



Diversification of Bromelioideae (Bromeliaceae) in the Brazilian Atlantic rainforest: A case study in *Aechmea* subgenus *Ortgiesia* [☆]



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ABSTRACT

Aechmea subgenus *Ortgiesia* comprises ca. 20 species distributed in Brazil, Argentina, Paraguay, and Uruguay, with a center of diversity in the Brazilian Atlantic rainforest. We examined interspecific relationships of *Ortgiesia* based on Amplified Fragment Length Polymorphisms (AFLP). Ninety-six accessions belonging to 14 species of *Ortgiesia* were sampled, and genotyped with 11 AFLP primer combinations. The neighbor joining (NJ) tree depicted two main genetic groups within *Aechmea* subgenus *Ortgiesia*, and four subgroups. The NJ tree showed short internal branches, indicating an overall shallow genetic divergence among *Ortgiesia* species as expected for the recently radiated subfamily Bromelioideae. Our results suggest that hybridization and/or incomplete lineage sorting may have hampered the reconstruction of interspecific relationships in *Aechmea* subgenus *Ortgiesia*. The mapping of petal color (yellow, blue, pink, or white), inflorescence type (simple or compound), and inflorescence shape (ellipsoid, subcylindric, cylindric, or pyramidal) against the NJ tree indicated that these characters are of limited taxonomic use in *Aechmea* subgenus *Ortgiesia* due to homoplasy. An analysis of the current distribution of *Ortgiesia* identified the southern region of the Brazilian Atlantic rainforest, between latitudes of 26° and 27°S, as the center of diversity for the subgenus.

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1. Introduction

The Brazilian Atlantic rainforest represents one of the top five biodiversity hotspots on Earth, with high levels of endemism (Myers et al., 2000). Despite its reduction to only 11–16% of its original area (Ribeiro et al., 2009), it is home to more than 15,000 plant species of which 45% are endemic to this biome (Stehmann et al., 2009). Bromeliaceae, the second largest family among vascular epiphytes in the Neotropics, are an important and characteristic element of the Atlantic rainforest, being represented by 816 species of which 651 are endemic (Stehmann et al., 2009). Bromeliads play an important ecological role due to their interaction with fauna, as sources for pollen, nectar, fruit,

and water, as well as for providing microhabitats for many invertebrates and even vertebrates (Benzing, 2000). For subfamily Bromelioideae (33 genera and 936 species; Luther, 2012) the Atlantic rainforest is the center of diversity with ca. 505 species (Martinelli et al., 2008) including ten genera endemic to this biome (Smith and Downs, 1979; Stehmann et al., 2009).

Subfamily Bromelioideae is morphologically and ecologically highly diverse, comprising terrestrial, lithophytic and epiphytic life-forms. Molecular phylogenetic studies have revealed that Bromelioideae comprise several small lineages, which diverged early in the evolution of the group, and the core bromelioid clade, which harbors the majority of species (Schulte and Zizka, 2008; Schulte et al., 2005, 2009; Silvestro et al., 2014). Recent molecular phylogenetic studies inferred an Andean origin of the subfamily in the late Miocene, at around 13 Ma, and an early migration to Eastern Brazil via a central South American corridor, at around 10 Ma (Schulte et al., 2005; Silvestro et al., 2014). Whereas the early diverging Bromelioideae lineages were terrestrials and lithophytes, the acquisition of a central water impounding structure, the tank, facilitated the core bromelioid's invasion of the epiphytic niche,

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especially within the Atlantic rainforest, where they diversified extensively (Givnish et al., 2011; Schulte et al., 2009; Silvestro et al., 2014).

Aechmea subgenus *Ortgiesia* belongs to the tank-forming core bromelioid clade (Sass and Specht, 2010; Schulte et al., 2009; Silvestro et al., 2014), and comprises ca. 20 species (Luther, 2010), most of which are endemic to the Atlantic rainforest (Smith and Downs, 1979). The subgenus occurs in south and south-eastern Brazil (Espírito Santo to Rio Grande do Sul states), mainly in the subtropical area, with several narrow endemic species, some only known from the type collection. Two more widespread species, *Aechmea calyculata* and *A. recurvata*, also expand to the west and reach into Argentina, Paraguay, and Uruguay (Smith and Downs, 1979). *Ortgiesia* species are encountered from sea level to 1200 m elevation as epiphytes, lithophytes, or terrestrials (Reitz, 1983; Smith and Downs, 1979; Wanderley and Martins, 2007).

Previous molecular phylogenetic studies included only a limited number of representatives of subgenus *Ortgiesia*. These studies demonstrated a close phylogenetic relationship between most of the investigated species, albeit the monophyly of the subgenus was not confirmed (Horres et al., 2007; Schulte and Zizka, 2008; Schulte et al., 2009; Silvestro et al., 2014). In several studies, *Aechmea racinae* L.B.Sm. (subgenus *Lamprococcus*) was nested within *Ortgiesia* (Horres et al., 2007; Schulte and Zizka, 2008; Schulte et al., 2005; Silvestro et al., 2014) or found in sister group position to an *Ortgiesia* clade (Schulte et al., 2009). In the molecular phylogenetic study of Sass and Specht (2010), the eight *Ortgiesia* species included formed a clade together, however *Billbergia zebrina* Lindl. was nested within this clade. So far, interspecific relationships within *Ortgiesia* remained unclear due to limited resolution of phylogenetic reconstructions, which may be due to the relative young age of the extant species of the subgenus. In a recent age estimate based on molecular dating analysis of a multi-locus phylogeny of the subfamily Bromelioideae, the crown diversification of *Ortgiesia* was estimated to have started during the late Pliocene, at around 2.5 Ma (Silvestro et al., 2014).

Floral features, in particular the petal color and the inflorescence branching pattern, have been given much emphasis in the taxonomy and systematics of *Aechmea* subgenus *Ortgiesia* (Smith and Downs, 1979). Petal color in *Ortgiesia* is mostly yellow or blue, sometimes pink or white, and intermediate colors are rare exceptions (M. Goetze, personal observation). A great diversity is found in inflorescence morphology, which varies from simple to compound, dense to lax, few to many flowered, with many intermediate forms, thus rendering the delimitation of species boundaries difficult (Faria et al., 2004; Smith and Downs, 1979; Wanderley and Martins, 2007). The lack of detailed molecular phylogenetic studies in *Ortgiesia* has hampered the evaluation of morphological characters and their taxonomic value.

Previous phylogenetic studies in Bromeliaceae have demonstrated very low DNA sequence variation of the plastid and nuclear markers used (e.g., Barfuss et al., 2005; De Sousa et al., 2007; Horres et al., 2007; Louzada et al., 2014; Maia et al., 2012; Schulte et al., 2009; Versieux et al., 2012), which has rendered the assessment of intra- and interspecific relationships difficult. Amplified fragment length polymorphisms (AFLP) have been successfully used to elucidate interspecific relationship in closely related Bromeliaceae (Heller et al., 2015; Horres et al., 2007; Jabaily and Sytsma, 2013; Rex et al., 2007; Schulte et al., 2010). The AFLP technique provides numerous genetic markers that are distributed across the whole genome and usually exhibit moderate to high rates of variability. AFLPs require no prior knowledge of the genome analyzed, and have been proven to be a time and cost efficient tool in assessing interspecific relationships in complex plant groups (Gaudeul et al., 2012; Meudt and Clarke, 2007).

