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Molecular phylogenetics of the Brazilian giant bromeliads (*Alcantarea*, Bromeliaceae): implications for morphological evolution and biogeography

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The genus *Alcantarea* comprises near 30 species endemic to rocky outcrops from eastern Brazil. Most species are ornamental and several are threatened due to habitat loss and over collection. In this paper we examine the phylogenetics of *Alcantarea* and its relationship with the Brazilian members of *Vriesea,* a genus of which *Alcantarea* has been treated as a subgenus. We discuss the morphological evolution of the stamen position and its implication for pollination and the occurrence of *Alcantarea* in the Espinhaço mountain range rocky savanna-like habitat vegetation. DNA sequence data derived from two plastid markers (*trn*K-*rps*16, *trn*C-*pet*N) and from a low copy nuclear gene (*Floricaula/Leafy*) together with 20 nuclear microsatellite loci were the data source to perform analyses and construct phylogenetic and Neighbor Joining trees for the genus. *Alcantarea* is well supported as monophyletic in both Bayesian and parsimony analyses, but sections of *Vriesea*, represented by the eastern Brazilian species, appear paraphyletic. Microsatellites delimit geographically isolated species groups. Nevertheless individuals belonging to a single species may appear related to distinct clusters of species, suggesting that hybridization and/or homoplasy and/or incomplete lineage sorting are also influencing the analysis based on such markers and may be the reasons for some unexpected results. *Alcantarea brasiliana* is hypothesized as putative hybrid between *A. imperialis* and *A. geniculata*. Spreading stamens, a morphological floral characteristic assumed to be related to Chiropterophily, apparently evolved multiple times within the genus, and invasion of rocky savanna-like habitat vegetation by Atlantic rainforest ancestors seems to have occurred multiple times as well.

1. Introduction

Bromeliaceae (58 genera, 3248 species) are almost exclusively Neotropical (Luther, 2010; Smith and Downs, 1974) and currently divided into eight subfamilies (Brochinioideae, Lindmanioideae, Tillandsioideae, Hechtioideae, Navioideae, Pitcairnioideae, Puyoideae and Bromelioideae) that are mainly characterized by the morphology of the flowers, fruit, seeds, and molecular data (Givnish et al., 2007, 2011). Subfamily Tillandsioideae have seed appendages that are finely divided forming a coma, entire leaves, dehiscent capsules, foliar trichomes that are generally radially symmetric and a predominantly epiphytic life style (Smith and Downs, 1974). This subfamily has been shown to be monophyletic in several recent molecular phylogenetic studies (e.g. Barfuss et al., 2005; Givnish et al., 2007, 2011; Horres et al., 2000; Schulte et al., 2005; Terry et al., 1997a,b). Tillandsioideae are divided into four lineages that are treated as tribes: *Catopsideae*, *Glomeropitcairnieae*, *Vrieseeae*, and *Tillandsieae* (Barfuss et al., 2005).

Tribe *Vrieseeae* W. Till & Barfuss comprises *Alcantarea* (~30 species endemic to eastern Brazil), *Vriesea* (~260 species distributed in two sections: sect. *Vriesea* and sect. *Xiphion*, with centers of diversity in eastern Brazil) and *Werauhia* (~85 species centered in Mesoamerica) (Barfuss et al., 2005; Luther, 2010; Smith and Till, 1998; Versieux, 2009). *Vrieseeae* includes four lineages arranged in two main clades showing sister relationships: (i) *Alcantarea* and the eastern Brazilian *Vriesea* species, and (ii) the remaining species of *Vriesea* (from Andean, Caribbean and Central American regions) and *Werauhia* (Barfuss et al., 2005). *Vriesea* and *Werauhia* are mostly epiphytes, whereas *Alcantarea* species are strictly rupicolous and occur on gneiss-granitic inselbergs (*insel* = island, *berg* = mountain) from eastern Brazil or, more rarely, on quartzite rocky outcrops in grasslands with rocky soils vegetation of the Espinhaço mountain range (e.g. *Alcantarea duarteana*, *A. hastchbachii*,

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A. turgida). The base chromosome number of *Alcantarea* is $n = 25$, and population genetic studies (below) indicate diploid behavior during meiosis.

Key morphological characters that distinguish *Alcantarea* from *Vriesea,* its closest relative, are the ephemeral, long lingulate and usually spiralescent petals, soon flaccid and drooping (versus longer-lived, short, elliptic, oblong or obovate petals, usually firm and remaining more or less erect or recurved but not spiraling after anthesis), seeds with both basal and apical appendages (versus apical appendages atrophied), and semi-inferior ovary (versus superior ovary) (Coffani-Nunes et al., 2010; Grant, 1995; Harms, 1930; Versieux and Wanderley, 2007a). *Alcantarea* has traditionally been treated as a subgenus of *Vriesea* (Mez, 1894; Smith and Downs, 1977) or as an independent genus (Grant, 1995; Harms, 1929, 1930). According to Harms (1930) the seeds with apical and basal appendages, long petals, large habit and bulky inflorescences were sufficiently distinct between these genera to elevate *Alcantarea* to the generic rank. Subsequent authors preferred to treat *Alcantarea* as a subgenus of *Vriesea* (Mez, 1934; Smith, 1934; Smith and Downs, 1977). Smith (1934, p. 465) explains his reluctance in accept the genus *Alcantarea* as delimited by Harms (1930): "Harms has made a separate genus of *Alcantarea*, but in view of the fact that his distinction of apical coma does not hold for all the species, it seems better to follow Mez and retain the group under *Vriesia*. The other distinction, the form of the petals, is not considered to be of generic value anywhere else in the family, even by Harms". Grant (1995) re-established the genus *Alcantarea*, after excluding two taxa from Central America and the West Indies that present distinct floral and seed morphology. Characters that were used by Grant (1995) to resurrect *Alcantarea* to the generic status are the linear-long (ca. 10–15 times longer than wide), ephemeral, distinctly flaccidescent, and spiralescent petals, and seeds with both basal and apical comas. Also, in Grant's (1995) circumscription, *Alcantarea* has a narrower geographical range, being restricted to the Northeastern (Bahia) and Southeastern (Espírito Santo, Minas Gerais, and Rio de Janeiro) states of Brazil. Recently, the genus was rediscovered in the state of São Paulo (Versieux and Wanderley, 2007a,b and references therein).

Alcantarea treated either as a genus or as subgenus has been investigated in some phylogenetic analyses, but only represented by few species. Gilmartin (1983) recovered a sister relationship between subgenera *Alcantarea* and *Vriesea* employing morphological data in different cladistics analyses. Horres et al. (2000) included *Alcantarea regina* in a broad Bromeliaceae phylogenetic analysis based on *trn*L (UAA) intron sequences and reported a well supported sister relationship between *A. regina* and *Vriesea racinae* L.B. Sm., both of them showing a 15 base pairs deletion. Costa (2002) included *Alcantarea farneyi* and *A. glaziouana* and other 66 species of *Vriesea* in a morphological phylogenetic analysis. Both *Alcantarea* species analyzed by this author appear in a clade supported by the presence of strongly recurved petals and seed bearing straight and long apical appendages. Barfuss et al. (2005) presented the most updated and complete molecular phylogeny for the Tillandsioideae that employed seven plastid markers and sampled two *Alcantarea* species – *A. duarteana* and *A. imperialis* – which formed a clade sister to the eastern Brazilian species of *Vriesea,* but not to the remainder *Vriesea* species that shared a more recent common ancestor with other species of *Werauhia.* Therefore, according to Barfuss et al. (2005) the separation of *Alcantarea* and *Werauhia* as independent genera would turn *Vriesea s.l.* paraphyletic. Horres et al. (2007) used *Alcantarea regina* in a molecular phylogeny and it appeared as sister to a clade that included *Guzmania* and *Tillandsia*, but in this analysis *Vriesea* species were not studied.

Molecular phylogenetic studies of Bromeliaceae go back to the 1990s with the first papers dealing with the relationships among subfamilies (e.g. Ranker et al., 1990). Subsequently, relationships within subfamilies started to be addressed through an increased sampling of genera and species within individual subfamilies (e.g. Barfuss et al., 2005; Givnish et al., 2004, 2007, 2011; Schulte et al., 2005; Terry et al., 1997a,b). More recent studies focused on intrageneric phylogenetic analyses through the use of combined morphological and molecular datasets (e.g. Sousa et al., 2007), AFLPs (Horres et al., 2007; Rex et al., 2007; Schulte et al., 2010) or nuclear genes (e.g. Chew et al., 2010; Jabaily and Sytsma, 2010; Sass and Specht, 2010; Schulte et al., 2009) in order to increase the amount of phylogenetically informative characters. These different markers have been used because the molecular studies conducted with Bromeliaceae have encountered surprisingly low variability of plastid DNA sequences (e.g. Horres et al., 2000; Schulte et al., 2005; Terry et al., 1997a,b).

