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Phylogenetic Relationships of the Pseudobulbous *Tillandsia* species (Bromeliaceae) Inferred from Cladistic Analyses of ITS 2, 5.8S Ribosomal RNA Gene, and ETS Sequences

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Abstract*—Tillandsia* subgenus *Tillandsia* as defined by Gardner includes 269 species in five groups. Within Group I, species in the *Tillandsia bulbosa* complex share a remarkable synapomorphy: a pseudobulb. We sampled the 13 species of pseudobulbous *Tillandsia* (including *T. intermedia*) in addition to 18 species from the five groups of subgenus *Tillandsia* and four species as outgroups to perform phylogenetic analyses of sequences from two nuclear ribosomal DNA markers (ITS 2 + 5.8S and ETS). Three combined parsimony analyses explored the effects of indels treated as missing data, fifth character states, and with the simple indel coding method. Results show that all 13 species of pseudobulbous *Tillandsia* are placed within a group that also includes five non-pseudobulbous species. The pseudobulb habit is optimized as a synapomorphy for this group although with three reversals. Our analyses also show that sequence variation at ITS 2 + 5.8S and ETS provided resolution and indels are an important source of phylogenetic information. All previously used chloroplast markers have been shown to be almost invariant even among distantly related species in *Tillandsia*. Therefore, both nuclear markers assayed here are promising sources of sequence variation for reconstruction of phylogenetic relationships among species of *Tillandsia* and other Bromeliaceae.

***Keywords*—**indels, intergenic spacer, myrmecophytic, nuclear ribosomal DNA markers, Tillandsioideae.

The search for variable molecular markers in the Bromeliaceae and especially among *Tillandsia* L. species has been difficult despite remarkable morphological variation among hundreds of species (Smith and Till 1998) and availability of a growing list of rapidly evolving genes or intergenic regions (Soltis et al. 2008). Chloroplast DNA has been extensively sequenced and used for the reconstruction of phylogenetic relationships within Bromeliaceae and to evaluate the evolution of characters like CAM photosynthesis or the epiphytic habit. Chloroplast markers studied include restriction site variation (Gilmartin et al. 1989; Ranker et al. 1990), coding regions like *rbcL* (Clark et al. 1993; Barfuss et al. 2005), *ndhF* (Terry and Brown 1996; Terry et al. 1997a, b), and *matK* (Crayn et al. 2000, 2004); and non coding regions like the *trnL* intron (Horres et al. 2000; Barfuss et al. 2005), *trnL–trnF* intergenic spacer, *trnK* intron, *atpB–rbcL* intergenic spacer, *rbcL–accD* intergenic spacer (Barfuss et al. 2005), and *rps16* (Crayn et al. 2004; Barfuss et al. 2005). Although valuable results have been found from these studies, a common drawback to them has been the poor resolution in the phylogenetic topologies found and low support for the few resolved clades. The lack of resolution and support could be explained by the low variability in the markers among the taxa considered. For example, in the study of Terry et al. (1997a) the *ndhF* gene has only 71 parsimoniously informative sites from 2,110 bp among 31 taxa sampled across three subfamilies of Bromeliaceae. Variation in previously studied markers is even less at lower taxonomic levels within subfamilies.

Subfamily Tillandsioideae are a monophyletic group (Gilmartin et al. 1989; Terry et al. 1997a, b; Horres et al. 2000; Barfuss et al. 2005). The subfamily is divided into ten genera: *Alcantarea* (E. Morren ex Mez) Harms, *Catopsis* Griseb., *Glomeropitcairnia* (Mez) Mez, *Guzmania* Ruiz & Pav., *Mezobromelia* L. B. Sm., *Racinea* M. A. Spencer & L. B. Sm., *Tillandsia* L., *Viridantha* Espejo, *Vriesea* Lindl., and *Werauhia* J. R. Grant (Spencer and Smith 1993; Grant and Zijlstra 1998; Smith and Till 1998; Espejo-Serna 2002). Relationships among these genera have been studied by Terry et al. (1997b), Horres et al. (2000), and Barfuss et al. (2005). Early attempts to elucidate relationships within subfamily Tillandsioideae found sequence homogeneity in the *trnL* UAA marker (Horres et al. 2000). A broader sample of 110 sequences from the ten genera of Tillandsioideae also revealed low sequence variation in each of seven chloroplast regions spanning intergenic and coding portions. Improved resolution and support were obtained only when considering all of these chloroplast markers combined (Barfuss et al. 2005). According to these studies, *Glomeropitcairnia* and *Catopsis* diverge early in the evolution of Tillandsioideae and are considered as two separate tribes by Barfuss et al. (2005). The rest of the genera are considered as the core Tillandsioideae, which Barfuss et al. (2005) divided into two tribes: Vrieseeae including *Alcantarea, Vriesea,* and *Werauhia,* and tribe Tillandsieae containing *Guzmania, Mezobromelia, Racinea, Tillandsia,* and *Viridantha*. Within this last lineage *Guzmania* and *Mezobromelia* are two clearly separate clades, but many species from *Racinea, Tillandsia,* and *Viridantha* are intermixed in one large clade with poor resolution.

