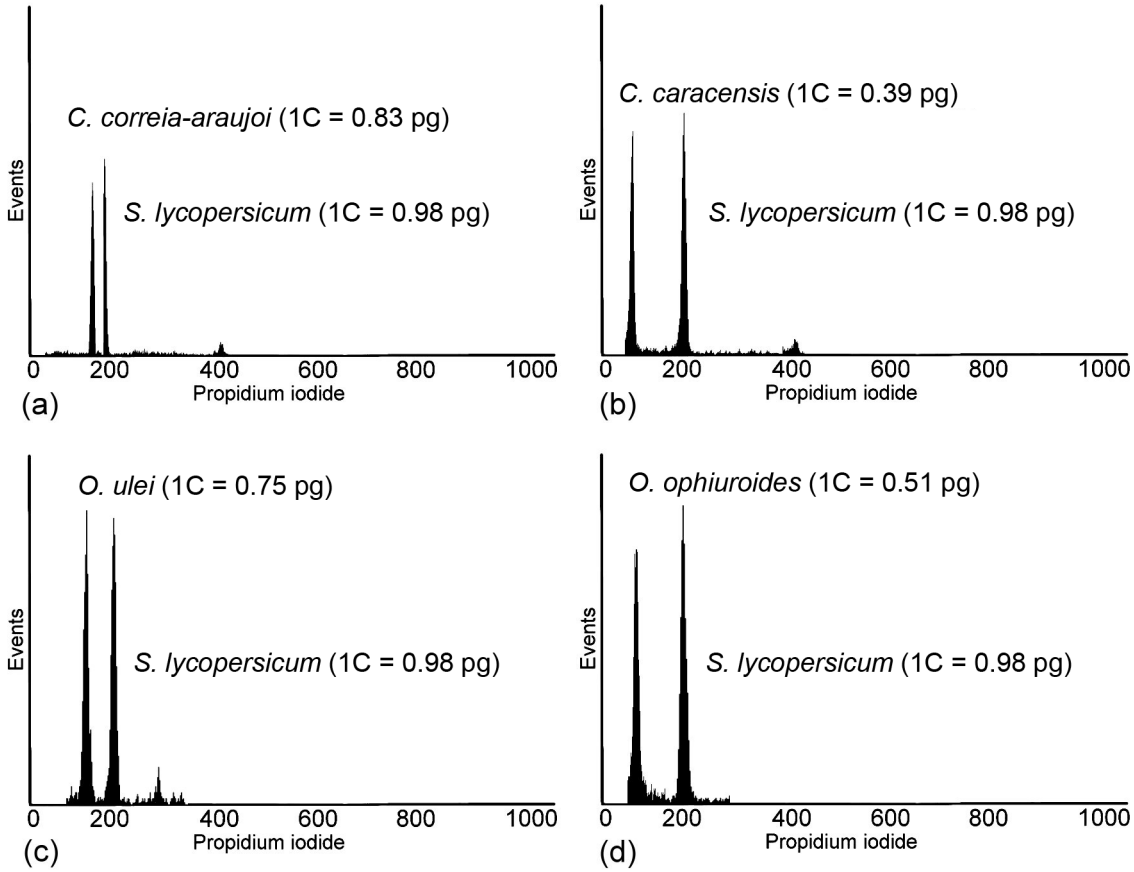

Figure 1: Fluorescence histograms of simultaneous analysis of propidium iodide stained nuclei isolated from fresh tissue of internal standard (*S. lycopersicum*) together with the species of both genera *Cryptanthus* (A and B) and *Orthophytum* (C and D), showed two major peaks corresponding to G0/G1 of each species.