Alcantarea includes ornamental bromeliads characterized by their large size (sometimes reaching up to 5 m high when flowering, Fig. 1) and by well-developed tanks (phytotelma) and they are popular garden plants. A few species show a much-reduced habit and almost lack the ability to impound any water (Benzing et al., 2000). Adaptation to life on inselbergs is a key feature of *Alcantarea* species (Martinelli, 1994). These adaptations include several anatomical characteristics in the leaves, such as the presence of a well-developed epicuticular stratum, water storage parenchyma, abundance of mesophyll fibers, as well as heterophylly (Versieux et al., 2010a). In many naturally fragmented rocky habitats of eastern Brazil this genus dominates (Porembski et al., 1998; Versieux and Wanderley, 2009, 2010b), sometimes being the only plants growing on vertical wall surfaces (Meirelles et al., 1999), and its diversification along rocky and harsh habitats represents a case of adaptive radiation. Due to their ability to hold a considerable volume of water, up to 45 l per rosette, as well as for establishing nucleation zones facilitating the establishment of other plants over the bare rock, *Alcantarea* species are key elements to the associated flora and fauna, particularly in an environment such as the inselbergs, where rock surface temperature can reach 61.5 °C during the summer (Benzing, 2000; Carauta and Oliveira, 1984). More recently, the cultivation of such large water-holding plants in gardens as well as their natural occurrence close to urban perimeters has raised a polemic debate in Brazil on the epidemiological importance of these plants to the proliferation of dengue mosquitoes inside their rosettes (e.g. Mocellin et al., 2009).

Elucidating relationships within such an ecologically important group of plants will help to understand interesting biogeographical and reproductive features that may have played some important role in the radiation of this genus. While many *Alcantarea* species present bat pollination syndrome characteristics (e.g. spreading stamens, nocturnal anthesis, abundant nectar production, cryptic coloration of the petals, Martinelli, 1994, 1997), other species present crepuscular or diurnal anthesis, smaller and bright yellow[2] perfumed flowers with bundled stamens (Fig. 1) and less developed septal nectaries that are suggestive of insect (sphingophily) or hummingbird pollination (Versieux, 2009; Vogel, 1969). Thus, the existence of a phylogenetic framework would be interesting to understand the evolution of these distinct floral morphologies and relate it to shifts in pollinators. Additionally, the species that grow on the quartzite stony grassland (*campo rupestre*) in the Espinhaço range of Bahia and Minas Gerais states (Fig. 1) have been hypothesized as derived from Atlantic forest mesic ancestors (Benzing et al., 2000, p. 498) or, alternatively, have been proposed as the early divergent lineages that retained a relict inland distribution associated with other plesiomorphic character states, such

[2] For interpretation of color in Figs. 1–3, the reader is referred to the web version of this article.

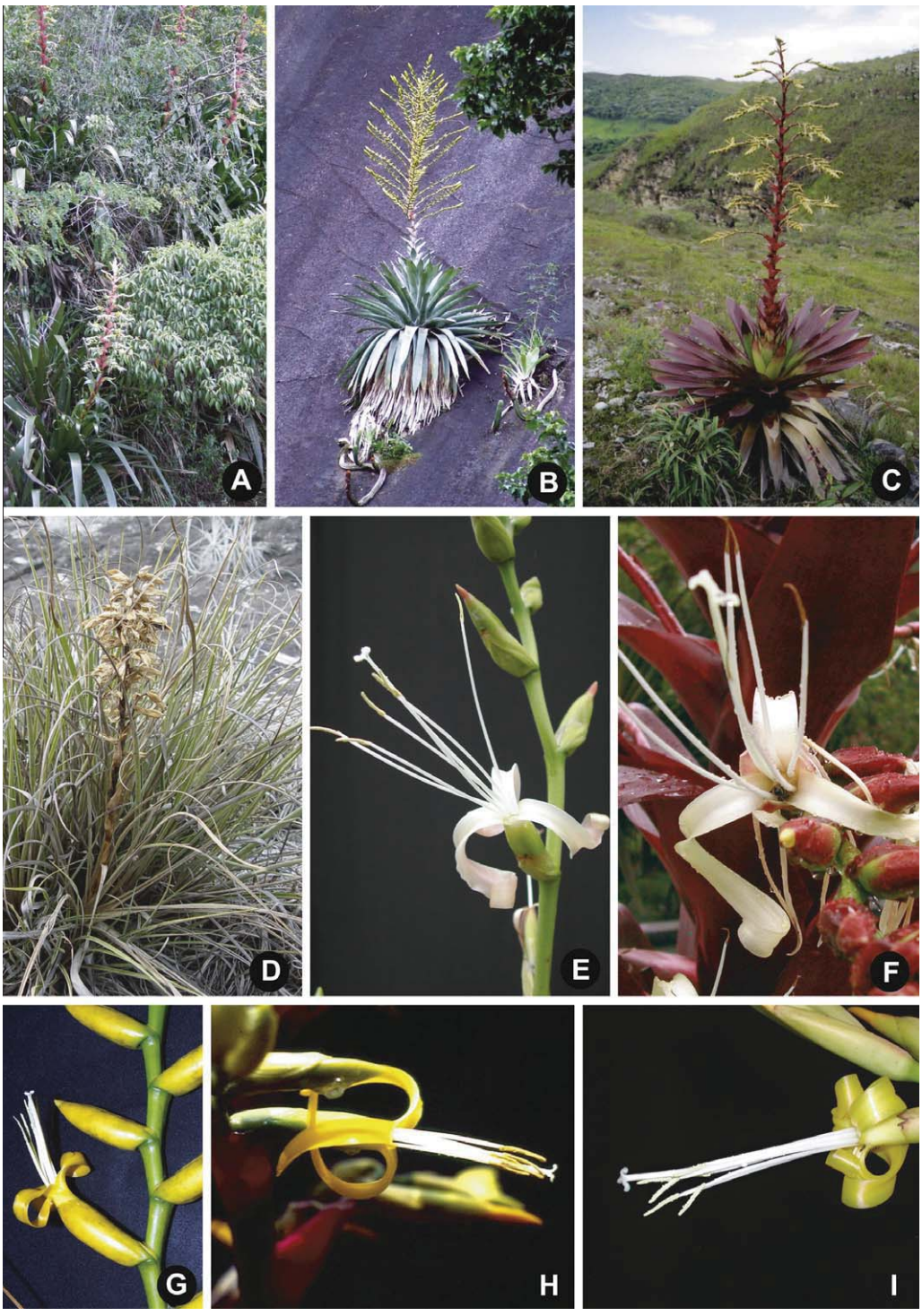

Fig. 1. *Alcantarea* habitat and floral morphology, showing position of stamens. (a and b) Gneiss-granitic inselbergs inside the Atlantic rainforest domain. (a) *Alcantarea geniculata* (b) *A. patriae* (c and d) Quartzite rocky outcrops in rocky savanna-like vegetation along the Espinhaço range. (c) *A. turgida*. (d) *A. duarteana*. (e–i) Morphology of the stamens. (e and f) Spreading. (g and i) Presented in a bundle. (e) *A. burle-marxii*. (f) *A. imperialis*. (g) *A. patriae*. (h) *A. heloisae*. (i) *A. tortuosa*.

as the rudimentary leaf rosettes (Leme, 1995, p. 23). Investigating these hypotheses related to the disjunctive occurrence between the Atlantic forest inselbergs versus the quartizitic outcrops of the savanna like habitats, as well as the morphological changes in *Alcantarea* flowers (Fig. 1), would be extremely useful as a baseline to address future questions and add evidences to discuss broader issues involving the evolution of eastern Brazil plants. Very little information is available in the literature regarding the appearance of the Espinhaço savanna-like habitats flora and their endemic plant species (e.g. Antonelli et al., 2010; Simon et al., 2009; Versieux and Wendt, 2007). By mapping such characters (i.e. occurrence in quatizitic outcrops of the grassland/or gneiss-granitic inselbergs of the Atlantic forest and the stamen position) over the phylogenetic trees, new insights may become available to help understand evolution within the genus.

Given the difficulty of finding molecular markers that present appropriate levels of variation for reconstructing phylogenetic relationships in Bromeliaceae, we used multiple molecular datasets

including plastid DNA markers, a low copy nuclear gene and microsatellite data to reconstruct intergeneric and infrageneric relationships in *Alcantarea* and allies. Both plastid regions used (*trn*K-*rps*16, *trn*C-*pet*N) have shown appropriate levels of variation for reconstructing relationships at this level in other plant groups (Kress et al., 2005; Lee and Wen, 2004; Ran et al., 2006; Whipple et al., 2007). *Floricaula/Leafy,* the nuclear marker employed in our sequencing analyses, is present as a single copy in most angiosperms, except in recent polyploids, and has also been effectively used in intrageneric phylogenetic investigations (Grob et al., 2004; Baum et al., 2005).