The neotropical genus *Tillandsia* is one of the largest within the Bromeliaceae with 551 species according to Smith and Till (1998). It is distributed from the southern U. S. A. to central Argentina and Chile (Till 1992). Traditionally the genus has been divided into seven subgenera (Mez 1935): *Allardtia* (A. Dietr.) Baker, *Anoplophytum* (Beer) Baker, *Diaphoranthema* (Beer) Baker, *Phytarrhiza* (Vis.) Baker, *Pseudalcantarea* Mez, *Pseudo-Catopsis* Baker, and *Tillandsia*. Several authors have recognized the need of a critical revision of these subgenera (Gardner 1982, 1986; Till 1992; Spencer and Smith 1993; Beaman and Judd 1996; Espejo-Serna 2002; Barfuss et al. 2005). Systematic works have already treated five subgenera: *Allardtia, Tillandsia* (Gardner 1982, 1986; Espejo-Serna 2002), *Diaphoranthema* (Till 1992), *Pseudo-Catopsis* (Spencer and Smith 1993), and *Pseudalcantarea* (Beaman and Judd 1996). Subgenus *Tillandsia* sensu Gardner (1986) is the largest with 269 species, including 147 species from subgenus *Allardtia* and 122 *Tillandsia* species previously recognized in subgenus *Tillandsia* by Smith and Downs (1977). The seven subgenera of *Tillandsia*, and their recent generic segregates, *Racinea* and *Viridantha*, were represented with 59 species in the most comprehensive phylogenetic study of the relationships within

Tillandsioideae (Barfuss et al. 2005). According to them, one of the well-supported groups was the clade identified in their paper as K, which included ten species from subgenus *Tillandsia* and two species from the subgenus *Allardtia*. These 12 exemplars in this clade are among the 269 species previously recognized as subgenus *Tillandsia* by Gardner (1986) in her preliminary classification.

According to Gardner (1986), *Tillandsia* subgenus *Tillandsia* consists of five groups (Groups I - V). Within Group I, she recognized a subgroup of 12 species, whose most important synapomorphy is a pseudobulb. The pseudobulb consists of involute leaves that form a bulbous rosette. Another species with a pseudobulb is *T. intermedia*, but this species was not included by Gardner (1986) in this subgroup. All 13 pseudobulbous *Tillandsia* are epiphytes and they all live in xeric habitats from sea level to 2,000 m and are distributed in Mexico, Central America, and northern South America. Most of the species are reported in association with ants (e.g. *T. butzii* and *T. caput-medusae*; Benzing 1970).

The aims of this study were, first, to elucidate whether pseudobulbous *Tillandsia* species indeed are a natural group and to investigate the evolution of such a conspicuous morphological habit. Second, we wanted to explore the relationships of these unique species among the five groups of *Tillandsia* subgenus *Tillandsia* sensu Gardner (1986). We also were looking for molecular markers with enough sequence variation for resolution at this taxonomic level. After a preliminary search for potential markers, we sequenced ITS 2, 5.8S ribosomal RNA gene (5.8S), and ETS from nuclear ribosomal DNA. No previous use of ETS and ITS has been reported for the Bromeliaceae. Our results show that these two markers contain potentially useful variation for a phylogenetic study of closely related species within Bromeliaceae.