This study aims to gain insights into the diversification of *Aechmea* subgenus *Ortgiesia* in the Atlantic rainforest. To this aim, we (1) reconstructed interspecific relationships in *Ortgiesia* based on AFLP data, (2) assessed the taxonomic value of key morphological characters, and (3) examined biogeographic patterns in *Ortgiesia*.

2. Materials and methods

2.1. Plant material

A total of 96 individuals belonging to 13 *Aechmea* species of subgenus *Ortgiesia*, one undescribed and potentially new taxon (here referred to as *Aechmea* sp.), plus *A. kleinii*, originally described as belonging to *Ortgiesia* but included in subgenus *Pothuava* by Smith and Downs (1979) and Luther (2010), were sampled along the geographical distribution of the group in the Brazilian Atlantic rainforest (Table 1; Fig. 1). As outgroup five species from the Nidularioid clade were included. Fresh leaves of 1–9 individuals per species were sampled and dried using silica gel. Voucher information is provided in Table 1.

2.2. AFLP procedures

Total genomic DNA was extracted with the CTAB method following Doyle and Doyle (1990). Amplified fragment length polymorphism protocols followed Vos et al. (1995) with modifications as outlined by Schulte et al. (2010). Briefly, 30 ng of genomic DNA were digested with the restriction enzymes *Hind*III and *Mse*I (10 U/μl; Fermentas) for 12 h at 37 °C in a final volume of 30 μl, and ligated to *Hind*III and *Mse*I adapters in the same reaction using T4 DNA ligase (5 U/μl; Fermentas). Pre-selective amplifications were carried out with primers with one selective base each (*Hind*III-A and *Mse*I-C, respectively) in a total volume of 10 μl containing 2 μl of the 1:10 diluted restriction-ligation product, 1 × *Taq* buffer (Kapa Biosystems, Boston, USA), 0.5 μM of each primer, 2 mM of dNTP mix, and 0.25 U of *Taq* DNA polymerase (Kapa Biosystems). Assays were subjected to 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. Selective amplifications were run with primers carrying three selective bases each (see below), and with fluorescently labeled *Hind*III primers (FAM, NED, or VIC; Applied Biosystems), with 2.5 μl of 1:20 diluted pre-selective PCR product, 1 × *Taq* buffer (Kapa Biosystems), 0.5 μM of each primer, 2 mM of dNTP mix, and 0.25 U of *Taq* DNA polymerase (Kapa Biosystems). Assays were subjected to a touchdown protocol as described by Schulte et al. (2010). Primer screening was carried out with 54 primer combinations and eight representative *Ortgiesia* samples. Based on the resulting banding patterns, eleven primer combinations were chosen for the generation of the final AFLP profiles: (1) *H*-ACA and *M*-CAA, (2) *H*-AGC and *M*-CGA, (3) *H*-AAC and *M*-CTG, (4) *H*-ACA and *M*-CTA, (5) *H*-AGC and *M*-CTG, (6) *H*-AAC and *M*-CGA, (7) *H*-ACC and *M*-CAG, (8) *H*-AGC and *M*-CTA, (9) *H*-ACT and *M*-CAG, (10) *H*-ACA and *M*-CTC, and (11) *H*-ACA and *M*-CAT. To assess the accuracy and reproducibility of the AFLP markers, a subset of samples was run independently for each primer pair. Comparisons of the results from the two independent runs were satisfactory, yielding highly similar and reproducible banding patterns. Amplification products were electrophoresed at the Australian Genome Research Facility Ltd (Brisbane, Australia) on an AB3730 DNA analyzer (Applied Biosystems). Molecular sizes in base pairs were determined using the GeneScan-500 LIZ size standard.

Table 1Accessions data for the populations of *Aechmea* subgenus *Orgiesia* collected.

Species (N)/Population code	Location	Sample ID	Voucher
<i>A. blumenavii</i> Reitz (8)			
BLP	Blumenau – SC	611, 612, 616, 617	FURB 13803
BLM	Blumenau – SC	632, 633, 640, 642	FURB 27979
<i>A. calyculata</i> (E. Morren) Baker (9)			
DER	Derrubadas – RS	325, 327	HAS 66416
CON	Concórdia – SC	530	FURB 34426
BAR	Barracão – RS	516	ICN 119812
PUT	Putinga – RS	581	HAVT 46
MAQ	Maquiné – RS	429, 431	HAS 36048
SFR	São Francisco de Paula – RS	480, 510	ICN 165253
<i>A. candida</i> E. Morren ex Baker (1)			
GUA	Guaratuba – PR	711	ICN 178781
<i>A. caudata</i> Lindm. (6)			
GAR	Guarujá – SP	08	SP 385012
ILH	Ilhota – SC	56	FURB 14688
LAU	Lauro Müller – SC	59	HAS 66425
ARA	Araquari – SC	170	FURB 28062
FLO	Florianópolis – SC	239, 266	FURB 22585
<i>A. coelestis</i> (K. Koch) E. Morren (9)			
UBA	Ubatuba – SP	09, 15, 17, 20	SP 39027
STE	Santa Teresa – ES	291, 292, 295	T.S. Coser 321 (R)
SMJ	Santa Maria de Jetibá – ES	305, 306	T.S. Coser 341 (R)
<i>A. comata</i> Baker (8)			
FLN	Florianópolis – SC	196, 199, 203, 205	ICN 165256
FLO	Florianópolis – SC	648, 649, 650, 655	FURB 22579
<i>A. cylindrata</i> Lindm. (1)			
SGR	Serra da Graciosa – PR	363	MBM 180463
<i>A. gamosepala</i> Wittm. (4)			
SDF	Serra Dona Francisca – SC	154, 156	ICN 191154
MAT	Matinhos – PR	421, 422	ICN 165259
<i>A. gracilis</i> Lindm. (9)			
PAR	Pariquera-Açu – SP	02, 03, 07	B.F. Abonanza 13 (SP)
IPO	Iporanga – SP	283, 284	B.F. Abonanza 23 (SP)
SGR	Serra da Graciosa – PR	386, 392, 396, 407	ICN 191151
<i>A. kertesziae</i> Reitz (9)			
ITA	Itajaí – SC	102, 588, 600	ICN 191153
BOM	Bombinhas – SC	659, 664	CESJ 62360
FLN	Florianópolis – SC	219, 734	UPBC 35253
LAG	Laguna – SC	40, 44	ICN 167498
<i>A. kleinii</i> Reitz (3)			
ANT	Antônio Carlos – SC	278, 279, 280	ICN 167501
<i>A. organensis</i> Wawra (8)			
TER	Teresópolis – RJ	731	B.F. Abonanza 17 (SP)
INT	Intervales – SP	732	B.F. Abonanza 26 (SP)
SGR	Serra da Graciosa – PR	388, 397, 398, 404, 406, 736	ICN 191150
<i>A. recurvata</i> (Klotzsch) L.B. Sm. (8)			
VIA	Viamão – RS	29, 20, 31, 32	ICN 115402
ITA	Itajaí – SC	119, 120, 121	FURB 14370
MON	Monte Castelo – SC	737	ICN 191149
<i>A. sp</i> (5)			
TAP	Tapiraí – SP	312, 313, 314, 315, 316	B.F. Abonanza 3 (SP)
<i>A. winkleri</i> Reitz (8)			
COR	Corupá – SC	130, 133, 140, 739	ICN 191152
SCR	Santa Cruz do Sul – RS	176, 178, 181, 735	ICN 189267
<i>Outgroup</i>			
<i>Neoregelia laevis</i> (Mez) L.B. Sm.	Serra da Graciosa – PR	705	ICN 190907
<i>Nidularium billbergioides</i> (Schult. f.) L.B. Sm.	Garuva – SC	710	UPCB 22872
<i>N. innocentii</i> Lem.	Serra Dona Francisca – SC	706	FURB 20521
<i>Wittrockia superba</i> Lindm.	Jacinto Machado – SC	726	FURB 25282

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

CESJ, Herbarium Leopoldo Kriger; FURB, Herbarium Dr. Roberto Miguel Klein; HAS, Herbarium Alarich Rudolf Holger Schultz; HAVT, Herbarium Vale do Taquari; ICN, Herbarium of Instituto de Ciências Naturais; MBM, Herbarium of Museu Botânico Municipal; R, Herbarium Museu Nacional; SP, Herbarium of Instituto de Botânica, São Paulo; UPBC, Herbarium Departamento de Botânica, Universidade Federal do Paraná.