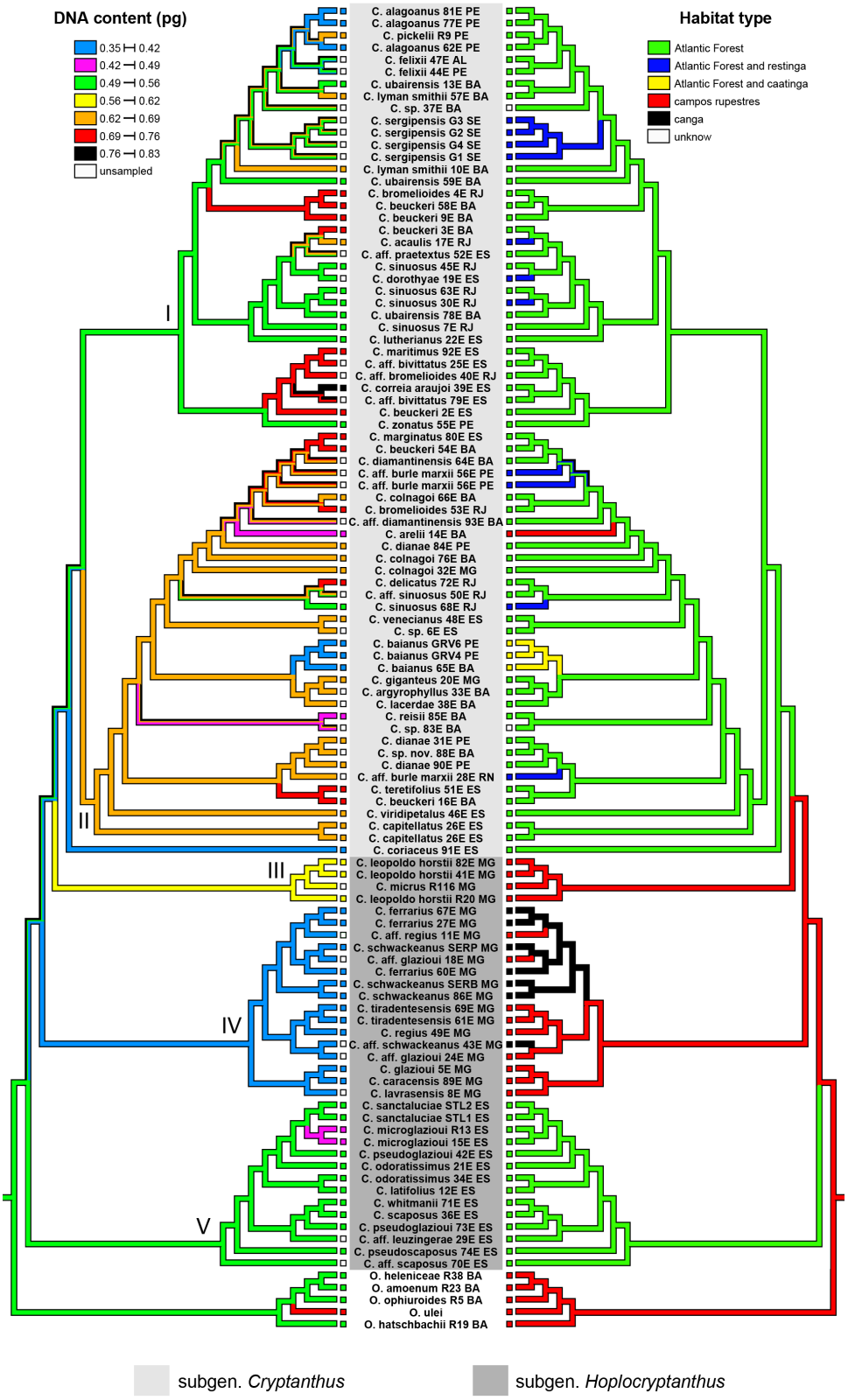

Figure 2: Most parsimonious reconstruction of the evolution of DNA content (on the left) and habitat type (on the right) in *Cryptanthus*, based on relationships revealed by the molecular phylogeny (Cruz *et al*. in prep.).

Appendix A. Supplementary material

Table S1. Chromossome numbers reported in the literature for *Cryptanthus* species, as compared to 1C genome sizes given in Table 1. Counts within parenthesis (carried out by Lindschau, 1933) were based on the microtome section technique that lead to misinterpretations of chromosome numbers, being therefore excluded from the discussion in the present work. [K]Kew Gardens database (Ramirez-Morillo and Brown, 2001).

Species	1C Value (pg)	Chromossome number (2n)	Reference
Cryptanthus bahianus L.B.Sm.	0.38[K]	34	Marchant, 1967
		34 + 1-4B	Cotias-de-Oliveira et al., 2000
		34 + 1-3B	Gitaí et al. (in prep.)
Cryptanthus beuckeri E.Morren	0.73	(54)	Lidschau, 1933
		ca. 34	Marchant, 1967
Cryptanthus bivittatus (Hook.) Regel	-	36	Sharma and Ghosh, 1971
Cryptanthus bromelioides Otto & Dietrich.	0.76	34	Sharma and Ghosh, 1971
Cryptanthus burle-marxii Leme	0.66	34	Gitaí et al. (in prep.)
Cryptanthus dianae Leme	0.68	32	Gitaí et al. (in prep.)
Cryptanthus marginatus L.B.Sm.	0.70	32	Gitaí et al. (in prep.)
Cryptanthus maritimus L.B.Sm.	0.74	34	Ceita et al., 2008
Cryptanthus praetextus E. Morren ex Baker	-	34	Sharma and Ghosh, 1971
Cryptanthus schwackeanus Mez	0.41	34	Ramirez-Morillo and Brown, 2001
		34	Gitaí *et al.* (in prep.)
Cryptanthus warren-loosei Leme	0.64	34	Ceita *et al.*, 2008
Cryptanthus zonatus (Visiani) Beer	0.52	(36)	Lindschau, 1933
		34	Marchant, 1967
		34	McWilliams, 1974

CAPÍTULO III

(Manuscrito publicado na Revista *Research in Plant Biology*)

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Short Communication

A set of variable plastid SSR markers for the genus *Cryptanthus* (Bromeliaceae)

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The genus *Cryptanthus* (Bromeliaceae) is endemic to Brazil. Many of its currently recognized 66 species are narrow endemics that are threatened by habitat destruction. Molecular markers are needed to evaluate the extent and distribution of genetic diversity in rare *Cryptanthus* species, which would be a prerequisite for taking appropriate conservation measures. Here we describe the development of plastid microsatellite markers (cpSSRs) for *Cryptanthus*. PCR primers specific for 34 cpSSR loci in *Dyckia marnier-lapostollei* were initially tested for their functionality in *Cryptanthus schwackeanus*. PCR was successful for 29 loci, and 13 loci were shown to harbour extended stretches of mononucleotide repeats. Seven loci were further characterized by genotyping *Cryptanthus* samples at the level of populations and species, and six loci proved to be polymorphic among 30 individuals of each of the two endangered species *C. schwackeanus* and *C. warren-loosei*, respectively. All primers cross-amplified in other genera from three subfamilies of Bromeliaceae.

Keywords : Bromeliaceae; Bromelioideae; *Cryptanthus*; cpSSR; microsatellites; population genetics

The genus *Cryptanthus* Otto & A. Dietr. (Bromeliaceae) comprises 66 species that are endemic to Brazil. *Cryptanthus* species can be found in a variety of habitats, ranging from semi-humid locations in Atlantic Forests to the semi-arid Campos Rupestres, which form exposed "rocky fields" in the Cerrado and Caatinga biomes of eastern Brazil. The genus is distributed from Rio Grande do Norte in the north to Rio de Janeiro in the south, forming a centre of biodiversity in the states of Minas Gerais and Espírito Santo (Ramirez-Morillo, 1996; Ramirez-Morillo & Brown, 2001; Luther, 2008; Forzza et al., 2011). In general, *Cryptanthus* species have narrow distribution areas, and many of them are micro-endemics. Due to ongoing deforestation, 26 *Cryptanthus* species have become quite rare and are now included in the list of endangered species, such as the particularly vulnerable *C. schwackeanus* Mez (Martinelli et al., 2008). *Cryptanthus* species from the Campos Rupestres of Minas Gerais and Bahia are additionally threatened by mining, anthropogenic fires, and grazing. Here, the ongoing habitat destruction is compromising the survival of e.g. *C. warren-loosei* Leme, a micro-endemic species of that habitat in Bahia (Versieux et al., 2008).

The successful implementation of conservation measures for *Cryptanthus*

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requires some basic knowledge about the extent and distribution of genetic diversity within and among species. Such knowledge can be collected via appropriate population genetic surveys. Suitable molecular genetic marker systems are however needed for this purpose. Here we describe the development of a set of highly polymorphic chloroplast microsatellite markers (cpSSRs) for *Cryptanthus*, based on previously analyzed plastome sequences from another bromeliad, *Dyckia marnier-lapostollei* L.B.Sm. (Krapp et al., 2012).