Materials and Methods

Taxon Sampling—The classification of five groups by Gardner (1986) was used as a basis for sampling exemplar species of *Tillandsia* subgenus *Tillandsia*. Her Group I includes 85 species, from which we chose 24 species including the 13 species of pseudobulbous *Tillandsia*. Her Group II includes 19 species, from which we sampled four. Her Group III consists of 11 species; we chose two species. Finally, her Group IV includes two species; we selected one. In total, we selected 31 *Tillandsia* species to represent diversity within subgenus *Tillandsia*. Her Group V was later segregated as *Viridantha,* and placed in the clade M identified by Barfuss et al. (2005; Fig. 3 in their paper).

In the study of Barfuss et al. (2005), *Racinea, Tillandsia,* and *Viridantha* species conform to what they called *Tillandsia* s. l., containing several distinct lineages (clades H-R; Fig. 3 in Barfuss et al. 2005). The relationships among these lineages are only partially resolved, but we can identify that clades N, O, P, Q, and R are in one big clade containing some species from subgenera *Allardtia, Anoplophytum, Diaphoranthema, Phytarrhiza, Pseudalcantarea,* and *Tillandsia*; clade K consists of species from subgenus *Allardtia* and *Tillandsia* (subgenus *Tillandsia* sensu Gardner 1986); and clades H, I, L, and M correspond to two or three-species-clades from subgenera *Allardtia, Phytarrhiza*, *Pseudo-Catopsis*, and from the genus *Vriesea.*

Therefore as outgroups, from the larger clade containing clades N to R we sampled two species, and from clade M we sampled *Viridantha atroviridipetala.* Additionally, as a distant outgroup we used *Catopsis nutans* (Appendix 1). Sequences of ETS were obtained for all 35 species. However, difficulties were encountered in obtaining sequence data for the ITS region in *Tillandsia seleriana* (a pseudobulbous species; Group I), *T. dasyliriifolia* and *T. utriculata* (Group II), *T. deppeana* (Group III), and *T. usneoides* (subgenus *Diaphoranthema*) despite adding DMSO and betaine to the PCR. In four out five of these species we were able to amplify the ITS region, but the sequence data was noisy on several sequencing reads we made for each species. For *T. deppeana*, it was not possible to amplify ITS. Consequently, this data matrix consisted only of 30 exemplars.

DNA Extraction, Amplification, and Sequencing—Genomic DNA was extracted from fresh leaves taken from specimens collected by the first author. Fresh samples for seven species, *Tillandsia achyrostachys*, *T. concolor, T. diguetti, T. erubescens,* and *T. flabellata* were donated by A. Espejo and his colleagues at the herbarium UAMIZ. One species, *Tillandsia arizajuliae,* was donated by the Bird Rock Tropical Garden. We used herbarium material for two species, *T. eizii* from MEXU and *T. dasyliriifolia* from XAL. All DNA was obtained following a modified protocol of the CTAB method of Doyle and Doyle (1987), as described by González and Vovides (2002). Vouchers were deposited at XAL and UAMIZ (Appendix 1).

Amplifications of ETS were performed first with primers "18S–IGS" and "26S–IGS", following Baldwin and Markos (1998). It was necessary to sequence three homologous fragments of ETS region for several *Tillandsia* spp. to design specific primers for the genus, and to avoid multiple PCR products. We named our primers "Till-1" (5′-TCgCACgCCCCgYgggCT CCCT-3′) and "Till-2" (5′-CTCCCTgCCTCCgCgCAgYCgA-3′). These are located on the 26S side of the marker about 20–30 bp within ETS. We were able to amplify only a single PCR product using the combination of primers 18S–IGS/Till-1 and 18S–IGS/Till-2.

We amplified the complete ITS region (ITS 1, 5.8S ribosomal RNA gene, and ITS 2) with primers NS7/ITS4 or ITS5/ITS4 (White et al. 1990). However, we were unable to sequence the PCR product with primers NS7 or ITS5 because a high GC content at the beginning of the ITS 1 that affected the sequencing reaction in several exemplars. Therefore, we decided to use only the sequences from the 5.8S ribosomal RNA gene and the ITS 2 region (ITS 2 + 5.8S) for our phylogenetic analyses.

Several amplification and sequencing samples were considered and compared from each species included in this study to corroborate the size and nucleotide composition of each sequence. These sizes are the maximum that could be obtained, due to the strong secondary structure present. Previous reports have also corroborated this (e.g. Baldwin et al. 1995). It was essential to add DMSO and betaine to the PCR reaction in order to facilitate the DNA strand separation. DMSO disrupts base pairing, whereas betaine equalizes the contribution of GC- and AT- base paring to the stability of the DNA duplex (Frackman et al. 1998).