Genotypes were automatically scored in GeneMarker version 1.97 (SoftGenetics, Pennsylvania, USA) using the AFLP analysis tool, carefully cross-checked, and manually edited where required.

Fragments of a size range between 90 and 400 base pairs and with signal strengths above an intensity score of 500 were scored as a binary presence/absence matrix (1/0).

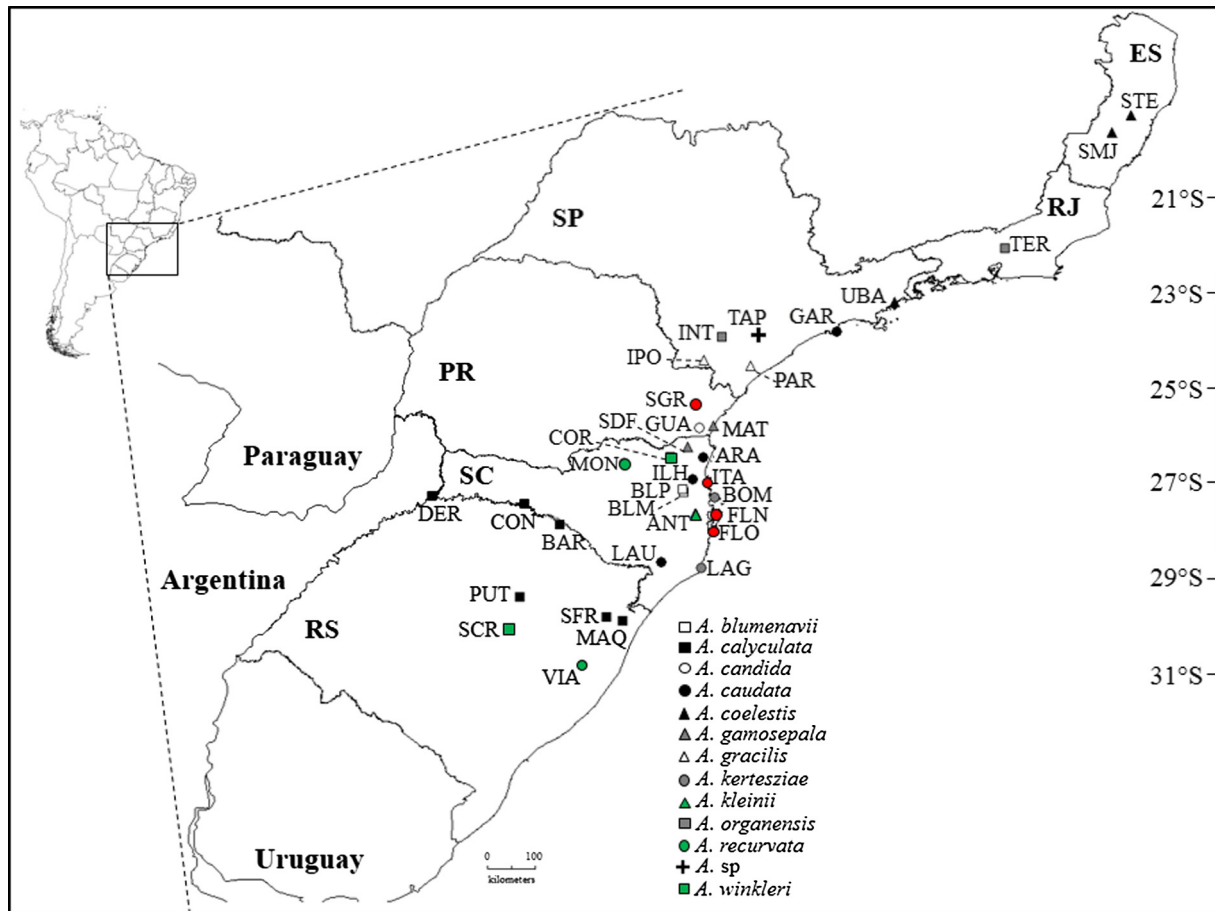


Fig. 1. Map showing the geographical location of *Aechmea* subgenus *Ortgiesia* collected in Brazilian Atlantic rainforest. The symbols correspond to those in the list of species. Red circles correspond to populations where more than one species was collected (see Table 1). Population codes correspond to those in Table 1. Brazilian federal states: ES, Espírito Santo; RJ, Rio de Janeiro; SP, São Paulo; PR, Paraná; SC, Santa Catarina; RS, Rio Grande do Sul. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.3. AFLP data analyses

AFLP matrices of all 11 primer combinations were concatenated to a single matrix and analyzed jointly. Interspecific relationships were reconstructed by neighbor-joining (NJ) analysis (Saitou and Nei, 1987) using Nei–Li's distance measure (Nei and Li, 1979) in PAUP* version 4.0b10 (Swofford, 2002). Statistical support values for nodes and clades were estimated by bootstrap analysis with 1000 replicates (Felsenstein, 1985).

Bayesian phylogenetic analysis was conducted in MrBayes 3.2 (Ronquist and Huelsenbeck, 2003), using the restriction site model and the prior lset coding = noabsencesites. Three simultaneous chains were run for 10 million generations each, sampled every 100th, and the default setting to discard the first 25% of the trees as burn-in. Convergence and stationarity of the MrBayes analysis were determined by evaluating the standard deviation of split frequency values of the end of the run, and by checking run convergence in TRACER 1.5 (available at <http://beast.bio.ed.ac.uk/Tracer>).

To quantify the amount of genetic differentiation among species, an Analysis of Molecular Variance (AMOVA) was performed in the program ARLEQUIN version 3.11 (Excoffier et al., 2005) under 10,000 permutations. Only species with more than one population sampled were included in this analysis (see Table 1).

2.4. Morphological character coding and mapping

Three morphological characters were evaluated to examine their taxonomic utility. Petal color (yellow, blue, pink, or white)

and inflorescence type (simple or compound) are important characters used in species identification of *Aechmea* subgenus *Ortgiesia* (Smith and Downs, 1979). The third character included in this study, inflorescence shape (ellipsoid, subcylindric, cylindric, or pyramidal), has not previously been emphasized in taxonomic treatments. Its potential usefulness was evaluated, as most of the other characters traditionally used to distinguish *Ortgiesia* species rely on measurements of flowers and sepals (Smith and Downs, 1979), which are highly polymorphic and lacking a clear pattern of variation among taxa (Wanderley and Martins, 2007). For each species, the morphological data were scored from published data (Reitz, 1983; Smith and Downs, 1979; Wanderley and Martins, 2007) and personal observations, and mapped against the NJ tree.