Materials and methods

DNA was isolated from leaves of individual plants following Tel-Zur et al. (1999). Thirty-four primer pairs that flank cpSSR loci in *Dyckia marnier-lapostollei* were initially tested on DNA from a single accession of *C. schwackeanus*. PCRs were carried out in 10 µL volumes in a Biometra T-Gradient cycler, using the indirect fluorescence labelling procedure described by Schuelke (2000). Each assay contained approximately 1 ng of template DNA, 1x Mango-*Taq* reaction buffer (Bioline), 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 1xTBE buffer, using an automated sequencer (Li-Cor 4300, Li-Cor Biosciences). Fragment sizes were determined by eye with the help of an external size standard, as outlined by Wöhrmann et al. (2012). Allele sizes were validated by repeated PCR amplification of selected samples. PCR products for one accession of *C. schwackeanus* were sequenced directly, using a Thermo Sequenase Primer Cycle Sequencing Kit (GE Healthcare) and an automated sequencer (Li-Cor 4300, Li-Cor Biosciences), following the protocols supplied by the manufacturers.

Results and discussion

Twenty-nine of the 34 loci showed distinct bands on agarose when tested with a single accession of *C. schwackeanus*. The presence and copy number of A/T mononucleotide repeats in the PCR products were validated by single nucleotide sequence analyses (Guicking et al., 2008). A/T repeats with N≥10 were found at 13 loci, which were further evaluated by amplifying an extended set of *Cryptanthus* individuals from three species, *C. schwackeanus*, *C. warren-loosei* and *C. sergipensis* I. Ramírez. Seven loci proved to be suitable for further study, based on the extent of variation within species and populations, the rate of successful PCR amplification, and the ease of sizing. Primer sequences, locus characteristics and GenBank accession numbers are shown in Table 1. These seven loci were further characterized by genotyping 30 individuals (three populations) of each of the two endangered species *C. schwackeanus* and *C. warren-loosei* as well as several samples from related species and genera. The results are summarized in Table 2. Six loci were polymorphic within *C. schwackeanus* with two or three alleles each, resulting in a total of eight different haplotypes. Three loci also showed polymorphisms within populations. Six loci were polymorphic in *C. warren-loosei*, with two or three alleles per locus combining into five different haplotypes. All six loci were polymorphic in at least one population. The seven cpSSR loci revealed between four and eight alleles within a total of five species of *Cryptanthus*, and all primers amplified well in ten other genera of Bromelioideae, five of Pitcairnioideae, and one of Puyoideae (Table 2).

Table 1 Characteristics of seven chloroplast microsatellite markers (cpSSRs) developed for *Cryptanthus* shown with GenBank accession numbers of the sequences of the locus in *C. schwackeanus* (sequenced for individual 01 of the population from Serra da Piedade). Primer sequences are derived from *Dyckia marnier-lapostollei* (Krapp et al., 2012)

Locus	Primer sequence (5'-3')	Position	Motif	Size (bp)	GenBank Accession No.
Crypt_cpSSR_01	fwd: 5'-CCTATTACAGAGATGGTGCG-3' rev: 5'-TTTCTCGTAAGACTGAGGGC-3'	*ycf3* intron 2/exon 3 intronic/exonic	$(T)_9$	69	KC111433
Crypt_cpSSR_02	fwd: 5'-GTTCCCAGTAAGAACCAACC-3' rev: 5'-CTCAATAATTTCACATTTCC-3'	*rpoC1* Intron intronic	$(T)_{23}$	104	KC111434
Crypt_cpSSR_03	fwd: 5'-TTGTTGTGGTATCTTTCGCC-3' rev: 5'-CAAGATTTCTCTGATACCCG-3'	*psbB-psbT* intergenic	$(T)_{10}$	64	KC111435
Crypt_cpSSR_04	fwd: 5'-TNAATCAATATGGCGAAGGC-3' rev: 5'-ATTCCCTCACGCTTGGCGCC-3'	*clpP* Intron 2 intronic	$(T)_{10}$	79	KC111436
Crypt_cpSSR_05	fwd: 5'-TTTTTGTTATGGGTATTCCC-3' rev: 5'-ACAAACAGAAAAGAGAGGGC-3'	*atpF* intron intronic	$(T)_{23}$	71	KC111437
Crypt_cpSSR_06	fwd: 5'-CTTCCATTTATCCATATCCC-3' rev: 5'-AAAATAAATCTGATTATGGG-3'	*rpl16* Intron intronic	$(T)_{14}$	67	KC111438
Crypt_cpSSR_07	fwd: 5'-GTGGATTTATTTTTTGTCCC-3' rev: 5'-AGACCCCCGGGCTCGACGACG-3'	*rps3-rpl22* intergenic	$(TA)_4(T)_{13}$	170	KC111439

Table 2. Observed allele sizes (bp) at seven chloroplast microsatellite loci in *C. schwackeanus* and *C. warren-loosei*, each represented by three populations with N=10. Allele numbers and allele size range among five different *Cryptanthus* species (*C. acaulis* Beer, *C. fosterianus* L.B. Sm., *C. microglazioui* I. Ramírez, *C. schwackeanus* and *C. warren-loosei* (between one and 30 individuals each) and cross-amplification in the subfamilies Pitcairnioideae, Bromelioideae (excluding *Cryptanthus*) and Puyoideae.

Locus	*C. schwackeanus*			*C. warren-loosei*			*Cryptanthus*	subfamily Pitcairnioideae	subfamily Bromelioideae	*Puya*
	Serra da Piedade*	Serra da Calçada*	Serra da Brígida*	Jacobina*	Buraco do Possidônio (MCH)*	Guaribu (MCH)*	allele number (size range in bp)			
Crypt_cpSSR_01	69, 71	69,71	69,71	80	75, 80	79, 80	8 (69-80)	2 (69-70)	5 (68-74)	1 (69)
Crypt_cpSSR_02	104	105	104	101	101, 104	101	6 (100-105)	3 (101-104)	5 (96-109)	1 (101)
Crypt_cpSSR_03	64	64	64	65	64, 65	63, 64	4 (61-65)	2 (63-64)	5 (62-69)	1 (64)
Crypt_cpSSR_04	79	80, 81	80	79	78, 79	78	5 (78-86)	3 (78-80)	5 (78-84)	1 (81)
Crypt_cpSSR_05	71, 72	72	73	68	68	68	4 (68-73)	4 (67-71)	4 (67-70)	1 (67)
Crypt_cpSSR_06	67	70	62	63	62, 63	63, 64	6 (61-70)	4 (60-65)	5 (60-65)	1 (63)
Crypt_cpSSR_07	170	172	176	170	169, 170	170	7 (161-176)	5 (156-163)	8 (161-179)	1 (159)