Polymerase chain reaction amplifications of ETS and ITS were performed in a 25 µl mixture containing 0.2 µl of Platinum Pfx DNA polymerase (Invitrogen, Carlsbad, California), 1 µl of $MgSO_4$ 50 mM, 2 µl of dNTP 2.5 mM, 2 µl of each primer 4 µM, 5 µl of betaine 1 µM, 1.25 µl of DMSO 10%, and 40 ng of genomic DNA. PCR reactions were heated to 96°C for 5 min for an initial denaturing step. Double stranded PCR products were obtained via 30 cycles of denaturation (96°C for 1 min 30 s), primer annealing (50°C for 1 min 30 s), and extension (72°C for 2 min 30 s). Because of two PCR bands of different size from ITS, prior to sequencing, PCR products were purified on a 1.2% low-melting-point agarose gel and recovered with Wizard SV Gel and PCR Clean-Up System kit (Promega, Madison, Wisconsin), following the manufacturer's instructions. We tried to sequence both ITS bands from 10 *Tillandsia* species, but only one of them gave us a readable sequence so later on we only recovered the correct size band for the rest of the species. For ETS, PCR products were purified using Sephadex G50 (Sigma-Aldrich, St. Louis, Missouri).

We used Big Dye Terminator Cycle Sequencing Kit version 1.1 (Applied Biosystems, Foster City, California). Primers for sequencing were the same as those used for amplification, in a 10 µM concentration. A total of 35 ETS sequences and 30 ITS 2 + 5.8S sequences were produced for this study. Vouchers and GenBank accession numbers are listed in Appendix 1. Sequences were edited and assembled with BioEdit Sequence Alignment Editor (Hall 1999).

Sequence Alignment and Indel Coding—Sequences of each marker were optimized by automatic multiple sequence alignments using Clustal W (Thompson et al. 1994) with BioEdit Sequence Alignment Editor (Hall 1999). Parameters for the multiple alignments were as follows: full multiple alignments, gap penalties were gapopen = 15, gapext = 6.66, and gapdist = 4. Manual adjustments to the Clustal alignments were made following procedures outlined by Simmons (2004), to obtain the maximum similarity among sequences following the criteria of Zurawski and Clegg (1987) in which the number of insertion or deletion events are minimized while simultaneously minimizing substitution events. Gaps at the beginning or ending of the sequences of both markers were left as missing nucleotides (N) because they are due to lack of information during the sequencing process. For ITS 2 + 5.8S, 18% of matrix cells are scored as missing. For ETS matrix cells only 2% were scored as missing.

Sequences alignment is still an issue to consider when dealing with sequences of different length as in ITS 2 + 5.8S. In these cases gaps are introduced to the data matrix to make homologous alignments; once there, we have to decide whether to include them or not in the phylogenetic analysis. There are still many studies in which gaps are simply deleted or

ignored as ambiguous characters (e.g. Olsen 1988; Olsen and Woese 1993), but there are increasing numbers of cases in which the phylogenetic information that could be provided by the indels is explored in several ways: as putative insertion or deletion events (Hibbett et al. 1995; Lutzoni et al. 2000; Simmons and Ochoterena 2000; Grubisha et al. 2002; Aagesen et al. 2005); treating multibase indels as binary characters (Milinkovitch et al. 1994; Kropp et al. 1997); including indels as fifth states (Coetzee et al. 2003); and using different coding schemes for indels according to the length of the indels (Vogler and DeSalle 1994; Hibbett et al. 1995; González 1996; Simmons and Ochoterena 2000; González et al. 2006).

In our study we decided to explore and compare the contribution of indels to the phylogenetic information of our sequences. For that reason we considered them in three different ways: as fifth states, under the simple indel coding method, and as missing information.

Five data sets were created: (1) ITS 2 + 5.8S with internal indels coded as fifth states, (2) ETS with internal indels also coded as fifth states, (3) combined ITS 2 + 5.8S and ETS matrix treating internal indels as missing data, (4) combined ITS 2 + 5.8S and ETS matrix with internal indels as fifth states, and (5) combined ITS 2 + 5.8S and ETS matrix treating internal indels under the simple indel coding method (Simmons and Ochoterena 2000). In this case we used SeqState version 1.32 (Müller 2005).