2.5. Biogeography

Habitat preference and occurrence data were studied to further explore and discuss biogeographical patterns in *Aechmea* subgenus *Ortgiesia*. Habitat preference of *Ortgiesia* species was coded following the different phyto-physiognomies recognized for the Brazilian Atlantic rainforest (IBGE, 2006) with some modifications, identifying five main areas of occurrence: ombrophilous forest, mixed forest, semi-deciduous forest, highland (grasslands), and sandy coastal plain ('restinga') (Fig. 2). Distribution data was obtained from field collections of each individual included in the analysis. These data were mapped against the NJ tree.

The number of species occurring in each latitudinal range was scored to identify the region that presents the greatest species

richness, based on the distribution data obtained from field collections and the online database of major Brazilian herbaria, *speciesLink* (<http://www.splink.org.br>). Based on the data collected we arbitrarily divided the species as occurring southern or northern 25°S, or in both regions, and mapped this information on the NJ tree.

3. Results

3.1. Interspecific relationships and genetic differentiation

AFLP profiles generated with eleven AFLP primer pairs for 96 *Ortgiesia* accessions yielded a data matrix comprising 942 fragments, and 1008 fragments including the outgroup. The number of scored fragments per primer pair ranged from 70 (*H*-ACC and *M*-CAG) to 123 (*H*-ACT and *M*-CAG), with an average of 93.8 fragments per primer combination.

The NJ analysis shows a tree with a backbone of short branches lacking bootstrap support greater than 50% (Fig. 3). The Bayesian analysis produced a consensus tree with limited resolution (Fig. S1). Overall, the same clusters as in the NJ tree were recovered in Bayesian analysis, and the majority of them with higher support values (posterior probabilities, PP). All accessions of *A. recurvata* formed a well-supported cluster (BS 90, PP 0.99). Likewise, all examined accessions of *A. comata* formed a cluster, receiving low statistical support in the Bayesian tree (PP 0.8). *A. winkleri* accessions clustered together in the NJ analysis with no statistical support, while in the Bayesian analysis five out of eight individuals formed a cluster with low posterior probability (PP 0.85). In the NJ tree, three accessions of *A. gamosepala* (SDF 154, MAT 421,

MAT 422) formed a supported cluster (BS 78, PP 0.99), and one examined accession (SDF 156) was found nested within a cluster comprised of three accessions of *A. caudata* (ILH 56, LAU 59, GAR 08), which remained unsupported. In the Bayesian tree, accession SDF 156 was recovered in a sister position (PP 0.88) to the clade formed by *A. caudata* described above, some *A. coelestis* accessions and one *A. candida*. Three other *A. caudata* accessions (FLO 266, FLO 239, ARA 170) formed a second cluster (PP 0.98). Relationships between the two clusters of *A. caudata* remained unclear due to lack of support in both NJ and Bayesian trees.

In the NJ tree six accessions of *A. gracilis* formed a cluster, yet three additional accessions (IPO 238, IPO 284, SGR 396) were found in a cluster together with one accession each of *A. cylindrata*, *A. kerteszieae*, and *A. organensis* (unsupported). Four accessions of *A. sp.* formed a weakly supported cluster (BS 53), and one accession of *A. sp.* (TAP 314) clustered together with one accession of *A. organensis* (TER). Six accessions of *A. organensis* formed a cluster, and two accessions were found in other clusters within the NJ tree (see above). In the Bayesian tree, three accessions of *A. gracilis* clustered together (SGR 386, SGR 392, and SGR 407; PP 1). This cluster was recovered in sister position (PP 0.8) to a group formed by five individuals of *A. coelestis* plus one of *A. organensis*. Two accessions of *A. gracilis* (PAR 02 and PAR 07) clustered together (PP 0.99) in a sister position to the clade formed by *A. gamosepala* (PP 0.98). *A. gracilis* accession IPO 283 clustered with *A. cylindrata* and one *A. organensis* (PP 0.75), while *A. gracilis* IPO 284 clustered with *A. winkleri* COR 130 (PP 0.61). Bayesian analysis recovered a cluster moderately supported (PP 0.78) formed by all *A. sp.* accessions, while no cluster with more than two individuals were observed for *A. organensis*.

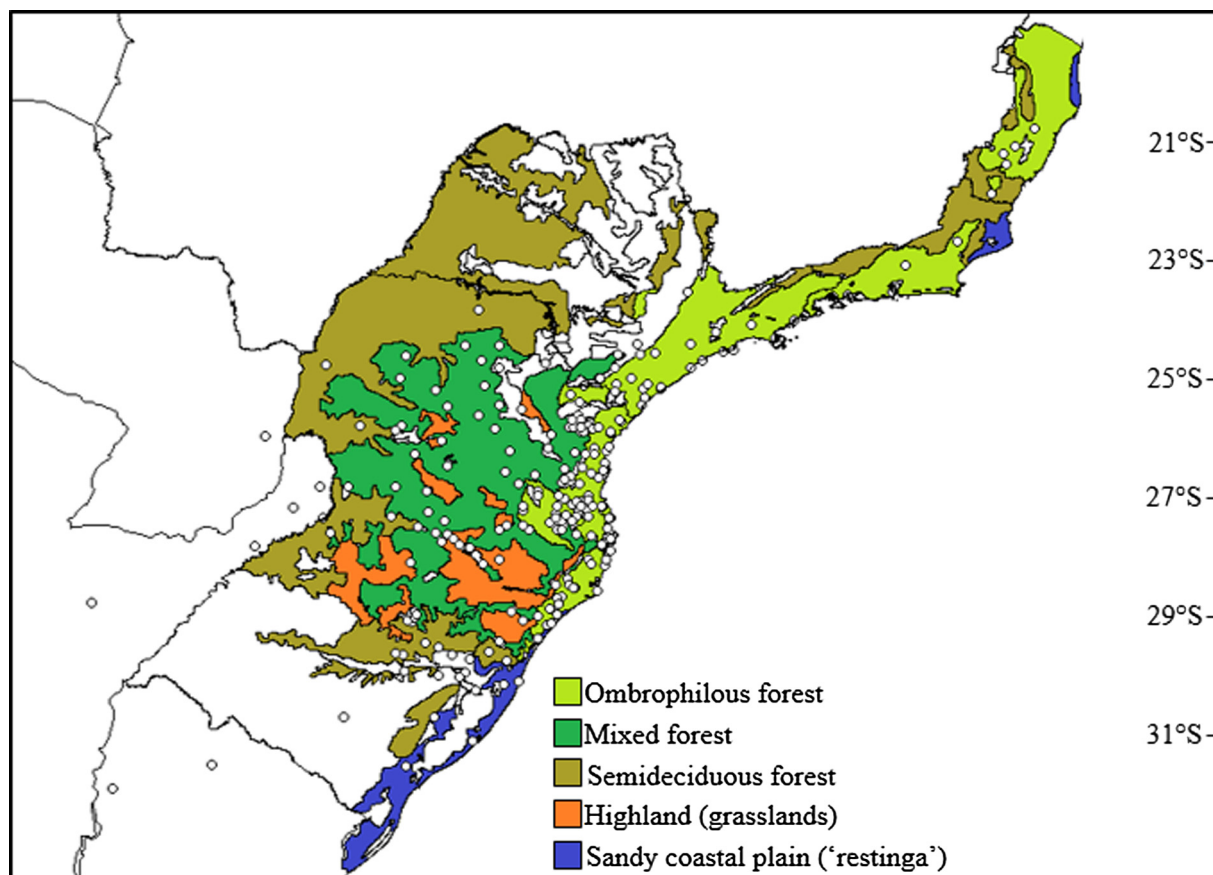


Fig. 2. Distribution of *Aechmea* subgenus *Ortgiesia* (represented by white circles according to herbaria records) in the different Atlantic rainforest phyto-physiognomies.