Nuclear microsatellites (Goldstein and Schlötterer, 1999) have not been used to reconstruct Bromeliaceae phylogenies thus far. Nuclear microsatellites represent important tools for measuring genetic distance between populations because they are extremely polymorphic (Chen et al., 2002), evolve neutrally and represent different genomic regions in a single dataset (Ochieng et al., 2007). The use of microsatellite markers to reconstruct phylogenies is a matter of debate due to rapid evolution and potential for homoplasy (Barkley et al., 2009; Jarne and Lagoda, 1996; Richard and Thorpe, 2001). Nevertheless, several recent publications have used microsatellites in order to understand relationships within plant genera, particularly when other markers have limited success in resolving species relationships (e.g. Barkley et al., 2009; Bowles et al., 2010; Yao et al., 2008). Specifically, discussions have focused on the best method for the analysis of this kind of data and on how to resolve uncertainties regarding mutation mechanisms and levels of homoplasy (Barkley et al., 2009; Chen et al., 2002; Ochieng et al., 2007; Petren et al., 1999). Three biological processes other than true genetic proximity can influence allele sharing patterns at microsatellite loci: (1) shared ancestral polymorphism = incomplete lineage sorting, (2) homoplasy and (3) hybridization. All of these processes must be carefully taken into consideration when interpreting the relationships suggested by these markers, particularly because all them could be interfering in the results obtained. Several population genetics studies based on microsatellite markers have been carried out in Bromeliaceae (reviewed in Barbará et al., 2007, 2008, 2009), and thus many primers sets are available (Boneh et al., 2003; Paggi et al., 2008; Palma-Silva et al., 2007; Sarthou et al., 2003).

Here we study the phylogenetics of *Alcantarea* in order to test its monophyly. We further use the phylogenetic trees obtained as a basis for evaluating the utility of morphological features traditionally used to diagnose taxa in the group and discuss the taxonomical, biogeographical and evolutionary implications of our results. In particular, we discuss the single versus repeated events for the colonization of rocky savanna-like habitats and the evolution of pollination syndromes, and we ask whether interspecific hybridization (discovered in previous population genetic studies) has played a role during the evolution of *Alcantarea*.

2. Materials and methods

2.1. Taxon sampling

Two different strategies for sampling were employed, one for the sequencing component of this study and the other for the microsatellite analyses. Sequencing for the plastid and nuclear markers included a broader sample of *Vriesea* (10 species belonging to the eastern Brazilian clade, as delimited by Barfuss et al., 2005) and *Alcantarea* (16 species plus three accessions belonging to the *Alcantarea extensa* complex, as defined by Versieux and Wanderley, 2010a: two of *A.* aff. *extensa* and one of *A.* aff. *burle-marxii*) and one species of *Werauhia* and *Tillandsia* (Table 1). We used *Tillandsia secunda* and *Werauhia ororiensis* as outgroups. The choice of the latter two species was based on a recent molecular phylogenetic analysis (Barfuss et al., 2005) of Tillandsioideae in which *Werauhia* appears as sister to the *Alcantarea* and the eastern Brazilian *Vriesea* clade, and *Tillandsia secunda* represents tribe *Tillandsieae*, sister to the tribe *Vrieseeae* in which *Alcantarea* is placed. The microsatellite study focused on the relationship among species of *Alcantarea*. A total of 47 individuals belonging to different populations of 19 species (73% of the total) were sampled (Table 1). Several attempts were made to extract DNA from herbarium specimens of *A. hatschbachii* but all failed.

2.2. DNA extraction

Genomic DNA was extracted from silica-dried leaves using the 2 × CTAB protocol (Doyle and Doyle, 1990) adapted for a scale-down protocol, with a final volume of 750 µl. DNA quality and quantity was assessed by measuring its absorbance at 260 and 280 nm with a Helena Biosciences Biophotometer (Eppendorf).

2.3. Amplification and microsatellite analyses

Twenty nuclear microsatellite loci were used in the present study. These loci were amplified using primers and PCR conditions described in their original publications: Ai4.03, Ai4.10, Ai5.18, VgA04, VgB10, VgC01, VgF01, VgF02, VgG02, VgG03, VgG05 (Palma-Silva et al., 2007), CT5, E6, E6b, E19, P2P19 (Boneh et al., 2003), PaA010, PaD07, PaZ01 (Paggi et al., 2008), Pit8 (Sarthou et al., 2003). Genotypes were analyzed in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems), using FAM or JOE fluorescent dyes. Molecular sizes in base pairs were determined using the GENESCAN-500 ROX size standard (Applied Biosystems). Alleles sizes were scored using Genotyper 3.7 software (Applied Biosystems). A matrix containing all alleles was analyzed in MSA software (Dieringer and Schlötterer, 2003) using the Pairwise Distance Between Individuals setting and the $(\delta\mu)^2$ genetic distance (Goldstein et al., 1995). The rationale for choosing such distance is that it was created specifically for microsatellite applications and depends on time of divergence and not on population size, thus being suitable to compare different species (Goldstein et al., 1995). A neighbor-joining (NJ) tree based on the matrix generated in MSA was built in PAUP 4.0 (Swofford, 2002). For the bootstrap (BS) analysis, 1000 matrix replicates were generated in MSA and used to build neighbor-joining trees and the major rule bootstrap consensus tree in PAUP.

2.4. Amplification for sequencing

A preliminary screening for molecular markers was conducted based on recently published primers, which have been successfully used for the order Poales overall. Primer selection was based on the ease of amplifying the target region after few trials, on the quality of the sequence obtained, and on the degree of variation observed among three morphologically distinct species. Also, regions that were not previously employed in Bromeliaceae phylogenetic investigations were preferred. The following primer pairs were tested for plastid loci: *ndh*F-*rpl*32, *trn*Q-*rps*16, *rpl*32-*trn*L, *trn*V-*ndh*C (Shaw et al., 2007); *trn*C-*pet*N1r, *trn*K5'r-*rps*16-4547mod (Whipple et al., 2007); *rpo*C1, *rpo*B, *acc*D, *mat*K x/5 (Ford et al., 2009); and nuclear loci: LFsxl-3-LFtxr (Frohlich and Meyerowitz, 1997); GScp687f-GScp994 (Emshwiller and Doyle, 1999); *PHYC* (Mathews and Donoghue, 1999); ms526Af-ms943r, prk488f-prk1167r (Lewis and Doyle, 2002); RPB2-INT23BF-RPB2-INT23-R (Roncal et al., 2005).

This preliminary screening allowed us to select two plastid markers, the *trn*K-*rps*16 and *trn*C-*pet*N. Amplifications were conducted using primers and PCR conditions described in Whipple

Table 1
List of taxa included in the microsatellite and/or phylogenetic sequencing analyses of *Alcantarea*, including origin, voucher (herbarium) and GenBank accession information. States of Brazil are abbreviated as follow: BA = Bahia, ES = Espírito Santo, MG = Minas Gerais, RJ = Rio de Janeiro, SP = São Paulo. IBt = Instituto de Botânica living collection, São Paulo, Brazil. Use of the accession in the microsatellite study: - = not used, used only for DNA sequencing analysis; γ = used only for microsatellite analysis; π used for both, sequencing and microsatellite analyses.