* Population (sampling location)

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CONCLUSÕES

- A filogenia molecular gerada pelo AFLP indicou que os subgêneros *Cryptanthus* e *Hoplocryptanthus* formam grupos não monofoléticos.
- A presença de vários nós sem suporte estatístico é provavelmente devido a característica evolução recente da família Bromeliaceae.
- É necessário investigações complementares das relações infragenéricas, já que os grupos morfológicos propostos foram definidos com base em caracteres homoplásicos, exceto o inconclusivo lacerdae.
- Aparentemente a ocupação da Floresta Altântica pelo gênero ocorreu várias vezes sendo esta bem definida em *Hoplocryptanthus*, constituindo mais uma evidência da condição polifilética deste subgênero.
- A reconstrução de estado de caracter ancestral mostrou a importância evolutiva das flores andromonóicas, as quais podem ter contribuído para a diversificação e colonização das espécies do subgênero *Cryptanthus* em diferentes ambientes no domínio da Floresta Altântica.
- Foi observado associação positiva entre a variação do tamanho genômico e a filogenia molecular entre os subgêneros *Cryptanthus* e *Hoplocryptanthus*. Adicionalmente, também foi observado diferença significativa dos tamanhos genômicos e preferência de ocupação dos habitats.
- Dentre as várias forças e processos evolutivos que moldam o tamanho genômico em *Cryptanthus,* as relações filogenéticas foram os mais determinantes.
- Em estudos populacionais, foi observado transferabilidade e polimorfismo de sete *loci* de microssatélites plastidiais em populações das espécies *C. schwackeanus* e *C. warren-loosei*. Portanto, os *loci* analisados apresentam-se como ferramentas informativas na determinação da estrutra genética populacional do grupo.

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Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

Reference style

Text: All citations in the text should refer to:
1. *Single author:* the author's name (without initials, unless there is ambiguity) and the year of publication;
2. *Two authors:* both authors' names and the year of publication;
3. *Three or more authors:* first author's name followed by 'et al.' and the year of publication.

Citations may be made directly (or parenthetically). Groups of references should be listed first alphabetically, then chronologically.
Examples: 'as demonstrated (Allan, 2000a, 2000b, 1999; Allan and Jones, 1999). Kramer et al. (2010) have recently shown ....'
List: References should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.
Examples:
Reference to a journal publication:
Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2010. The art of writing a scientific article. J. Sci. Commun. 163, 51–59.
Reference to a book:
Strunk Jr., W., White, E.B., 2000. The Elements of Style, fourth ed. Longman, New York.
Reference to a chapter in an edited book:
Mettam, G.R., Adams, L.B., 2009. How to prepare an electronic version of your article, in: Jones, B.S., Smith , R.Z. (Eds.), Introduction to the Electronic Age. E-Publishing Inc., New York, pp. 281–304.

Journal abbreviations source

Journal names should be abbreviated according to
Index Medicus journal abbreviations: http://www.nlm.nih.gov/tsd/serials/lji.html;
List of title word abbreviations: http://www.issn.org/2-22661-LTWA-online.php;
CAS (Chemical Abstracts Service): http://www.cas.org/content/references/corejournals.

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You can enrich your online articles by providing phylogenetic tree data files (optional) in Newick or NeXML format, which will be visualized using the interactive tree viewer embedded within the online article. Using the viewer it will be possible to zoom into certain tree areas, change the tree layout, search within the tree, and collapse/expand tree nodes and branches. Submitted tree files will also be available for downloading from your online article on ScienceDirect. Each tree must be contained in an individual data file before being uploaded separately to the online submission system, via the "phylogenetic tree data" submission category. Newick files must have the extension .new or .nwk (note that a semicolon is needed to end the tree). Please do not enclose comments in Newick files and also delete any artificial line breaks within the tree data because these will stop the tree from showing. For NeXML, the file extension should be .xml. Please do not enclose comments in the file. Tree data submitted with other file extensions will not be processed. Please make sure that you validate your Newick/NeXML files prior to submission. For more information please see http://www.elsevier.com/phylogenetictrees.

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Ensure that the following items are present:

One author has been designated as the corresponding author with contact details:

• E-mail address
• Full postal address
• Phone numbers

All necessary files have been uploaded, and contain:

• Keywords
• All figure captions
• All tables (including title, description, footnotes)

Further considerations

• Manuscript has been 'spell-checked' and 'grammar-checked'
• References are in the correct format for this journal
• All references mentioned in the Reference list are cited in the text, and vice versa
• Permission has been obtained for use of copyrighted material from other sources (including the Web)
• Color figures are clearly marked as being intended for color reproduction on the Web (free of charge) and in print, or to be reproduced in color on the Web (free of charge) and in black-and-white in print
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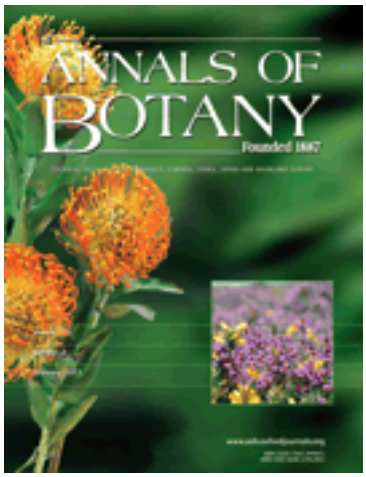
Anexo II. Instrução para autores: revista *Annals of Botany*



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(Always consult a recent issue of *Annals of Botany* for layout and style)

Text should be typed using size 12 Times New Roman or Courier, double-spaced throughout and with an approx. 25 mm margin. All pages should be numbered sequentially. Each line of the text should also be numbered, with the top line of each page being line 1. The article file should be in PC-compatible Microsoft Word - file type DOC [please make sure the "Language" is "English (U.K)" via Tools → Language → Set Language]. RTF files are also acceptable. Please do not use the Windows Vista DOCX format: if you have created the text in this format, please save the files as RTF before submitting them. Please do *not* submit PDFs, desktop publishing files or LaTeX files. The article file should *include* a list of any figure legends but*exclude* any figures themselves – these should be submitted separately, with each figure in a separate file. Tables should be included at the end of the article file, in a Word format and *not* embedded as an image/picture. For more details see below under **PREPARING TABLE and FIGURE FILES, SUPPLEMENTARY INFORMATION FILES AND VIDEO FILES**.