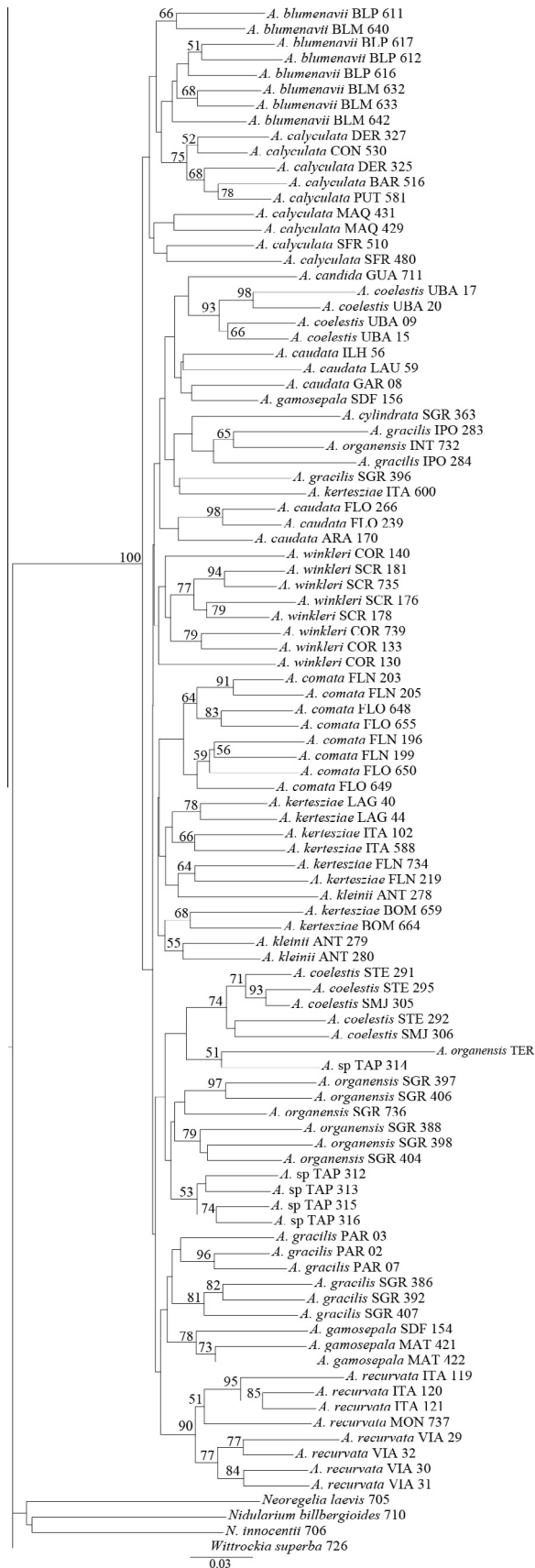


Fig. 3. Neighbor-joining tree of 96 *Aechmea* subgenus *Ortgiesia* accessions plus outgroup based on Nei and Li (1979) distances of 1008 AFLP characters obtained with eleven primer pair combinations. Bootstrap values (>50) are shown above branches. The similarity scale is indicated by a horizontal bar.

Aechmea coelestis formed two supported clusters in both trees. One of these clusters received high statistical support (BS 93, PP 1), while the other received moderate support in the NJ tree (BS 74) and high in Bayesian tree (PP 1), although one accession of *A. organensis* was nested within it. However, relationships between these two clusters of *A. coelestis* remained unclear due to a lack of statistical support. *Aechmea candida* was recovered in sister group position to one of the *A. coelestis* clusters (PP 0.86). Six accessions of *A. kertesziae* clustered together, with one accession of *A. kleinii* (ANT 278) nested within this cluster in the NJ tree. A second cluster of *A. kertesziae* comprised of two accessions (BOM 659, BOM 664) formed a cluster with two other accessions of *A. kleinii* (ANT 279, ANT 280), yet these relationships remained unsupported. In the Bayesian tree, neither *A. kertesziae* nor *A. kleinii* formed a cluster with more than two individuals. *Aechmea calyculata* formed two clusters in the NJ analysis and one in the Bayesian analysis. The cluster which was moderately supported (BS 75) in the NJ was highly supported in the Bayesian tree (PP 0.92). The relationships of the two clusters of *A. calyculata* recovered in the NJ tree remained unclear due to a lack of support. In the NJ analysis, six accessions of *A. blumenavii* clustered together, and other two accessions (BLP 611, BLM 640) formed a moderately supported cluster (BS 68). Relationships between these two *A. blumenavii* clusters remained unclear due to a lack of statistical support. In the Bayesian tree, five out of eight accessions of *A. blumenavii* clustered together, with moderate statistical support (PP 0.62), yet a second cluster formed by individuals BLP 611 and BLM 640 (PP 1) was recovered, but relationships between these two clusters remained unclear due to a lack of support.

AMOVA analysis revealed that only 10.4% of the total genetic variation was found among species ($F_{CT} = 0.104$; $P < 0.001$), 13.9% among populations within species ($F_{ST} = 0.244$; $P < 0.001$) and 75.7% within populations ($F_{SC} = 0.156$; $P < 0.001$).

3.2. Morphological character mapping

The three selected morphological characters were mapped against the NJ tree to examine their taxonomic value (Fig. 4). A majority of species in *Aechmea* subgenus *Ortgiesia* have yellow (seven species) or blue petals (six species). *Aechmea candida* possesses white petals while *A. recurvata* has pink petals (Fig. 4). Most of the species in *Ortgiesia* possess simple inflorescences (eight species), and five species have compound inflorescences. For two species, *A. gracilis* and *A. sp.*, both character states have been reported (Smith and Downs, 1979; Wanderley and Martins, 2007). Simple inflorescences can be ellipsoid, subcylindric, or cylindric in shape in *Ortgiesia* whereas compound inflorescences are always pyramidal. Pyramidal is the most frequent inflorescence shape within the subgenus (five species) (Fig. 4).

3.3. Biogeography

All *Ortgiesia* individuals sampled in our study were found in ombrophilous forest with the exception of *A. winkleri*, which is restricted to mixed and semi-deciduous forests. The only individual of *A. cylindrata* included in our analyses is from mixed forests. Eight species were found only in ombrophilous forest (*A. blumenavii*, *A. candida*, *A. coelestis*, *A. comata*, *A. gamosepala*, *A. kertesziae*, *A. kleinii*, and *A. sp.*). *A. recurvata* inhabits four habitats included in this study (all but semi-deciduous forests), and *A. calyculata* occurs in all habitats except for sandy coastal plains (Fig. 5).

Aechmea subgenus *Ortgiesia* is found from latitude 19°S to 32°S where a lot of the species co-occur (Fig. 6). However, most of the species are distributed at latitudes between 26° and 27°S, which indicates this region as the center of diversity for the subgenus.