Phylogenetic Analyses—The maximum parsimony criterion was implemented using PAUP* version 4.0b10 (Swofford 2001). We used beta software, version 1 of PAUPRat (Sikes and Lewis 2001) to implement the ratchet strategy (Nixon 1999) with ten independent searches for each of the five data sets. The shortest trees from each ratchet search were compared in length and resolution to see if they were of the same length and resolution or if there was variation among them, in order to try further independent ratchet searches. In all analyses stability was reached within the tenth independent ratchet search. ETS and ITS 2 + 5.8S were analyzed separately (matrices 1 and 2) to compare the optimal topologies from each marker, and to reveal the contribution of each character suite to the combined analysis.

Several evaluation methods were considered. Bootstrap (BS) analyses (Felsenstein 1985) were implemented with two strategies; the first strategy used the fast BS search option and consisted of 12 independent runs of 100,000 replicates, each BS replicate consisting of heuristic searches with random stepwise addition (100 replicates). Bootstrap trees from each independent search were saved and then loaded all together to compute the consensus tree of all searches. The second strategy was a single extensive search, with 500 BS replicates, each BS replicate consisting of 3,000 random stepwise-addition replicates. Jackknife support (JK) was calculated under the same two strategies as above with 36% of characters deleted (Farris et al. 1996). Bremer support (B; Bremer 1988) was evaluated using PRAP version 1.21 (Müller 2004) using the ratchet search option and TreeRot (Sorenson and Franzosa 2007) using 20 replicate heuristic searches with random addition of taxa for each constrain statement. Bayesian posterior probabilities were estimated using MrBayes version 3.1 (Huelsenbeck and Ronquist 2001), excluding indels and considering flat prior probabilities. Selection of best model of sequence evolution, GTR + I + G, was achieved with Modeltest version 3.06 (Posada and Crandall 1998) using the Akaike information criterion (Akaike 1974). Four Markov chains starting with a random tree were run simultaneously for 1,000,000 generations, where we reached an average standard deviation of split frequencies of 0.0094. We sampled trees every 100th generation and discarded initial samples applying a "burnin" value of 250,000 generations before calculating the majority consensus tree and posterior probabilities for clades.

Levels of resolution on strict consensus trees from each of the five data matrices were quantified using the Colless weighted consensus fork index (FI; Colless 1980) as calculated in PAUP*. Other properties of data and trees were described with the data decisiveness score (DD; Goloboff 1991), ensemble consistency index (CI; Kluge and Farris 1969), ensemble retention index (RI; Farris 1989a, b), number of parsimony informative sites, and number of optimal trees.

We optimized the occurrence of pseudobulb habit among the *Tillandsia* species sampled in the single most parsimonious tree from analysis 5 using Mesquite version 2.01 (Maddison and Maddison 2007). We also ran an analysis in PAUP* using matrix 5 with the pseudobulb habit coded as one additional character. The five matrices and trees were posted to TreeBASE (Study number S2428).

Results

ITS 2 + 5.8S and ETS Characteristics—Sequences of ETS from *Tillandsia* range in length from 398 bp in *Tillandsia balbisiana* to 409 bp in *T. ionantha*. Similarly, ITS 2 + 5.8S sequences range in length from 455 bp in *Tillandsia paucifolia* to only 69 bp in *T. heterophylla* (we made four amplifications of ITS 2 + 5.8S for this species and in all of them we obtained the same sequence length). Note that most length variation found in ITS 2 + 5.8S sequences is due to incomplete sequences at the 5′ or 3′ ends.

To determine the identity of our ETS sequences with other plant species we performed BLAST searches (Stephen et al. 1997) on GenBank, with our longest ETS sequences. Sequences corresponded to *Tillandsia palmasolana* and *T. streptophylla*. These species were sequenced and amplified with primers 18S–IGS and 26S–IGS. The sequences obtained included a segment of 30 nucleotides inside the 26S subunit and a segment of 15–20 nucleotides inside the 18S subunit. Similar hits obtained with the BLAST searches corresponded to other monocot sequences such as part of the full-length *Zea mays* cpDNA, a small region of the ETS and 18S ribosomal RNA gene of several *Homalomena* species, a fragment of the ETS of *Philodendron bipinnatifidum*, and with some regions for the complete genome of *Oriza sativa*. The same BLAST procedure was done for our ITS sequences and the most similar hits corresponded to other monocot ITS sequences.