Taxa	Locality	Voucher (herbarium)	Use in the microsatellite analysis	GenBank accession		
				*trn*C-*pet*N	*trn*K-*rps*16	*FLO/LFY*
Alcantarea aff. burle-marxii (Leme) J.R. Grant	Brazil, MG, Pedra Grande	*Versieux 421* (*SP*)	π	JQ685250	JQ685314	JQ685343
Alcantarea aff. extensa (L.B. Sm.) J.R. Grant	Brazil, MG, Carangola	*Pinheiro 27 Indiv. 1*	γ			
		Pinheiro 27 Indiv. 2 (*SP*)	γ			
‘‘	Brazil, MG, Alto Caparaó, cultivated	*Versieux 358* (*SP*)	γ			
		Versieux 360 (*SP*)	π	JQ685251	JQ685315	JQ685344
‘‘	Brazil, MG, Padre Paraíso	*Versieux 431 Indiv. 1*	π	–	JQ685316	JQ685345
		Versieux 431 Indiv. 2 (*SP*)	γ			
Alcantarea brasiliana (L.B. Sm.) J.R. Grant	Brazil, RJ, Petrópolis, Serra da Estrela	*Versieux 451* (*SP*)	γ			
		Versieux 452 (*SP*)	π	JQ685252	JQ685317	JQ685346
		Versieux 453 (*SP*)	γ			
Alcantarea burle-marxii (Leme) J.R. Grant	Brazil, MG, Pedra Azul	*Versieux 420* (*SP*)	π	JQ685253	JQ685318	JQ685347
Alcantarea duarteana (L.B. Sm.) J.R. Grant	Brazil, MG, Diamantina	*IBt 1962*	γ			
		Versieux 255 (*SP*)	π	JQ685254	JQ685319	JQ685348
Alcantarea extensa (L.B. Sm.) J.R. Grant	Brazil, ES, Cachoeiro do Itapemirim	*Versieux 380* (*SP*)	γ			
‘‘	Brazil, ES, Castelo, Forno Grande	*Versieux 372* (*SP*)	γ			
Alcantarea farneyi (Martinelli & A. F. Costa) J.R. Grant	Brazil, RJ, Santa Maria Madalena	*Versieux 245* (*SP*)	γ			
		Versieux 247 (*SP*)	γ			
Alcantarea geniculata (Wawra) J.R. Grant	Brazil, RJ, Teresópolis	*Versieux 285* (*SP*)	γ			
		Versieux 456 (*SP*)	π	JQ685255	JQ685320	JQ685349
Alcantarea glaziouana (Lem.) Leme	Brazil, RJ, Rio de Janeiro, Prainha	*Versieux 183* (*SP*)	γ			
‘‘	Brazil, RJ, Rio de Janeiro, Urca	*Versieux 342* (*SP*)	γ			
		Versieux 343 (*SP*)	π	JQ685256	JQ685321	JQ685350
Alcantarea heloisae J.R. Grant	Brazil, RJ, Santa Maria Madalena	*Versieux 238* (*SP*)	γ			
		Versieux 239 (*SP*)	γ			
Alcantarea imperialis (Carrière) Harms	Brazil, RJ, Nova Friburgo, Lumiar	*Versieux 215* (*SP*)	γ			
‘‘	Brazil, RJ, Nova Friburgo, Macaé de Cima	*Versieux 221* (*SP*)	γ			
		Versieux 222 (*SP*)	γ			
‘‘	Brazil, RJ, Nova Friburgo, Macaé de Cima, green color morph	*Barbará s.n.* (*K*)	π	–	JQ685322	JQ685351
Alcantarea martinellii Versieux & Wand.	Brazil, RJ, Petrópolis, Pedra do Oratório	*Moraes 164* (*RB*)	–	–	–	JQ685352
Alcantarea nahoumii (Leme) J.R. Grant	Royal Botanic Gardens, Kew	*Barbará 25c* (*K*)	γ			
‘‘	Brazil, BA, Santa Teresinha	*Versieux 415* (*SP*)	γ			
		Versieux 417 (*SP*)	π	JQ685257	JQ685323	JQ685353
Alcantarea nevaresii Leme	Royal Botanic Gardens, Kew	*Barbará 7* (*K*)	π	JQ685258	JQ685324	JQ685354
‘‘	Brazil, RJ, Nova Friburgo, Macaé de Cima	*Versieux 223* (*SP*)	γ			
Alcantarea nigripetala Leme & L. Kollmann	Brazil, ES, Pancas	*Louzada 22* (*SP*)	γ			
Alcantarea odorata (Leme) J.R. Grant	Brazil, MG, Além Paraíba	*Versieux 353* (*SP*)	γ			
‘‘	Brazil, MG, Muriaé	*Versieux 432* (*SP*)	π	JQ685259	JQ685325	JQ685355
‘‘	Brazil, MG, Além Paraíba	*Versieux 433* (*SP*)	γ			
Alcantarea patriae Versieux & Wand.	Brazil, ES, Jerônimo Monteiro	*Versieux 365* (*SP*)	π	JQ685260	JQ685326	JQ685356
Alcantarea regina (Vell) Harms	Brazil, RJ, Mangaratiba	*Versieux 265* (*SP*)	γ			
		Versieux 270 (*SP*)	π	JQ685261	JQ685327	JQ685357
Alcantarea roberto-kautskyi Leme	Brazil, ES, Baixo Guandú	*Versieux 398* (*SP*)	γ			
‘‘	Brazil, MG, Pedra Azul	*Versieux 419* (*SP*)	γ			
‘‘	Brazil, MG, Santa Maria do Salto	*Versieux 430* (*SP*)	π	JQ685262	JQ685328	JQ685358
Alcantarea tortuosa Versieux & Wand.	Brazil, RJ, Santa Maria Madalena	*Versieux 240* (*SP*)	γ			
Alcantarea trepida Versieux & Wand.	Brazil, ES, Baixo Guandú	*Versieux 395* (*SP*)	π	JQ685263	JQ685329	JQ685359
Alcantarea turgida Versieux & Wand.	Brazil, MG, Conceição do Mato Dentro	*Versieux 260* (*SP*)	π	JQ685264	JQ685330	JQ685360
‘‘	Brazil, MG, Entre Itabira e João Monlevade	*Versieux 41* (IBt)	–	JQ685265	JQ685331	JQ685361
Alcantarea vinicolor (E.Pereira & Reitz) J.R. Grant	Brazil, ES, Domingos Martins	*Versieux 376* (*SP*)	π	JQ685266	JQ685332	JQ685362
Tillandsia secunda Kunth	Ecuador, Loja, Oña	*Versieux 459* (*SP*)	–	JQ685267	JQ685333	JQ685363
Vriesea atropurpurea Silveira	Brazil, MG, Santana do Riacho	*Versieux 296* (*SP*)	–	JQ685268	JQ685334	JQ685364
Vriesea bahiana Leme	Brazil, BA, Santa Teresinha	*Versieux 413* (*SP*)	–	JQ685269	–	JQ685365
Vriesea crassa Mez	Brazil, MG, Alto Caparaó	*Versieux 355* (*SP*)	–	JQ685270	JQ685335	JQ685366

(*continued on next page*)

Table 1 (*continued*)

Taxa	Locality	Voucher (herbarium)	Use in the microsatellite analysis	GenBank accession		
				*trn*C-*pet*N	*trn*K-*rps*16	*FLO/LFY*
Vriesea densiflora Mez	Brazil, MG, Santo Antônio do Itambé	*Versieux 332* (*SP*)	–	JQ685271	JQ685336	JQ685367
Vriesea itatiaiae Wawra	Brazil, SP, Cruzeiro	*IBt 2055*	–	JQ685272	JQ685337	JQ685368
Vriesea pauperrima E. Pereira	Brazil	*Kew living collection* (*2003-1506*)	–	–	–	JQ685369
Vriesea procera var. tenuis L.B. Sm.	Brazil, MG, Santo Antônio do Itambé	*Versieux 257* (*SP*)	–	JQ685273	JQ685338	JQ685370
Vriesea pseudoatra Leme	Brazil, RJ, Nova Friburgo	*Versieux 234* (*SP*)	–	JQ685274	JQ685339	JQ685371
Vriesea scalaris E. Morren	Brazil, ES, Santa Teresa	*Versieux 388* (*SP*)	–	JQ685275	JQ685340	JQ685372
Vriesea segadas-viannae L.B. Sm.	Brazil, MG, Santana do Riacho	*Versieux 440* (*SP*)	–	JQ685276	JQ685341	JQ685373
Werauhia ororiensis (Mez) J.R. Grant	Costa Rica, Saavegre	*Herbarium SP 399345*	–	JQ685277	JQ685342	JQ685374

et al. (2007). Additionally, we sequenced the second intron of *Floricaula/Leafy* (*FLO/LFY*) using primers and PCR conditions presented by Frohlich and Meyerowitz (1997). New primers were designed when necessary (Supplementary data). Reactions containing a final volume of 20 μl were performed using: 2 μl of 5× Go taq Promega Buffer, 2 μl of BSA, 1 μl of 25 μM MgCl, 1 μl of each 10 μM primer, 2u of Promega Go Taq, 0.4 μl of 10 mM dNTPs and 5 ng of genomic DNA, with the final volume completed with ddH_2O. For the amplification of *FLO/LFY,* 0.8 μl of DMSO was added to each reaction. PCR products were run on 1% agarose gels to check for amplification and quality. Amplified fragments were purified using NucleoSpin Extract II purification kits (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol, and sequenced using the dideoxy chain-termination method.