It is NOT journal style to have footnotes within articles. Any such notes must be incorporated into the main text, for example within brackets or as a separate paragraph.

The **first page** should state the type of article (e.g. Original Article, Technical Article) and provide a concise and informative full **title** followed by the names of all authors. Where necessary, each name should be followed by an identifying superscript number ([1, 2, 3] etc.) associated with the appropriate institutional address to be entered further down the page. For papers with more than one author, the corresponding author's name should be followed by a superscript asterisk*. The institutional address(es) of each author should be listed next, each address being preceded by the relevant superscript number where appropriate. A running title of not more than 75 characters, including spaces, should also be provided, followed by the e-mail address of the corresponding author. Please follow the layout used for the first page of papers published in *Annals of Botany*.

The **second page** should contain a structured **Abstract** not exceeding 300 words made up of bulleted headings. For ‘ORIGINAL ARTICLES’ these heading will normally be as follows:
•*Background and Aims*
• *Methods*
• *Key Results*
• *Conclusions*
Alternative bulleted headings, such as ‘*Background*’, ‘*Scope*' and '*Conclusions*', are also acceptable for ‘REVIEWS’, ‘INVITED REVIEWS’, ‘BOTANICAL BRIEFINGS’, ‘TECHNICAL ARTICLES’ papers and ‘VIEWPOINT’ papers.

INFORMATION FOR AUTHORS

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INTRODUCTION

Scope of the journal

Annals of Botany is published for the Annals of Botany Company by Oxford University Press. Experimental, theoretical and applied papers on all aspects of plant science are welcome. The submitted manuscript or its essential content must not have been published previously or be under consideration for publication elsewhere. To merit publication in *Annals of Botany*, contributions should be substantial, written in clear English and combine originality of content with potential general interest. Submission of manuscripts that report small incremental advances or are of geographically local interest only is discouraged unless the implications of the findings are wide-reaching. Agronomic papers are expected to contain a substantial amount of basic plant biology. In general, a paper is unlikely to be accepted unless the referees and editors involved in its evaluation are enthusiastic about the science. The Covering Letter is an essential part of all submissions. It should include an ~60 word summary of the scientific strengths of the paper that the author(s) believe qualify it for consideration by *Annals of Botany*.

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It is NOT journal style to have footnotes within articles. Any such notes must be incorporated into the main text, for example within brackets or as a separate paragraph.

The **first page** should state the type of article (e.g. Original Article, Technical Article) and provide a concise and informative full **title** followed by the names of all authors. Where necessary, each name should be followed by an identifying superscript number ($^{1, 2, 3}$ etc.) associated with the appropriate institutional address to be entered further down the page. For papers with more than one author, the corresponding author's name should be followed by a superscript asterisk*. The institutional address(es) of each author should be listed next, each address being preceded by the relevant superscript number where appropriate. A running title of not more

than 75 characters, including spaces, should also be provided, followed by the e-mail address of the corresponding author. Please follow the layout used for the first page of papers published in *Annals of Botany*.

The **second page** should contain a structured **Abstract** not exceeding 300 words made up of bulleted headings. For 'ORIGINAL ARTICLES' these heading will normally be as follows:
• *Background and Aims*
• *Methods*
• *Key Results*
• *Conclusions*
Alternative bulleted headings, such as '*Background*', '*Scope*' and '*Conclusions*', are also acceptable for 'REVIEWS', 'INVITED REVIEWS', 'BOTANICAL BRIEFINGS', 'TECHNICAL ARTICLES' papers and 'VIEWPOINT' papers.

The Abstract should be followed by between three and 12 **Key words** that include the complete botanical name(s) of any relevant plant material. If many species are involved, species groups should be listed instead. Note that essential words in the title should be repeated in the key words since these, rather than the title, are used in some electronic searches. **Title**, **Abstract** and **Key words** should be self-explanatory without reference to the remainder of the paper.

The **third and subsequent pages** should comprise the remaining contents of the article text. 'ORIGINAL ARTICLES' and 'SHORT COMMUNICATIONS' will usually have the structure INTRODUCTION, MATERIALS AND METHODS, RESULTS, DISCUSSION, ACKNOWLEDGEMENTS and LITERATURE CITED followed by a list of captions to any figures.

The RESULTS section should not include extensive discussion and data should not be repeated in both graphical and tabular form. The DISCUSSION section should avoid extensive repetition of the RESULTS and*must* finish with some conclusions.

Abbreviations are discouraged *except* for units of measurement, standard chemical symbols (e.g. S, Na), names of chemicals (e.g. ATP, Mes, Hepes, NaCl, O_2), procedures (e.g. PCR, PAGE, RFLP), molecular terminology (e.g. bp, SDS) or statistical terms (e.g. ANOVA, s.d., s.e., *n*, *F*, *t*-test and r^2) where *these are in general use*. Other abbreviations should be spelled out at first mention and all terms must be written out in full when used to start a sentence. Abbreviations of scientific terms should not be followed by a full stop. Use the minus index to indicate 'per' (e.g. m^{-3}, L^{-1}, h^{-1}) except in such cases as 'per plant' or 'per pot'. If you decide that a list of abbreviations would help the reader, this should be included as an Appendix.

Units of Measurement. Use the *Systéme international d'unités* (SI) wherever possible. If non-SI units have to be used, the SI equivalent should be added in parentheses at first mention. For units of volume, expressions based on the cubic metre (e.g. 5×10^{-9} m^3, 5×10^{-6} m^3 or 5×10^{-3} m^3) or the litre (e.g. 5 μL, 5 mL, 5 L) are acceptable, but one or other system should be used consistently throughout the manuscript. Typical expressions of concentrations might be 5 mmol m^{-3}, 5 μM (for 5 μmol L^{-1}), or 25 mg L^{-1}. The Dalton (Da), or more conveniently the kDa, is a permitted non-SI unit of protein mass.

Names of plants must be written out in full (Genus, species) in the abstract and again in the main text for every organism at first mention (but the genus is only needed for the first species in a list within the same genus, e.g. *Lolium annuum*, *L. arenarium*). The authority (e.g. L., Mill., Benth.) is *not* required unless it is controversial. Guidance for naming plants correctly is given in The International Plant Names Index and in *The Plant Book: a Portable Dictionary of the Vascular Plants* (1997) by D.J. Mabberley (Cambridge: Cambridge University Press. ISBN 0521-414210-0). After first mention, the generic name may be abbreviated to its initial (e.g. *A. thaliana*) except where its use causes confusion.