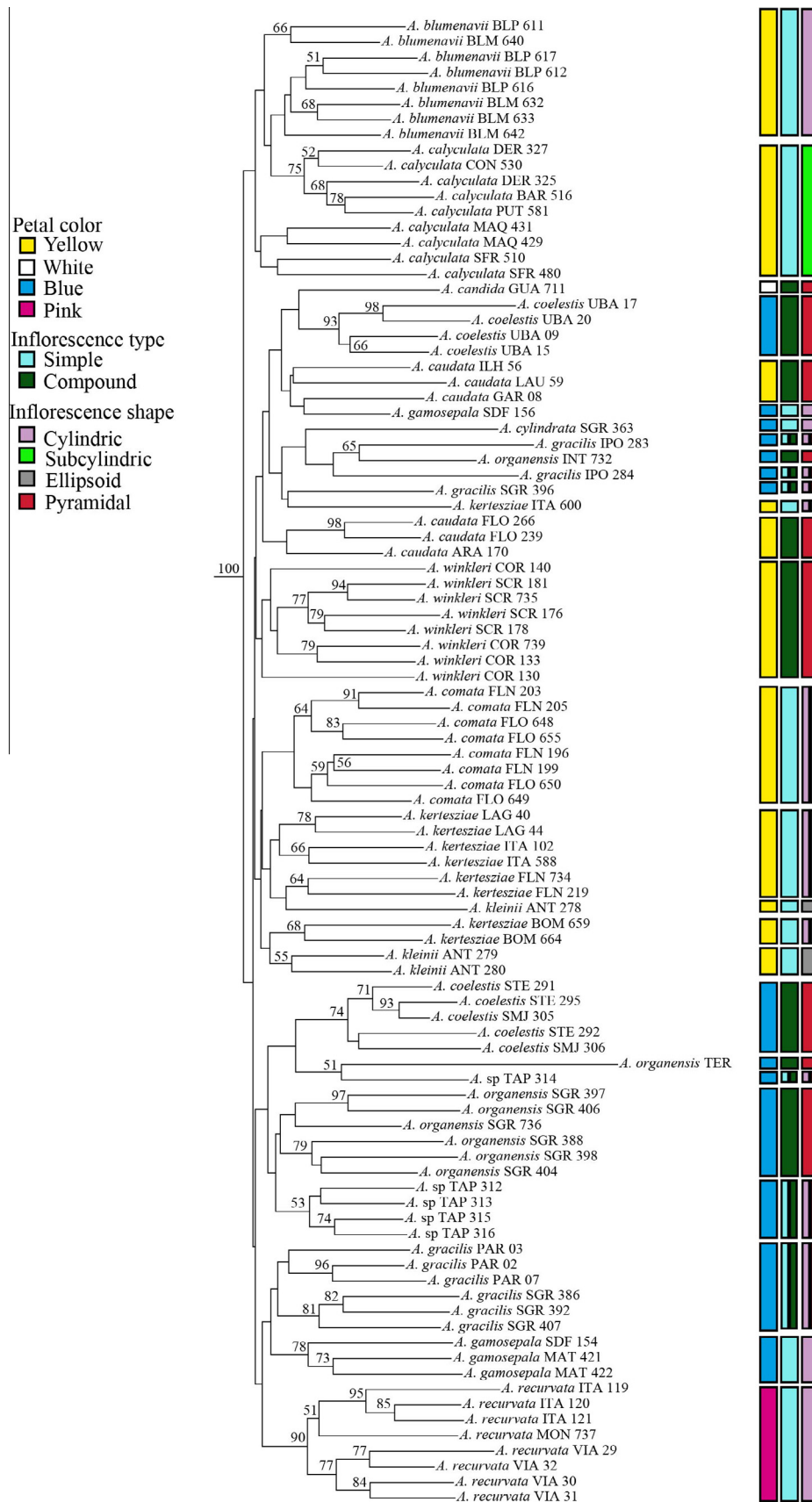


Fig. 4. Character mapping of petal color, inflorescence type, and inflorescence shape in *Aechmea* subgenus *Ortgiesia* against the neighbor-joining tree obtained from the analysis of 1008 AFLP characters. Symbols along the tree correspond to those in the key in the left side. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

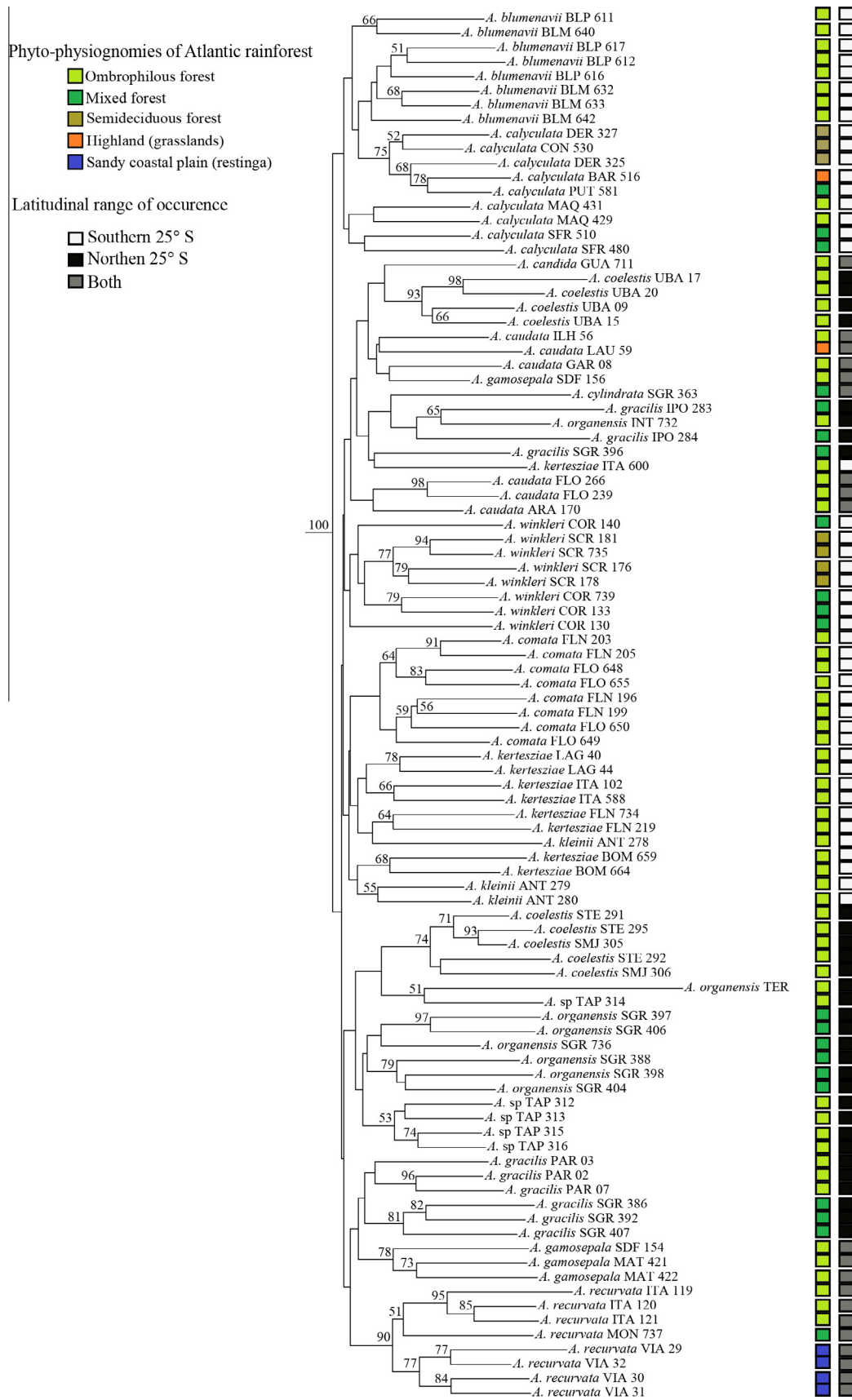


Fig. 5. Habit preferences of *Aechmea* subgenus *Orgtiesia* mapped against the neighbor-joining tree obtained from the analysis of 1008 AFLP characters. Symbols along the tree correspond to those in the legend at the left side.