A comparison of general features of data and trees from the five analyses performed is summarized in Table 1. The four trees shown in Figs. 1 and 2 are the strict consensus of each analysis (analyses 1–4). The two trees shown in Figs. 3 and 4 are the single most parsimonious tree from analysis 5.

Individual ITS 2 + 5.8S and ETS Analyses—Singleton and multiposition ITS 2 + 5.8S indels are both informative. Most of the singleton ETS indels are informative, but only a few multiposition ETS indels are informative. Logically, in both markers the number of informative sites goes down when indels are not considered. The few groups recovered from the strict consensus of ITS 2 + 5.8S alone are compatible with the groups on the ETS strict consensus (Fig. 1; Table 1), although resolution of tree topology from each marker is poor

Table 1. Comparison of results from five analyses of ITS 2 + 5.8S and ETS under different combinations of data sets and indel coding schemes. Analyses 1–5 correspond to those described in the text. Number of characters correspond to the nucleotides and indels included in each analyses (#Char). We report the number of parsimony informative characters (Inf) and the percentage of informative sites with respect to the total (Inf/#Char); number of most parsimonious trees (#MPT) found on each analysis, length of the most parsimonious trees (Length), ensemble consistency index (CI), ensemble retention index (RI), data decisiveness score (DD), and Colless weighted consensus fork index (FI).

Analysis	#Char	Inf	#MPT	Length	CI	RI	DD	FI
1. ITS 2 + 5.8S indels as fifth states	492	91 (18%)	394	490	0.624	0.512	0.477	0.032
2. ETS indels as fifth states	453	101 (22%)	166	502	0.567	0.581	0.540	0.196
3. ITS 2 + 5.8S-ETS indels as missing	944	131 (14%)	8	687	0.532	0.495	0.451	0.319
4. ITS 2 + 5.8S-ETS indels as fifth states	944	177 (19%)	5	972	0.553	0.448	0.403	0.462
5. ITS 2 + 5.8S-ETS indels simple indel coding	1,064	199 (19%)	1	952	0.502	0.423	0.372	0.538