Any cultivar or variety should be added to the full scientific name e.g. *Solanum lycopersicum* 'Moneymaker' following the appropriate international code of practice. For guidance, refer to the ISHS *International Code of Nomenclature for Cultivated Plants* (2004) edited by C.D. Brickell, B. R. Baum, W. L. A. Hetterscheid, A. C. Leslie, J. McNeill, P. Trehane, F. Vrugtman, J. H. Wiersema (ISBN 3-906166-16-3).

Once defined in full, plants may also be referred to using vernacular or quasi-scientific names without italics or uppercase letters (e.g. arabidopsis, dahlia, chrysanthemum, rumex, soybean, tomato). This is often more convenient.

Items of **Specialized Equipment** mentioned in MATERIALS AND METHODS should be accompanied by details of the model, manufacturer, and city and country of origin.

Numbers up to and including ten should be written out unless they are measurements. All numbers above ten should be in numerals except at the start of sentences. **Dates** should be in the form of 10 Jan. 1999, and**Clock Time** in the form of 1600 h.

Mathematical equations must be in proper symbolic form; word equations are not acceptable. Each quantity should be defined with a unique *single character* or symbol together with a descriptive subscript if necessary. Each subscript should also be a *single*

character if possible, but a short word is permissible. For example, a relationship between plant dry mass and fresh mass should appear as $M_d = 0.006M_f^{1.461}$, where M_d is plant dry mass and M_f is plant fresh mass; and not as $DM = 0.006FM^{1.461}$.

The meaning of terms used in equations should be explained when they first appear. Standard conventions for use of *italics* only for variables should be followed: normal (Roman) font should be used for letters that are identifiers. Thus in the above example, *M* is the *variable quantity* of mass, the subscripts d and f are identifiers for dry and fresh respectively.

Special note regarding 'Equation Editor' and other software for presentation of mathematics. Symbols and equations that are imported into Word documents as embedded objects from other software packages are generally incompatible with typesetting software and have to be re-keyed as part of the proof-making process. It is therefore **strongly advisable** to type symbols and equations directly into MS Word wherever possible. Importing from other software should ideally be confined to situations where it is essential, such as two-line equations (i.e. where numerators and denominators cannot be set clearly on a single line using '/') and to symbols that are not available in Word fonts. This will minimize the risk of errors associated with rekeying by copyeditors.

Summary statistics should be accompanied by the number of replicates and a measure of variation such as standard error or least significance difference. Analysis of variance is often appropriate where several treatments are involved. Presentation of an abridged ANOVA table is permissible when its use illustrates critical features of the experiment.

Chemical, biochemical and molecular biological nomenclature should be based on rules of theInternational Union of Pure and Applied Chemistry (IUPAC) and the International Union of Biochemistry and Molecular Biology (IUBMB). Chapter 16 of *Scientific Style and Format. The CBE Manual for Authors, Editors, and Publishers 6th edn.,* by Edward J. Huth (Cambridge: Cambridge University Press. ISBN 0-521-47154-0) gives guidelines.

Sequence information. Before novel sequences for proteins or nucleotides can be published, authors are required to deposit their data with one of the principal databases comprising the International Nucleotide Sequence Database Collaboration: EMBL Nucleotide Sequence Database, GenBank, or the DNA Data Bank of Japan and to include an accession number in the paper. Sequence matrices should only be included if alignment information is critical to the message of the paper. Such matrices can be in colour but should not occupy more than one printed page. Larger matrices will only be printed by special agreement but may more readily be published electronically as **Supplementary Information** (see below).

Gene nomenclature. Species-specific rules on plant gene nomenclature are available for:

maize; rice; wheat and arabidopsis.

Otherwise, *Annals of Botany* adopts the following conventions for abbreviations: each gene abbreviation is preceded by letters identifying the species of origin. Lower-case italics should be used for mutant genes (e.g.*Rp-etr1*); upper-case italics (e.g. *Le-ACO1*) for wild-type genes; upright lower-case for proteins of mutated genes (e.g. Le-adh1); and upright upper-case for proteins of wild-type genes (e.g. At-MYB2). It may often be helpful to readers if the names of genes or gene families are spelled out in full at first mention.

Citations in the text. These should take the form of Felle (2005) or Jacobsen and Forbes (1999) or (Williamson and Watanabe, 1987; Rodrigues, 2002*a*, *b*) and be ordered chronologically. Papers by three or more authors, even on first mention, should be abbreviated to the name of the first author followed by et al. (e.g. Zhang *et al*., 2005). If two different authors have the same last name, give their initials (e.g. NH Kawano, 2003) to avoid confusion. Only refer to papers as 'in press' if they have been accepted for publication in a named journal, otherwise use the terms 'unpubl. res.', giving the initials and location of the person concerned. (e.g. H Gautier, INRA, Lusignan, France, unpubl. res.) or 'pers. comm.' (e.g. WT Jones, University of Oxford, UK, 'pers. comm.')

The **LITERATURE CITED** should be arranged alphabetically based on the surname of the first or sole author. Where the same sole author or same first author has two or more papers listed, these papers should be grouped in year order. Where such an author has more than one paper *in the same year*, these should be ordered with single authored papers first followed by two-author papers (ordered first alphabetically based on the second author's surname, then by year) , and then any three-or-more-author papers (in year order only). Italicized letters '*a*', '*b*', '*c*', etc., should be added to the date of papers with the same first authorship and year.

For papers with *six* authors or fewer, please give the names of *all* the authors. For papers with *seven* authors or more, please give the names of the *first three* authors only, followed by *et al*.

Each entry must conform to one of the following styles according to the type of publication.

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Öpik H, Rolfe S. 2005. *The physiology of flowering plants. Physicochemical and environmental plant physiology*, 4th edn. Cambridge: Cambridge University Press.

Chapters in books

Scandalios JG. 2001. Molecular responses to oxidative stress. In: Hawkesford MJ, Buchner P, eds. *Molecular analysis of plant adaptation to the environment*. Dordrecht: Kluwer, 181-208.

Research papers

Popper ZA, Fry SC. 2003. Primary cell wall composition of bryophytes and charophytes. *Annals of Botany***91**: 1–12.