2.5. Sequence analyses

Sequencing was carried out at the Jodrell Laboratory (Kew) using an ABI 3730 DNA Analyzer or at the Macrogen Inc. (South Korea). Sequences were edited in Sequencher v. 4.8 (Gene Codes Corporation) and aligned manually in MacClade v. 4.06 (Maddison and Maddison, 2003). Six indels (one in *trn*C-*pet*N, and five in the *FLO/LFY* data sets) were coded separately using the simple indel coding method (Simmons and Ochoterena, 2000). Few short regions (one in the *trn*K-*rps*16 and nine in the *LFY* intron, including a difficult to align microsatellite in the beginning of the sequences) with ambiguous alignment and uncertain homology were excluded from the analysis. Maximum parsimony (MP) and MP bootstrap (BS) analyses were performed in PAUP 4.0 (Swofford, 2002) with 1000 replicates of heuristic searches each. Heuristic searches were set to use a stepwise-addition starting tree and TBR branch swapping, saving a maximum of 20 trees per replicate. The Partition Homogeneity Test (PHT) implemented in PAUP was performed for the search of incongruence between datasets. Evolutionary models for the Bayesian analyses (BA) were estimated for each dataset in MrModeltest v. 2 (Posada and Crandall, 1998). The following models of evolution were selected using the Akaike Information Criterion: HKY for *trn*C-*pet*N, GTR for *trn*K-*rps*16 and HKY for *FLO/LFY*. The standard discrete model was employed for a separate partition containing the six coded indels information (Lewis, 2001; Ronquist and Huelsenbeck 2003). Bayesian analyses were conducted using 10 million generations sampled every 100 generation and using two simultaneous runs, each with four simultaneous chains in MrBayes v. 3.1.1 (Ronquist and Huelsenbeck, 2003). The standard deviation of split frequencies (i.e. the convergence diagnostic) was analyzed to access convergence in both runs. Burn-in was determined after ploting –*ln*L versus generations and the first 473 samples were discarded in the analysis containing plastid and nuclear data combined. A 50% majority rule consensus tree was generated with the remaining trees. Maximum Parsimony and Bayesian analyses were carried out on Bioportal (www.bioportal.uio.no).

2.6. Morphological and biogeographical characterization

Selected characters related to geographical distribution, habitat preference and inflorescence morphology were studied to further explore and discuss the relationship found among clades and clusters of *Alcantarea* species. Characters and character states considered for each species were: (i) habitat occurrence (inselberg within the Atlantic rainforest versus rocky outcrops within the Espinhaço range rocky savanna-like habitat), (ii) position of the stamens during the anthesis (spreading versus presented in a bundle), and (iii) branching pattern of the inflorescence (unbranched versus branched). Colored symbols mapped along the side of the Bayesian and NJ trees indicate the states used for each species. Stamen position and inflorescence branching pattern were mapped only for *Alcantarea* species, whereas habitat preference was also mapped for *Vriesea* species.

3. Results

3.1. Phylogenetic analyses of the plastid DNA dataset

Phylogenetic analyses for plastid DNA data were carried in single dataset including *trn*K-*rps*16 and *trn*C-*pet*N sequence data. The final aligned plastid dataset included 1104 characters and 30 taxa, as we were unable to obtain sequences of *Alcantarea martinellii* and *Vriesea pauperrima* for both plastid markers. In addition, we were unable to obtain *trn*C-*pet*N sequences for one specimen of *A. aff. extensa* and for *A. imperialis* and *trn*K-*rps*16 sequences for *Vriesea bahiana*. A total of 41 characters were variable and 13 potentially parsimony-informative in the plastid dataset. The parsimony analysis of molecular data yielded 644 most parsimonious trees (Supplementary material) of 43 steps (CI 0.9767, RI 0.9783; Table 2). The Bayesian analysis consensus tree resulted in a very similar topology (data not shown). The plastid analyses resulted in poorly resolved topologies containing a large polytomy in which all species of *Alcantarea* are found together with one well-supported clade with all *Vriesea* species (BS = 85, posterior probabilities (PP) = 1.00). Therefore, the exact relationship between these two genera could not be reconstructed based on this dataset. CI and RI values indicate low homoplasy, which is also associated with the low levels of variation encountered. Furthermore, within *Alcantarea*, one well-supported clade (BS = 86, PP = 1.00) hold the sister species *A. duarteana* and *A. turgida*.

3.2. Phylogenetic analysis of nuclear FLO/LFY

PCR amplifications resulted in a single band in all taxa and no evidence of intraindividual polymorphism was found. The aligned molecular matrix for this marker included a total of 32 taxa and 625 characters, of which 97 were variable and 33 were potentially parsimony informative. The parsimony analysis of this dataset

yielded 3527 most parsimonious trees of 108 steps (CI 0.92, RI 0.95; Table 2). The Bayesian analysis consensus tree resulted in a very similar topology (data not shown). The topologies obtained with the second intron of *FLO/LFY* strongly indicates reciprocal monophyly of *Alcantarea* (BS = 91, PP = 1.00) and of the Eastern Brazilian *Vriesea* (BS = 96, PP = 1.00) and that these two groups are sister lineages (BS = 97, PP = 1.00). Within *Alcantarea* resolution is improved at least for few lineages. *Alcantarea roberto-kaustkyi* is the first divergent lineage, followed by a polytomy (BS = 88, PP = 0.97) containing one well-supported (BS = 95, PP = 1.00) clade (*A. regina* (*A. brasiliana*, *A. imperialis*)), a polytomy (BS = 78, PP = 0.99) clade (*A. vinicolor*, *A. turgida*, *A. trepida*, *A. patriae*, *A. odorata*, *A. nahoumii*, *A. glaziouana*, *A. burle-marxii* and *A. extensa* complex) and four species (*A. duarteana*, *A. geniculata*, *A.nevaresii* and *A. martinellii*).

3.3. Phylogenetic analyses of the combined datasets

The partition homogeneity test indicated that the different data partitions of plastid DNA and the low copy nuclear gene were not significantly incongruent ($P = 0.079$). The individual datasets were thus combined in a single matrix with 32 taxa. This combined matrix had a total of 1729 characters with 137 variable and 46 potentially parsimony informative characters. The parsimony analysis of this molecular dataset yielded 5410 most parsimonious trees of 155 steps (CI 0.91, RI 0.94; Table 2). The Bayesian and the parsimony analyses with the combined dataset resulted in very similar topologies, however the Bayesian topology offers greater resolution (Fig. 2; Supplementary data).

The topologies obtained with the plastid and nuclear combined datasets corroborate the monophyly of *Alcantarea* (BS = 91, PP = 1.00) and of eastern Brazilian *Vriesea* (BS = 99, PP = 1.00) generating higher support values than those obtained for the datasets individually. In both MP and BA the topology obtained for the clade of eastern Brazilian *Vriesea* is identical (Fig. 2). This topology is also the same as that obtained from *FLO/LFY* alone. In the *Alcantarea* clade *A. roberto-kautskyi* is the earliest diverging lineage sister to the remaining *Alcantarea* (BS = 86, PP = 0.99), which form three clades. The first one is well supported (BS = 96, PP = 1.00) and comprises (*A. regina* (*A. brasiliana*, *A. imperialis*)). The remaining two clades were only obtained with the bayesian analysis and show: (i) a subclade formed by *A. geniculata, A. nevaresii* and *A. martinellii* (PP = 0.94), and (ii) a polytomy containing the remaining *Alcantarea* species (core *Alcantarea*, PP = 0.98). Core *Alcantarea* (Fig. 2, in red) includes two additional clades, one comprising *A. duarteana* and *A. turgida* (PP = 0.97) and the other comprising *A.* aff. *burle-marxii, A.* aff. *extensa* and *A. burle-marxii* (PP = 0.97).

3.4. Microsatellite analyses

The NJ tree of individuals based on microsatellite genetic distance (Fig. 3) revealed relationships among 22 species of *Alcantarea*. Most species were grouped together although *A. odorata*, *A. nevaresii*, *A. regina, A. roberto-kautskyi* did not cluster. Individuals treated here as *A. aff. extensa* appear in different positions in the NJ tree. *Alcantarea odorata* shares alleles with *A. heloisae* and *A. duarteana. Alcantarea roberto-kautskyi* also shares alleles with *A. regina*. Groups of species detected with the microsatellites may share biogeographical areas and/or morphological characters, although these may not be exclusive of them. Some species that occur mainly in the Serra dos Órgãos appear united: *A. brasiliana*, *A. geniculata*, *A. imperialis*, *A. nevaresii* and *A. regina*. Another cluster of species groups *A. farneyi*, one individual of *A. odorata* and *A. tortuosa*. The *Alcantarea extensa* complex appears divided into three subgroups.

Explicit conflicts among microsatellite results and the sequencing plastid and low copy nuclear gene are not easy to detect due to the different sampling employed in each case and the long polytomy that embraces most *Alcantarea* species in the phylogenetic BA tree. Nevertheless, where resolution allows, comparisons can be made. The 'Serra dos Órgãos' group appears well delimited in the NJ tree and as two clades showing uncertain relationship in the BA tree. Most clusters of species do not have BS support.

3.5. Morphological and biogeographical characterization

The habitat preference and position of stamens were mapped on the Bayesian and on the NJ trees. For the habitat preference of *Alcantarea*, it is observed that most of the species occur in the Atlantic forest. *Alcantarea duarteana* is endemic to rocky savanna-like habitats, while *A. turgida* and *A. nahoumii* can grow in both kinds of habitats (Figs. 2 and 3). Considering *Vriesea*, taxa that occur in rocky savanna-like habitat appear in a more derived clade. Apparently independent events for the evolution of bundled stamens took place, and this is an indicator of non-bat pollination. Also, unbranched inflorescence can be observed in distantly related taxa (Figs. 2 and 3).