Aechmea comata and *A. kleinii* are narrow endemics, only occurring at the latitude of 27°S, while *A. winkleri* exhibits a disjunct geographical distribution (occurring at latitudes of 26° and 29°S). *Aechmea coelestis* is the species which extends most to the north-east (19°S) whereas *A. recurvata* was the most southerly distributed species with a latitude of 32°S (Fig. 6). There is some evidence of geographical separation in subgenus *Ortgiesia* when the results of latitudinal range occurrence are analyzed (Fig. 5). *A. coelestis*, *A. gracilis*, *A. organensis*, and *A. sp.* only occur northern of 25°S, while *A. blumenavii*, *A. calyculata*, *A. comata*, *A. kertesziae*, *A. kleinii*, and *A. winkleri* are found southern this latitude. This pattern suggests that species with yellow petals are predominantly found in southern Atlantic rainforest, while species with blue petals are more frequent at the northern region.

4. Discussion

In this study we used 11 AFLP primer combinations to investigate interspecific relationships in *Aechmea* subgenus *Ortgiesia*. Our results showed that *Ortgiesia* species exhibit a shallow genetic divergence, which was evidenced by short internal branches in the NJ tree (Fig. 3). Although *Ortgiesia* species possess considerable morphological and ecological differences (Reitz, 1983; Smith and Downs, 1979), only three of the 14 included species were recovered as monophyletic lineages, and from these three clusters, only *A. recurvata* received statistical support in both Bayesian and NJ trees (Fig. 3, Fig. S1). *Aechmea recurvata* is a morphologically distinctive taxon within *Ortgiesia*, being the only species that possesses pink flowers and a nested inflorescence (Reitz, 1983; Smith and Downs, 1979).

AMOVA analysis revealed that only 10.4% of the genetic variation found in the data set is due to species-level differences whereas 75.7% was found within populations. This low ratio of among- versus within-species differentiation in the context of considerable morphological and ecological divergence is indicative of a recent diversification (Gaudeul et al., 2012; Turner et al., 2013). In fact, *Aechmea* subgenus *Ortgiesia* diversification started as recent as 2.5 Ma (Silvestro et al., 2014).

Bromeliaceae represents a well known example of a species radiation (Benzing, 2000). Several groups found in the Brazilian Atlantic rainforest have been shown to have undergone a rapid diversification process e.g., *Aechmea* (Schulte et al., 2005, 2009), *Alcantarea* (Versieux et al., 2012), *Dyckia* (Krapp et al., 2014), and *Lymania* (De Sousa et al., 2007). Establishing interspecific

relationships in groups where the diversification process was fast is challenging, especially if it was a recent phenomenon. Our NJ tree showed a backbone lacking bootstrap support greater than 50% (Fig. 3). In groups that have recently radiated, evolutionary processes such as introgression/hybridization and/or incomplete lineage sorting/retention of ancestral polymorphism may hamper the reconstruction of interspecific relationships and can lead to low support values in phylogenetic reconstructions (e.g., Glor, 2010; Hinsinger et al., 2014; Sauer and Hausdorf, 2010; Viales et al., 2014).

Although artificial hybrids are easily produced within Bromeliaceae (e.g., Vervaeke et al., 2004), the occurrence of natural hybrids are rare within the family (Wendt et al., 2008). So far, little is known about the extent of natural hybridization or introgression in *Aechmea* subgenus *Ortgiesia*. However, hybrid species were recognized during the evolution of other species of Bromeliaceae, as in the genus *Puya* (Jabaily and Sytsma, 2010; Schulte et al., 2010). In the presence of weak reproductive isolation and shallow genetic divergence in *Aechmea* subgenus *Ortgiesia*, the occurrence of hybridization cannot be ruled out.

Retention of ancestral polymorphisms between species increases with decreased time between speciation events (Maddison, 1997; Rosenberg, 2003). *Aechmea* species underwent rapid and recent diversification (Givnish et al., 2011; Schulte et al., 2009; Silvestro et al., 2014) and the occurrence of incomplete lineage sorting may have been a common feature, leading to retention of ancestral polymorphisms. In plant species with long generation times and large population size, the retention of ancestral polymorphism would be favored (Bouillé and Bousquet, 2005). Species of *Aechmea* are known as investing in clonal reproduction (Benzing, 2000; Izquierdo and Pinero, 2000; Loh et al., 2015), which thus increases generation time, promoting the overlap of many generations (Orive, 1993; Young et al., 1996). Clonal reproduction then may slow down the loss of genetic diversity through genetic drift (as suggested for some bromeliads; Goetze et al., 2015; Izquierdo and Pinero, 2000; Loh et al., 2015; Ribeiro et al., 2013), also favoring the retention of ancestral polymorphisms (Bouillé and Bousquet, 2005). Furthermore, early in the speciation process, lineages may share ancestral polymorphisms and the differences that exist may be in a few genes with large effect, which may be responsible for morphological and ecological adaptation (Seddeek et al., 2014; Wu, 2001). Therefore, these evolutionary processes discussed might be responsible for the low resolution of the phylogenetic reconstructions recovered for *Aechmea* subgenus *Ortgiesia*.

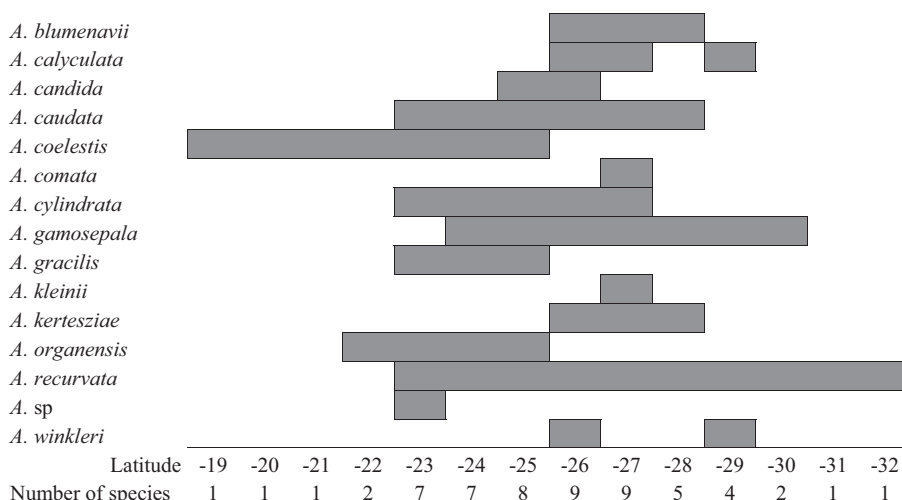


Fig. 6. Number of *Aechmea* subgenus *Ortgiesia* species occurring in each latitudinal range and their distribution.

In spite of relatively shallow genetic divergence and low bootstrap support for the main clusters recovered in the NJ tree, and the low resolution of the Bayesian tree, most of the accessions of each species clustered together. Some of these clusters are characterized by morphology and/or ecological data. For example, *Aechmea comata*, *A. kertesziae* and *A. kleinii* clustered together. These species present similar morphology and their geographical distribution is restricted to Santa Catarina state (Reitz, 1983; Smith and Downs, 1979). While *A. comata* and *A. kertesziae* thrive at sea level and can be found in sympatry, *A. kleinii* occurs at altitudes above 1000 m from sea level and is the only *Ortgiesia* species with nocturnal anthesis (Reitz, 1983).