Papers published online ahead of print

Forster MA, Ladd B, Bonser SP. 2011. Optimal allocation of resources in response to shading and neighbours in the heteroblastic species, *Acacia implexa. Annals of Botany*, in press. doi:10.1093/aob/mcq228.
NB include the doi number: a search for the doi will always be directed to the most recent version, so the reader will be able to find the final published paper as soon as it appears.

Online-only journals

Aizen MA, Morales C, Morales JM. 2008. Invasive mutualists erode native pollination webs. PloS Biology 6: e31. doi:10.1371/journal.pbio.0060031.
NB include the doi number after the volume and article number.

Theses

Tholen D. 2005. *Growth and photosynthesis in ethylene-insensitive plants*. PhD Thesis, University of Utrecht, The Netherlands.

Anonymous sources

Anonymous. **Year.** *Title of booklet, leaflet, report, etc.* City: Publisher or other source, Country.

References to websites should be structured as: **Author(s) name, author(s) initial(s). year.** *Full title of article*. Full URL. Date of last successful access (e.g. 12 Jan. 2003)

Acknowledgements. In the ACKNOWLEDGEMENTS, please be brief. 'We thank . . .' (not 'The present authors would like to express their thanks to . . .').

Funding information. Details of all funding sources for the work in question should be given in a separate section entitled 'Funding'. This should appear before the 'Acknowledgements' section.

The following rules should be followed:

- The sentence should begin: ‘This work was supported by …’
- The full official funding agency name should be given, i.e. ‘the National Cancer Institute at the National Institutes of Health’ or simply 'National Institutes of Health' not ‘NCI' (one of the 27 subinstitutions) or 'NCI at NIH’ (full RIN-approved list of UK funding agencies)
- Grant numbers should be complete and accurate and provided in brackets as follows: ‘[grant number ABX CDXXXXXX]’
- Multiple grant numbers should be separated by a comma as follows: ‘[grant numbers ABX CDXXXXXX, EFX GHXXXXXX]’
- Agencies should be separated by a semi-colon (plus ‘and’ before the last funding agency)
- Where individuals need to be specified for certain sources of funding the following text should be added after the relevant agency or grant number 'to [author initials]'.

An example is given here: ‘This work was supported by the National Institutes of Health [P50 CA098252 and CA118790 to R.B.S.R.] and the Alcohol & Education Research Council [HFY GR667789].

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Appendix.
If elaborate use is made of units, symbols and abbreviations, or a detailed explanation of one facet of the paper seems in order, further details may be included in a separate APPENDIX placed after the LITERATURE CITED.
For more detail and information on types of files required for text, graphics and tables etc., please see the next section.

PREPARING TABLE FILES, FIGURE FILES, SUPPLEMENTARY INFORMATION FILES AND VIDEO FILES

Each table, figure, video and set of supplementary information should be prepared as a separate file on your computer in preparation for online submission. Towards the bottom of the first submission screen of the online submission system, you should enter the appropriate number of files you have in each category. This creates the spaces (boxes) that will accommodate the files when they are uploaded later. The files are categorized as ‘Colour Figures’, ‘Black and White Figures’, ‘Tables’, ‘Supplemental Material’ and ‘Video’.

Tables. The best guide for laying out tables and diagrams are papers in a recent issue of *Annals of Botany*. Tables should be placed at the end of the main text file after the Literature Cited, and include a complete caption above the table and be numbered Table 1, Table 2 etc. according to the order in which they are first mentioned in the text. When preparing tables, adopt the 'Tables' set-up in MS Word, using one cell for each datum cluster (e.g. 12.2 ± 1.65) and avoid the use of the 'return' key. If the tables have been prepared in MS Excel, please paste them into the Word document as text, not as an object: i.e. it should be possible in Word to select and edit the text within the table.

Figures. All images (e.g. line diagrams, drawings, graphs, photographs, plates) are considered to be ‘Figures’. Each figure should be in a separate file and be numbered (Fig. 1, Fig. 2 etc.) according to the order in which they are first mentioned in the text. Electron and light **photomicrographs** should have internal scale markers. **Colour images** are encouraged and printed without charge where they enhance significantly the clarity of the scientific information. **Line diagrams** will normally be black on white and boxed with inward scale markings. Use of colour in line diagrams may sometimes be agreed where this enhances clarity significantly. Use open and/or closed circles, squares and triangles for symbols in line graphs. Height and width should be chosen for either single (8.4 cm wide) or double (up to 17.3 cm wide) column reproduction. Grouping of related graphics into a single figure is strongly encouraged. When a block of illustrative material consists of several parts, each part should be labelled A, B, C, etc. and not treated as separate figures. Note that graphs and diagrams of finally accepted papers are normally redrawn by the publisher to ensure a consistent house style and should be inspected carefully by authors at the proof stage.

Simple black and white **line drawings and graphs** should be supplied as approx. 300 dpi JPG files or MS PowerPoint files. The publisher will almost always redraw all such material if the paper is accepted. More complicated drawings, such as detailed botanical illustrations will not be redrawn and should be supplied as 600 dpi JPG files. For continuous tone images (e.g. **photographs**), please supply JPG files at 300 dpi (or 600 dpi if the image is a mix of pictures and text and/or has thin lines). Keeping total files sizes down will lessen up- and downloading times. To help achieve this *all images should be submitted at approximately the physical size they would appear in the Journal*. Scaling, sizing and cropping are best carried out within image handling programs such as Adobe PhotoShop or Corel PhotoPaint. Please do not supply photographic images as PowerPoint files as these are generally of poor resolution. Note that PDF files are not acceptable. Also, please ensure that images that do NOT contain colour are saved as ‘grayscale’ and that any layers have been flattened – taking these steps can make the file size up to 10 times smaller. Note that a JPG file should not be repeatedly saved as this reduces quality.

Large amounts of additional information can be submitted for publication electronically as **Supplementary Information** provided that it is not essential for a basic understanding of the main paper. Supplementary material will be refereed along with the core paper. At appropriate positions in the main text authors should indicate what details are being made available, followed by the words [**Supplementary Information**] in bold and between square brackets. The online submission system provides space for supplementary information to be uploaded in “Supplemental Material” files. The appropriate number of these types of file can be selected towards the bottom of the first submission screen. Similarly, if you are including a **video** you should enter [**Supplementary Information - Video**] in bold and between square brackets at the appropriate place(s) in the text. A video can be uploaded after selecting a “Video” file on the first submission screen. The movie should be created in a widely available program such as Windows MediaPlayer. A short paragraph describing the contents of any Supplementary Information or Video should also be inserted in the main text immediately before ACKNOWLEDGEMENTS.