4. Discussion

In this study we used three different sets of molecular genetic markers to investigate the relationships among members of tribe *Vrieseeae*, focusing on *Alcantarea*. In the following paragraphs we discuss our main findings with respect to the systematics of *Alcantarea* taxa relative to members of the Eastern Brazilian species of the genus *Vriesea*. We also use our phylogenetic hypotheses to discuss specific questions related to the nature and mechanisms of adaptive radiation in this species-rich subfamily of bromeliads: Does *Alcantarea* represent a monophyletic group, indicating recent common ancestry for species of this primarily inselberg radiation? Did stamen position, a key trait involved in pollinator shifts, evolve more than once during the evolution of *Alcantarea*? If yes, this would indicate that pollinator shifts arise rather easily in this genus. Do the species of *Alcantarea* that occur in rocky savanna-like form a clade? Or, were rocky savanna-like habitats colonized more than once by *Alcantarea* and *Vriesea* species during their evolutionary history? If yes, this would indicate a role for multiple, temporarily separated radiation episodes during species diversification in the rocky savanna-like habitats biodiversity hotspot. We also use patterns of allele sharing to discuss the potential role of hybridization during the evolution of *Alcantarea* species.

4.1. Phylogenetic relationships of Alcantarea based on plastid DNA markers

Both plastid markers provided low resolution in reconstructing phylogenetic relationships in *Alcantarea*, and the low DNA sequence variation in the plastid DNA markers used was insufficient to resolve relationships among genera. The level of sequence variability for Bromeliaceae in general is notoriously low (Smith and Donoghue, 2008) indicating a slow evolutionary rate for the plastid genome (Terry et al., 1997a) or young age of the study group. This low level of variation is in stark contrast with the degree of morphological diversification in the family and has been thought to be associated with a rapid radiation and speciation within Bromeliaceae (e.g. Schulte et al., 2005; Sousa et al., 2007).

4.2. Phylogenetic relationships of Alcantarea based on a low copy nuclear gene

The higher level of DNA sequence variation observed among the species of *Alcantarea* and the Eastern Brazilian *Vriesea* for this marker was more useful in resolving relationships than plastid DNA.

Table 2
Summary statistics of plastid markers (*trn*C-*pet*N, *trn*K-*rps*16) and *FLO/LFY* second intron data matrices and parsimony analyses.

	Plastid markers	*FLO/LFY*	Combined
Aligned length	1104	625	1729
Sequence length	360–408 (*trn*C-*pet*N); 536–668 (*trn*K-*rps*16)	562–901	–
Variable characters/%	41/3.7	97/15.5	137/7.9
No. of potentially parsimony-informative characters/%	13/1.2	33/5.2	46/2.7
No. of MP trees	644	3527	5410
Length of MP trees	43	108	155
Consistency index (CI)	0.98	0.92	0.90
CI excluding uninformative characters	0.93	0.80	0.78
Retention index (RI)	0.98	0.95	0.94

Data presented in Table 2 indicate that *FLO/LFY* had near five times more potentially parsimony-informative characters than the plastid DNA markers. The phylogenetic utility of the second intron of *FLO/LFY* has been demonstrated in several recent publications, in which it was used to resolve intrageneric relationships (e.g. Grob et al., 2004; Oh and Potter, 2003) and here we suggest that it may also be useful for broader analyses within Bromeliaceae.

4.3. Phylogenetic relationships of Alcantarea based on combined analysis

The analyses based on the combined datasets of plastid and nuclear markers strongly support (PP = 1) *Alcantarea* as sister to the eastern Brazilian *Vriesea* species (Fig. 2) corroborating previous molecular studies (Barfuss et al., 2005). *Alcantarea* was previously treated as an 'ancestral group' in relation to *Vriesea*, due to its almost uniformly compound inflorescences assumed by Grant (1995) to be a plesiomorphic character state. This hypothesis is not corroborated by previous works that used a broader sampling of *Vriesea* species (Barfuss et al., 2005) nor by this study.

Alcantarea roberto-kautskyi, the first diverging lineage within the genus is unique in regard to several of its morphological features, such as the inflorescence shape (in candelabrum format), versatile anthers and pollen grain shape (Versieux, 2009). In the well-supported clade formed by (*A. regina* (*A. imperialis, A. brasiliana*)) (Fig. 2), all species are restricted to the Serra dos Órgãos, except *A. regina*, which has a broader distribution, but still confined to the Atlantic rainforest of Rio de Janeiro and adjacent areas in

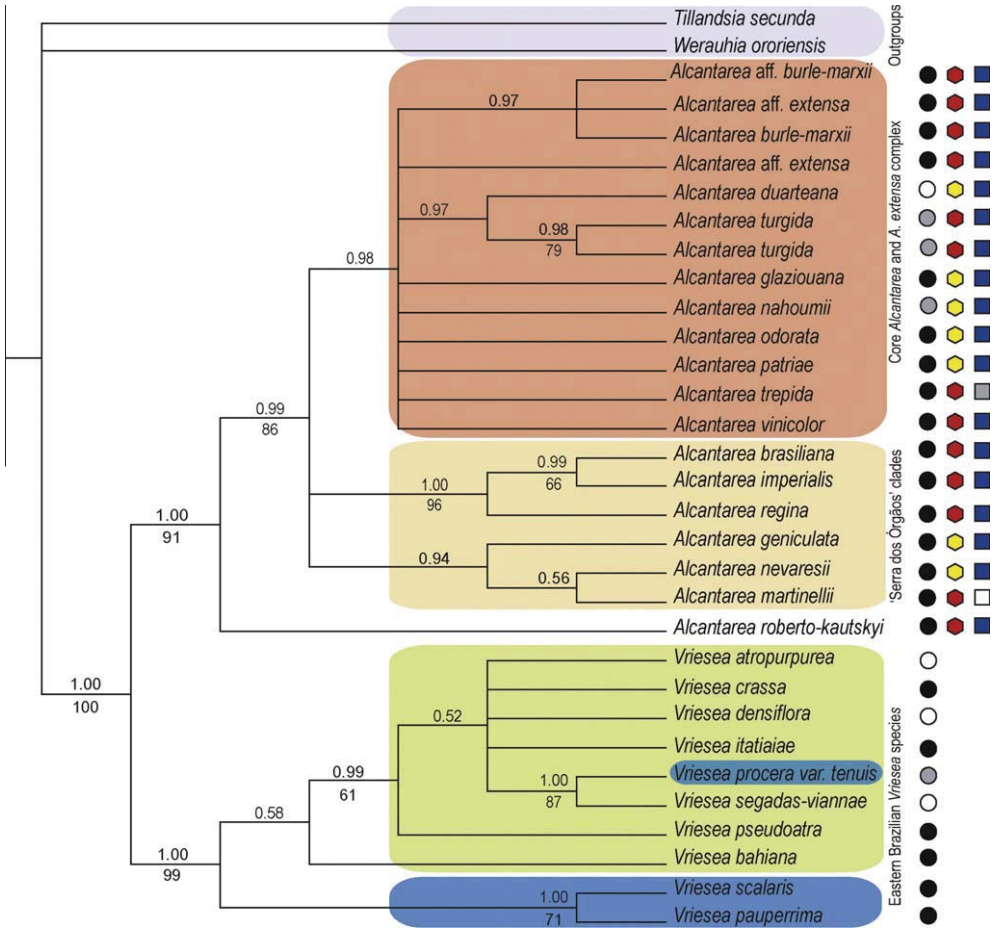

Fig. 2. 50% Majority-rule consensus of 99,538 Bayesian trees from three concatenated regions from plastid DNA (*trn*C-*pet*N, *trn*K-*rps*16) and a low copy nuclear gene (*FLO/LFY*) of *Alcantarea* and *Vriesea*. PP > 0.50 are indicated above branches. Sections of *Vriesea* are indicated by dark blue (Sect. *Vriesea*) and green (Sect. *Xiphion*). Symbols after the taxon name indicate: circle = Habitat (black = inselbergs within the Atlantic rainforest domain; white = outcrops within rocky savanna-like vegetation; gray = inselbergs and outcrops); hexagon = position of stamens at anthesis (red = spreading; yellow = forming a bundle); square = type of inflorescence (blue = branched; white = unbranched; gray = unbranched or branched). Refer to Fig. 1 to understand characters states.

São Paulo and Minas Gerais states. A possible morphological synapomorphy of this clade is the presence of secund flowers. This distinct position of flowers during anthesis was already employed by Harms (1930) to subdivide *Alcantarea* into two groups. Also, these species have bat-pollinated flowers that produce abundant nectar and have petals that fade from yellow while in bud to whitish at anthesis (Martinelli, 1994, 1997).