Aechmea winkleri accessions clustered together in the NJ tree. *Aechmea winkleri* and *A. caudata* are the only yellow-flowered species with compound inflorescences within *Ortgiesia*, but the former is reduced in size in many morphological characters when compared to the latter and is not found in coastal regions (Smith and Downs, 1979; Fig. 4). Only two populations of *A. winkleri* are known to date, which seem to be geographically isolated from the remaining *Ortgiesia* species (Fig. 1). Despite of the geographical isolation from the other *Ortgiesia*, *A. winkleri* wild population from Rio Grande do Sul has high levels of genetic diversity and no signs of demographic contractions were observed (Goetze et al., 2015).

Almost all accessions of the possible new taxon included in this study were recovered in a weakly to moderate supported clade (Fig. 3, Fig. S1). *Aechmea* sp. is seen as distinct taxon from the closely related *A. organensis* especially because of the inflorescence type, in general simple, rarely compound, and due to differences in placentation, with placentae positioned along the ovary in *A. sp.* while in *A. organensis* it is almost always restricted to the central region of the ovary (Wanderley and Martins, 2007). These authors stated *A. sp.* is possibly a new taxon, although further studies are required before conclusions can be drawn about its validity.

The AFLP technique is a multi-locus approach, covering the entire genome, which is a desirable feature. Moreover, AFLPs have been used successfully to resolve interspecific relationships in recently diverged and radiated groups (Althoff et al., 2007; Bussell et al., 2005; García-Pereira et al., 2010; Koopman, 2005), especially when other molecular marker failed to resolve them (Vitales et al., 2014, and references therein). The patterns recovered in the present study may reflect the recent and rapid diversification of *Aechmea* subgenus *Ortgiesia* as already observed for the entire core bromelioids (e.g., Schulte et al., 2009; Silvestro et al., 2014). Markers with a deeper sampling in the genome, as those generated by next generation sequencing, may be used in further studies to clarify interspecific relationships in *Aechmea* subgenus *Ortgiesia*. The next step would be to further elucidate the phylogenetic placement of *Ortgiesia* within the core bromelioids including the identification of monophyletic lineages, which will require a broader sampling within the group.

4.1. Evaluation of morphological characters

The mapping of three selected traits against the NJ tree suggests that independent evolution events may have occurred (Fig. 4). Petal color and inflorescence type may not constitute synapomorphies, although they have been used as the main characters to distinguish between *Aechmea* subgenus *Ortgiesia* species (Smith and Downs, 1979). Similarly, inflorescence shape showed substantial homoplasy when mapped against the NJ tree, which implies that this character has limited taxonomic value in the subgenus. High levels of homoplasy in characters often used to distinguished species of *Aechmea* were previously highlighted in a morphological study (Faria et al., 2004), which have been considered a consequence of the rapid diversification of the group (Faria et al., 2004; Schulte et al., 2009).

4.2. Biogeography

Our analysis of habitat preferences showed that the majority of *Ortgiesia* species occur in ombrophilous forest (Fig. 5). This phytophysiognomy is found near the Brazilian coastal line and receives the greatest amount of rainfall per year in the Atlantic rainforest domain (Oliveira-Filho and Fontes, 2000). Therefore, the occurrence of most of the *Aechmea* subgenus *Ortgiesia* in this region is probably a result of a higher humidity, a pattern already observed in other Brazilian plant groups, which often are floristically more diverse close to the sea (Oliveira-Filho and Fontes, 2000). The number of *Ortgiesia* species found in other phytophysiognomies decreases with increasing distance from the coastal line and which thus receive less rainfall (e.g., mixed forest presents seven species and semi-deciduous forest three). In the highlands (grasslands) the number of species is also smaller (where only three *Ortgiesia* species are found), and this pattern may occur not only due to drier conditions but also because of the decrease in temperature (the highlands are the coolest regions in Brazil; Safford, 1999). *Aechmea recurvata* is the only species that occurs in all phytophysiognomies, which thus highlights the ability of this species to deal with different climatic conditions.

Today, the greatest species richness in *Ortgiesia* is found at latitudes between 26° and 27°S, thus rendering the south of Brazilian Atlantic rainforest the center of diversity for the subgenus (Fig. 6). In previous studies examining the patterns and processes that promoted diversification within the Brazilian Atlantic rainforest, the southern part of the biome had received little attention. Among the studies which consider diversification processes in Brazilian Atlantic rainforest, and which mainly focused on animal taxa, several point to a recent colonization of the southern region (e.g., Carnaval and Moritz, 2008; D'Horta et al., 2011; Fitzpatrick et al., 2009; Palma-Silva et al., 2009; Turchetto-Zolet et al., 2012), whereas others found early divergent lineages occurring in the south (e.g., Grazziotin et al., 2006; Thomé et al., 2010; Valdez and D'Elia, 2013). Our results favor the second scenario for *Ortgiesia*, as most of the species occur in the south as well as many endemics and narrow endemic species can be found in this region of the Brazilian Atlantic rainforest (Fig. 6).

The latest molecular phylogeny of subfamily Bromelioideae suggests that the crown diversification in *Ortgiesia* started at around 2.5 Ma, which implies that Pleistocene climatic oscillations may have affected the distribution pattern of the species (Silvestro et al., 2014). Palynological studies revealed that during this period in south and southeastern Brazil, forest distribution was strongly affected by the decrease in temperature and drier conditions, restricting the occurrence of forest to small refugial areas in deep valleys or to more humid areas along rivers. At the beginning of the Holocene there was a significant increase in temperature and humidity, which allowed forest expansion (Behling and Negrelle, 2001; Behling and Pillar, 2007; Behling et al., 2004). These shifts in vegetation range may have fragmented species distributions leading to geographical isolation and in some cases population differentiation. The topography of southern Brazil, with many mountain chains and valleys probably provided many areas for Atlantic rainforest species to persist during the climatic oscillations of the Pleistocene. Therefore, the geographical isolation imposed by this scenario could have been a driver for allopatric speciation in *Ortgiesia* and for other species. This would explain the narrow endemics (*A. comata*, *A. kleinii*, and *A. sp.*) as well as the many species with restricted distribution (*A. blumenavii*, *A. gracilis*, *A. kertesziae*, *A. organensis*, and *A. winkleri*) and the disjunct pattern of occurrence observed for *A. winkleri*.

5. Conclusions

Our analysis with 11 AFLP primer combinations showed that *Aechmea* subgenus *Ortgiesia* exhibits a shallow genetic divergence among species, despite morphological and ecological divergence. This study provided the first insights into *Ortgiesia* evolution, confirming its recent origin and revealing that hybridization and/or incomplete lineage sorting may hamper the reconstruction of interspecific relationships. Further studies should make use of a deeper genome sampling as well as increase the sampling inside the core bromelioid clade to investigate the position of *Ortgiesia* within this lineage, assess its monophyly and to further elucidate interspecific relationships.

Mapping petal color, inflorescence type and inflorescence shape against the NJ tree indicated that these characters possess different levels of homoplasy, which implies a limited taxonomic value within *Ortgiesia*.

The biogeographic characterization showed that most of the *Ortgiesia* species occur at ombrophilous forest. The southern region of Brazilian Atlantic rainforest was considered the center of diversity for the group, as most of the species occur between latitudes of 26° and 27°S. The shifts in vegetation ranges imposed by the climatic oscillation of the Pleistocene may have fragmented the distribution of the species and likely caused geographical isolation, which might have been a driver for allopatric speciation in *Aechmea* subgenus *Ortgiesia*.

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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.2016.03.001>.

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