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Anexo III. Instrução para autores: revista *Research in Plant Biology*

General Guidelines

Submission of Papers

All submissions to Research in Plant Biology (RPB) must be made submit as an email attachment to resplantbiol@gmail.com. Word document is acceptable format, it is recommended that all tables and figures be assembled into a single file together with the main text when submitted.

The manuscript must be accompanied by a cover letter stating the title of the manuscript, names of each author, and complete mailing address(es), telephone and fax number(s) of the corresponding author, electronic mail address(es). Manuscripts should be single-spaced and all pages, including the abstract, figures, and tables, should be numbered in sequence. Manuscript pages must have margins of at least 2.5 cm on all four sides.

Editorial Policy

Originality

Only papers that report novel and significant scientific findings in Plant Biology will be considered and accepted for publication. Manuscripts submitted to RPB must represent reports of original research. A manuscript will be accepted on the conditions that the presented work was not published previously, and is not under consideration for publication elsewhere.

Authorship

Anyone who made a substantial contribution to the work may be included in the author list. All authors of each manuscript are responsible for the entire paper and must have agreed that the corresponding author has the authority to act on their behalf on all matters pertaining to publication of the manuscript. To avoid any possible dispute during processing, authorship changes including the order of authors' names during revision must be agreed upon by all of the authors and brought to the editor's attention in the cover letter submitted with the revised version.

Ethical Aspects

Manuscripts dealing with any experimental work on human or animal materials should meet the relevant regulations or requirements imposed by institutional or governmental authorities, and this should be clearly stated in the manuscript. The editor reserves the right to reject papers if ethical aspects are in doubt. Nucleotide and Amino Acid Sequences any novel nucleotide or amino acid sequences described should be deposited in a public database, such as GenBank, EMBL or DDBJ, and the accession numbers should be included in a separate paragraph in the Materials and Methods section. It is expected that the sequence data will be publicly available no later than the publication of the article.

Review Process

All manuscript is reviewed confidentially by members of the editorial board and qualified reviewers. The reviewers operate under the guidelines for reviewers and are expected to complete their reviews as soon as possible.

Notification of Acceptance

When an editor has decided that a manuscript is acceptable for publication, the corresponding author will be notified. The editorial office will check if the manuscript was prepared according to the guidelines, however the authors are primarily responsible for the format and quality of the paper.

Galley Proofs

The editorial office sends a galley proof and Agreement for publication form to the corresponding author. Galley proof should be corrected, signed by the corresponding author and send back to the editorial office within 5 days, however extensive corrections, additions, or deletions should not be made during the proof stage. Important new information or references of unpublished data or personal communications that have become available in the time between acceptance of the manuscript and receipt of the proofs may be inserted. Otherwise, changes are limited to correction of spelling errors, incorrect data, grammatical errors and updated information regarding references.

Organization and Format

General Organization The most desirable plan for the organization of a paper is as follows: (a) Abstract, in less than 250 words, (b) Introduction, in less than two typed pages, (c) Materials and Methods, (d) Results, (e) Discussion, (f) Acknowledgments, (g) References. In some cases the presentation might be more effective if you combined some sections, e.g. Results and Discussion. This is particularly true in short papers. The Journal imposes no lower limit on the size of regular papers.

The **title** should be informative and as short as is consistent with clarity.

List full **names** of all authors. A footnote to an author, indicating a change of address, should be given on the title page using one of the following superscript: 1, 2, 3. The asterisk symbol * should be reserved for the author to whom correspondence should be addressed.

List the **institutions** in which the work was carried out. Identify the affiliations of all authors and their institutions, departments, or organizations by use of superscript lower case alphabets.

The **Abstract** should not exceed 250 words, and should concisely summarize the basic content of the paper. Experimental details should not be presented in the abstract. Avoid specialized terms, abbreviations, diagrams, and references.

Introduction states the purpose of the investigation and its relation to other works in the same field, but should not present an extensive review of the literature.

The descriptions in **Materials and Methods** should be brief, but sufficiently detailed to permit repetition of the work by a qualified operator. Refer to published procedures by citing both the original description and pertinent published modifications. Do not include extensive details unless they constitute a significant new modification.

Results should describe the results of the experiments. Present the results as concisely as possible in one of the following: text, table(s), or figure(s). Avoid presenting essentially similar data in both table and figure form. Also avoid extensive use of graphs to present data that might be more concisely presented in the text or tables. Limit photographs to those that are absolutely necessary for presenting the experimental findings. Number figures and tables according to the order of citation in the text.

Discussion should be concise and provide an interpretation of the results in relation to previously published work and to the experimental system at hand. It should not contain extensive repetition of the Results section or reiteration of the Introduction.

Acknowledge personal assistance and financial assistance in the same paragraphs.

Citations of revelant published work in the text, from **Introduction** to **Discussion** should read John (2010) or (John, 2010). When a paper cited has three or more authors, use the style Kumar *et al*. (2012) of (Kumar *et al*., 2012).

References section must be in alphabetical order by the first author. Use the following style:

Article in a periodical:
Lee S, Jeon JS, Jung KH, An G. 1999. Binary vectors for efficient transformation of rice. J. Plant Biol. 42: 310-316.

Article in a book:
Daniell, H., Camrmona-Sanchez, O., and Burns, B. 2004. Chloroplast derived antibodies, biopharmaceuticals and edible vaccines. In *Molecular Farming*, Eds. R. Rischer and S. Schillberg, Weinheim, Germany: Wiley-VCH Verlag, pp. 113-133.

An entire book:
Wickner, R.B., Esteban, R., and Suzuki, N. 2000. *Virus Taxonomy,* California: Academic Press.

References should include only articles that are published or in press. **Do not** list Unpublished data, submitted manuscripts, abstracts, and personal communications.

Responsibility for the accuracy of bibliographic references rests entirely with the author(s).

Tables should be numbered consecutively with Arabic numbers in order of appearance in the text. Type each table single-spaced on a separate page with short descriptive title typed directly above and with essential footnotes below. Footnotes to tables should be identified with the italic superscript lower case and placed at the bottom of the table.

Figures should be approximately the same size as you would like to appear in press. Prepare and save your figures as .gif, .jpeg more than 300 dpi resolution. Number figures consecutively with Arabic numbers. Figure legends should provide enough information for the figure to be understandable without frequent reference to the text. Define all symbols and abbreviations used in the figure that have not been defined elsewhere.

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