The position of *A. duarteana* in a well-supported clade with *A. turgida* may have some relation to the geographical proximity of the distribution of these two taxa and an ancient event of gene flow between them. Morphologically, *A. duarteana* is quite distinct from *A. turgida*, and is characterized by some unique traits. It is a medium-sized plant, which has bright yellow petals, and leaves densely covered by trichomes on both faces, which is a rare feature in the genus (Versieux et al., 2010a). In addition, it is restricted to the far inland rocky savanna-like habitat vegetation in the Diamantina Plateau, state of Minas Gerais (Versieux and Wendt, 2006; Versieux et al., 2010a,b). This relationship is not present in the analysis using microsatellites. So, future studies should clarify the position of *A. duarteana* by adding more samples of other potentially related species (such as *A. farneyi* and *A. heloisae*) and also by including one recently described (*A. compacta* Leme) and one rediscovered sympatric species (*A. hatschbachii*) that were not available at the time this study was conducted (Coffani-Nunes et al., 2010).

Morphologically based taxonomy of Bromeliaceae has suffered from high levels of homoplasy and many traditionally delimited taxa are para- or polyphyletic (Costa, 2002; Sass and Specht, 2010; Schulte et al., 2005). In spite of morphological distinctiveness observed within *Alcantarea* taxa, a low molecular divergence was noticed for both plastid markers and the second intron of *FLO/LFY*. This could indicate a relatively recent and rapid radiation, as observed for other genera of Bromeliaceae (Sousa et al., 2007; Schulte et al., 2005) after the evolution of adaptations to survive in the harsh environments of the rocky outcrops of eastern Brazil.

4.4. The use of microsatellites in phylogenetic studies of Alcantarea

The NJ analysis based on microsatellite loci indicates distinct grouping patterns for the different species of *Alcantarea*, though most of them are weakly supported by BS values. *Alcantarea duarteana*, *A. farneyi*, *A. geniculata*, *A. brasiliana*, *A imperialis*, *A heloisae*, *A. glaziouana*, and *A. nahoumii* form well defined groups based on the microsatellite data. A biogeographically well-defined group includes most taxa from the Serra dos Órgãos mountain range and this group can be compared to two clades that were seen in the BA tree, showing congruence. All *A. imperialis* individuals sampled throughout this mountain range appear clustered, independently of the distance among populations or differences in color pattern of leaves and bracts (red and green color morphs). *Alcantarea brasiliana*, which has been considered a doubtful taxon, appears related to both *A. nevaresii* and *A. geniculata* (Fig. 3). *Alcantarea farneyi* and *A. tortuosa* occur in sympatry in Santa Maria Madalena, along the Desengano mountain range and show several morphological similarities (Versieux and Wanderley, 2007b). These taxa are shown to be close to *A. duarteana*, which is a species that occurs isolated in rocky savanna-like vegetation, ca. 450 km further north-west. This relationship is not comparable to the sequence analysis, where *A. duarteana* appears in a clade together with *A. turgida*. Nevertheless, these three species (*A. duarteana*, *A. farneyi* and *A. tortuosa*) occur in high altitude habitats (rocky savanna-like habitat or high altitude grasslands) and show a reduction in size of the rosette and inflorescence. Another group revealed by the microsatellite data are *A. heloisae* and *A. odorata*, that share close areas of occurrence (northern Rio de Janeiro),

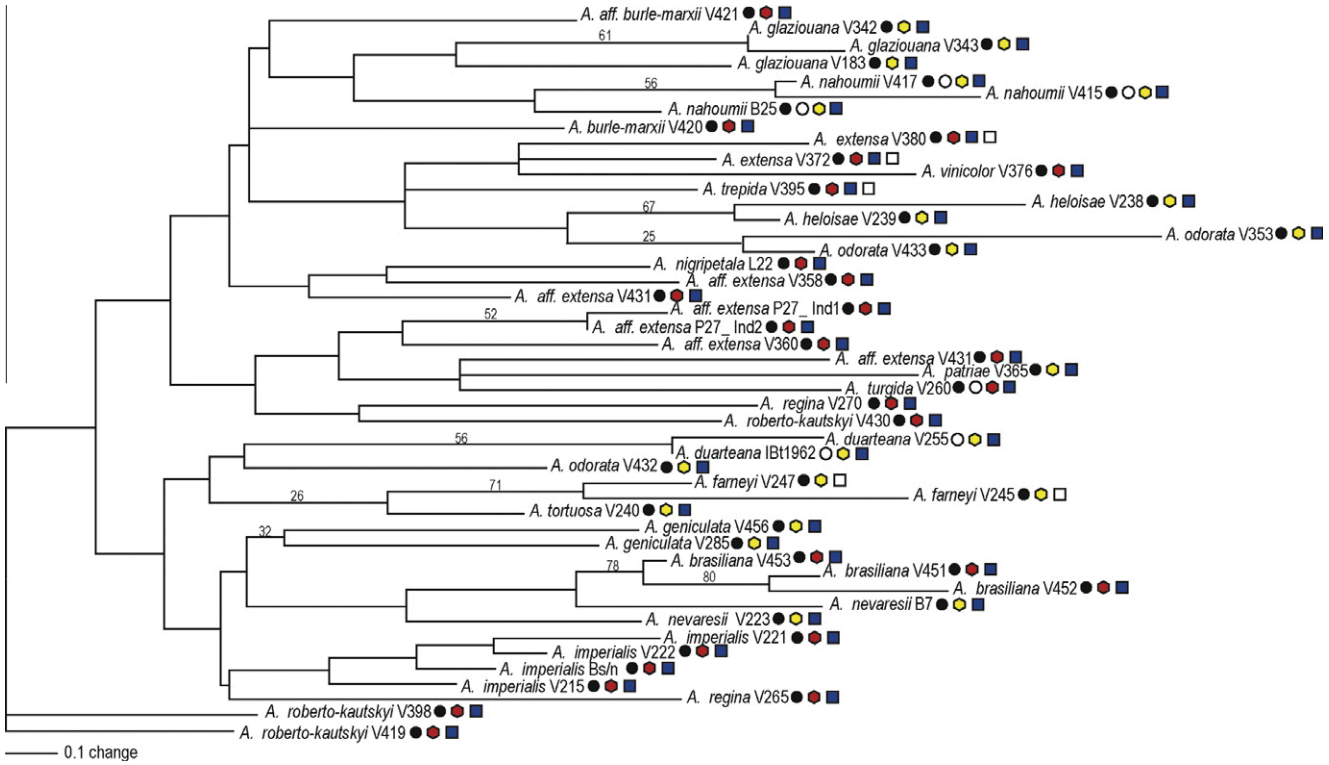

Fig. 3. Neighbor joining dendrogram based on the Pairwise Distance Between Individuals (Goldstein et al., 1995) for 20 microsatellite loci from 47 *Alcantarea* individuals. *Alcantarea roberto-kautskyi* was used as outgroup. Bootstrap values (>20) are indicated above the branches. Letters and numbers after the taxon name indicate collector initials and number (Table 1). Symbols after the taxon name indicate: circle = Habitat (black = inselbergs within the Atlantic rainforest domain; white = outcrops within rocky savanna-like vegetation); hexagon = position of stamens at anthesis (red = spreading; yellow = forming a bundle); square = type of inflorescence (blue = unbranched; white = branched). Refer to Fig. 1 to understand characters states.

leaves and bracts covered by epicuticular wax and similar flower morphology.

An intriguing group involves *A. glaziouana*, a narrowly endemic species that occurs only close to the sea in Rio de Janeiro and Niterói, and *A. nahoumii*, which is the species with the most northern distribution in the genus, occurring ca. 1250 km away from Rio de Janeiro in the state of Bahia (Versieux and Wanderley, 2010b). Future studies are necessary to elucidate if this is a plausible relationship or an artifact of the microsatellite method, since convincing morphological or biogeographical data do not support this relationship. Based on morphology, *A. nahoumii* could be compared to *A. odorata*, but the sequence data are not informative here because all these three species appear inside the polytomy.

The microsatellite patterns support the view that *Alcantarea extensa* should be treated as a species complex, as already mentioned by Versieux and Wendt (2006) and Versieux and Wanderley (2010a). The lack of clustering/paraphyly of several individuals treated here as *A.* aff. *extensa* indicates either extensive hybridization and/or incipient speciation and/or other difficulties associated with the microsatellite methodology, especially the amount of sampling for each taxon. We should rigorously evaluate these results, particularly because there was no clustering of two individuals of *Alcantarea* aff. *extensa*, coming from the same population (*Versieux 431*). This *Alcantarea extensa* complex group could be considered as a recently diverged lineage that has not had the necessary time to accumulate apomorphies and reproductive isolation, leading to unclear species boundaries (Versieux and Wanderley, 2010a). Alternatively, different phenomena could explain the non-monophyly of species and this may have practical implications (e.g. species conservation, red list assessments), particularly due to the concept of species to be adopted, since gene flow is an important determinant for some species concept (Edwards et al., 2008). Microsatellites may be exposed to size homoplasy due to rapid evolution (Jarne and Lagoda, 1996). Population sampling and the number of loci employed should be maximized in order to alleviate homoplasy problems in such analyses. As shown by Barbará et al. (2007) for each microsatellite locus in *Alcantarea* there is a size range. This range is only known by sampling several individuals per species/population. Most species are represented in our NJ tree by only two individuals what can lead to a biased sampling of the gene pool, due to the genetic drift. However, according to Kalinowski (2005) even small sample sizes (one or two individuals) would lead to similar results as larger samples for estimating genetic distances using microsatellites when there is a large differentiation among the sampled taxonomic units.

It is expected that the microsatellite NJ tree shows groups of species or suggests relationships that are also present in the phylogenetic tree based on combined plastid and low copy nuclear gene sequences as was already observed in other similar works (e.g. Ochieng et al., 2007). Barkley et al. (2009) demonstrated that microsatellites could be used to make phylogenetic inferences in closely related species of citrus, although after sequencing some alleles that were similar in size they found that the sequences were not identical neither contained the same microsatellite repeats. So, our conclusions regarding the microsatellite use in Bromeliaceae are that such studies are valid but should be based in large samples, maximizing the number of populations per species and also the number of loci genotyped.

4.5. Implications for morphological evolution

When the phylogenetic trees derived from DNA sequencing are interpreted in the light of the microsatellite NJ tree it becomes clear that selected morphological traits are encountered in distantly related taxa. Limited data are available regarding reproductive biology of most species of *Alcantarea* (Martinelli, 1994, 1997; Matallana et al., 2010; Vogel, 1969). Despite that, the floral morphology, particularly the bundled or spreading stamens may reflect the pollination syndrome (see Introduction). Our results suggest that bat pollination, indirectly inferred by the position of the stamens, has evolved several times. The presence of bright yellow petals and bundled stamens in several other species groups suggests pollination by moths/hummingbirds. Inflorescence branching patterns have been widely employed in the taxonomy of Bromeliaceae. Unbranched inflorescences, for instance, also exist in distantly related taxa and possibly have multiple origins as well. This character is present in *A. martinellii, A. farneyi*, *A. trepida*, *A. extensa* and other species, including *A. benzingii* and *A. hatschbachii* that were not sampled.

4.6. Biogeography notes

The Espinhaço mountain range and its associated rocky savanna-like habitats are thought to be one of the floristically richest regions in the world (Giulietti et al., 1997). Nevertheless, only limited data are available in the literature regarding the origins of the rocky savanna-like habitats flora. Bromeliaceae that occur in rocky fields show a high degree of endemism (Versieux et al., 2008). However, some taxa from rocky savanna-like habitats also occur in the Atlantic rainforest domain, indicating a phytogeographical connectivity between these neighboring domains (Versieux and Wendt, 2007). In our analysis of habitat we noted that most species occur in the Atlantic rainforest. Based on microsatellite data, occupation of the rocky savanna-like vegetation has occurred more than once. Considering only the species that occur in rocky savanna-like vegetation, *Alcantarea duarteana* and *A. turgida* do form a clade but *A. nahoumii* appears to be distantly related to this clade by genetic distance. However, we cannot rule out the possibility that *A. nahoumii* may be closely related to the clade of *A. duarteana* and *A. turgida* using only sequence data. The addition of few species endemic to outcrops that were not sampled in sequence analysis (e.g. *A. hatschbachii*), as well as resolving polytomies will allow us to get a more conclusive answer to this biogeographical question. *Alcantarea duarteana* is restricted to rocky savanna-like habitats, whereas the other two species grow either in rocky savanna-like habitats or in outcrops (inselbergs) in the Atlantic rainforest domain. For *Vriesea* species a clearer pattern is observed, in which rocky savanna-like habitats endemics are younger than the Atlantic forest lineages. This finding is concordant to a recent biogeographical study conducted by Simon et al. (2009) that indicates that the Brazilian savanna, which is closely similar to the rocky savanna from the Espinhaço range, was recently assembled.

4.7. Hybridization hypothesis

Interspecific hybridization, with or without subsequent introgression from one species into another, is a widespread phenomenon in plants (Rieseberg and Carney, 1998). Artificial hybrids are abundant in Bromeliaceae and may indicate that genetic incompatibility systems are of minor importance in isolating species under natural conditions (Smith and Till, 1998). Despite this, studies conducted in areas of high diversity of Bromeliaceae indicate that natural hybrids are rare in the face of the weak prezygotic isolation mechanisms for avoiding interspecific pollination (Wendt et al., 2002, 2008). Recently conducted molecular studies have shown that hybridization occurs and play an important, but still difficult to prove, role on the process of bromeliads speciation (Jabaily and Sytsma, 2010; Palma-Silva et al., 2011; Schulte et al., 2010). Fragmentation of species ranges during cycles of climatic change may have led to expansions and contractions in area of occurrence and these shifts and the subsequent secondary contacts after some period of isolation may have caused extensive hybridization (Benzing,

2000). This process of secondary contact has been evoked to explain the radiation of species that nowadays occupy naturally fragmented habitats such as mountain tops, as in the genera *Dyckia*, *Encholirium* (Benzing, 2000) and *Puya* (Schulte et al., 2010). In the case of *Alcantarea*, the great overlap of distribution and flowering period (concentrated during summertime) indicates opportunities for hybridization or gene introgression especially within the Serra dos Órgãos clades and in the *Alcantarea extensa* species complex. Previous work on *Alcantarea* (Barbará et al., 2007, 2009) indicates that there is a very low level of interspecific gene flow between sympatric populations of *Alcantarea geniculata* and *A. imperialis* in Serra dos Órgãos. Possibly, this process has led to the many confusing intermediate forms that can be seen under cultivation (e.g. Wright, 1915). While *A. geniculata* has predominantly green foliage and green bracts, narrower leaf blades and triangular leaf apex, *A. imperialis* foliage and bracts vary from green to wine-red, leaf blades are much wider and the apex is round and acuminate. Inflorescence and flowers are also quite distinct between these two species (Versieux, 2009). Nevertheless, considering molecular phylogenetic analyses presented here and morphological data (Versieux, 2009), we hypothesize that *Alcantarea brasiliana* is a hybrid taxon. Based on morphology it is treated as a synonym of *A. imperialis* (Versieux, 2009), due to very limited variation between these taxa. Actually several Bromeliaceae specialists get confused when identifying specimens of *A. brasiliana* in herbaria, which commonly appears as *A. imperialis*. In our microsatellite analysis, all individuals of *A. imperialis* sampled cluster together whereas *A. brasiliana* individuals appear more related to *A. nevaresii* and *A. geniculata*. In contrast, the Bayesian analysis reconstructs a sister relationship of *A. brasiliana* and *A. imperialis* with high PP support, and no differences between sequences for the second intron of *FLO/LFY* were observed. Examinations of populations of *A. brasiliana* in the field, revealed that it occurs in sympatry with *A. geniculata*. Though Versieux (2009) treated *A. brasiliana* as a new synonym of *A. imperialis*, there are a few differences in the inflorescence morphology of *A. brasiliana* that also suggest it may be close to *A. geniculata* as well. In fact, when Smith (1943) described *A. brasiliana*, he mistakenly included in his description a specimen of *A. geniculata* (*Glaziou 11685*) indicating some difficulty in separating these taxa. Based on our field observations, earlier morphological and field works (Smith, 1943; Versieux, 2009) and in the position occupied by this species in NJ tree (i.e. clustered together with *A. nevaresii* and *A. geniculata*) and in the BA (in a clade with *A. imperialis* and *A. regina*) we hypothesize that *A. brasiliana* may be a natural hybrid between *A. geniculata* and *A. imperialis*, species that co-occur around Petrópolis in Serra dos Órgãos, particularly close to the collection locality were our samples of *A. brasiliana* were obtained (i.e. Meio da Serra). This hypothesis should be carefully investigated in the future, employing a much broader sampling of all these species.

5. Conclusions

Our phylogenetic analyses have shown that *Alcantarea* is monophyletic and sister to a clade that comprises Brazilian species of both sections of *Vriesea*: *Vriesea* and *Xiphion*. The infrageneric classification of *Vriesea* deserves further investigations, since its sections, as traditionally defined, are paraphyletic. Different datasets provided distinct levels of resolution, and microsatellites emerged as possible tools for inferring relationships among closely related species of *Alcantarea*. The colonization of rocky savanna-like habitats and the chiropterophilous syndrome, inferred by the spreading position of the stamens, seems to have evolved several times, thus suggesting that pollinator shifts occur easily in this group of bromeliads. Rocky savanna-like habitat species of *Alcantarea* and *Vriesea* apparently evolved from Atlantic rainforest ancestors. The multi-species microsatellite data support the view that hybridization may have played a role during bromeliad diversification, as predicted by theory on the evolutionary genetics of adaptive radiation (Seehausen, 2004). Both hybridization and the evolution of particular traits in the genus will be better understood with the addition of taxa and genomic regions to resolve polytomies and clearly document gene flow.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2012.03.015.

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