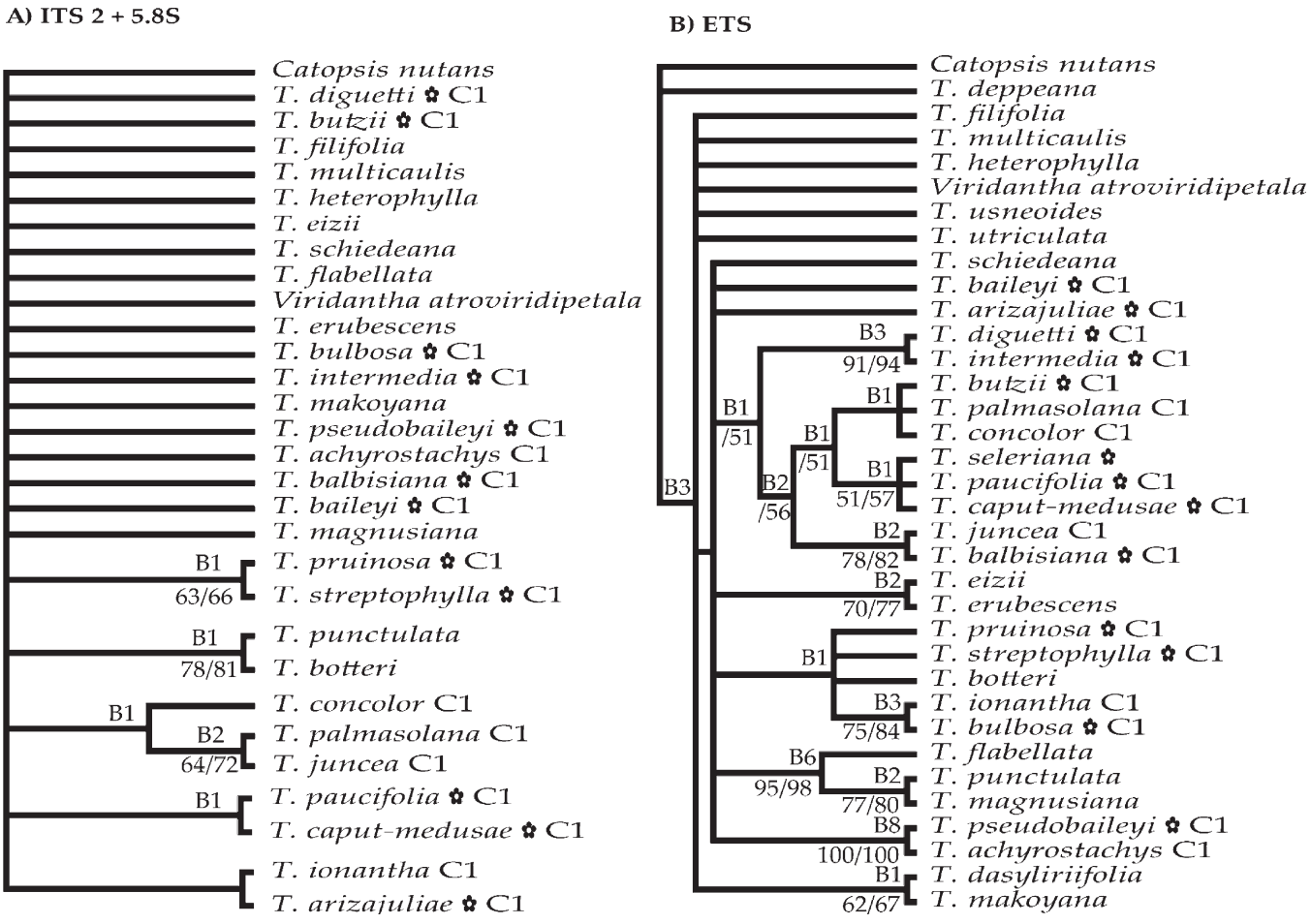

FIG. 1. Strict consensus trees based on multiple ratchet searches on ITS 2 + 5.8S and ETS sequences analyzed separately, with indels treated as fifth states (Analyses 1 and 2). A. Strict consensus of 394 equally most parsimonious trees (490 steps; CI = 0.624; RI = 0.512) found in analysis of ITS 2 + 5.8S sequences for 30 species (Analysis 1). B. Strict consensus of 166 equally most parsimonious trees (502 steps; CI = 0.567; RI = 0.581) found in analysis of ETS sequences for 35 species (Analysis 2). Pseudobulbous *Tillandsia* species are identified with the rosette symbol; C1 indicates species belonging to Clade 1 in Fig. 3. Numbers above branches are estimated values of Bremer support. Numbers below branches are BS/JK ≥ 50%; in some clades there is no BS value but there is JK support, in these cases the value below branches appears as "/xx".

(ITS 2 + 5.8S FI = 0.032; ETS FI = 0.196; Table 1) compared with the resolution of topologies from combined matrices.

Combined ITS 2 + 5.8S and ETS Analyses—Analysis of matrix 3 (indels as missing data) contains 14% informative characters and produces the least resolved strict consensus tree (FI = 0.319; Table 1) of the three combined analyses. Whereas matrix 4 (indels as fifth states) and matrix 5 (indels coded with the simple indel coding method) each contain 19% informative characters. The strict consensus tree from analysis of matrix 4 is better resolved than the tree from matrix 3, but analysis of matrix 5 was the best resolved, producing a single most parsimonious tree (Figs. 2, 3; Table 1). All groups recovered from the three combined analyses are congruent. Therefore, we selected the tree obtained with matrix 5 as the best phylogenetic hypothesis explaining the relationships between pseudobulbous and other *Tillandsia* species we sampled (Fig. 3). We also selected this tree as the best hypothesis because the indel coding followed by this method, as single characters, is less susceptible to be biased by an excess of indel signal.

Basically the same clades with ≥ 50% support on the BS and JK analyses are resolved by the three combined data sets (analyses 3, 4, and 5). Most of the supported clades correspond to 2-terminal clades and have higher support values (≥ 70%; Figs. 2 and 3). Bremer support values range between one and seven, but the majority of supported clades have values of two or three. As in the case of BS and JK values, most of the supported clades for Bremer and Bayesian posterior probabilities are 2-terminal clades. Well supported clades are not contradictory between the different analyses (Figs. 2 and 3).

The most parsimonious tree from analysis 5 depicts a large clade with most of the exemplars sampled from subgenus *Tillandsia* according to Gardner (1986). One exemplar from subgenus *Tillandsia*, *T. heterophylla*, is outside of this clade (Fig. 3). Three main clades are resolved: ((Clade 1, Clade 2), Clade 3)). Clade 1 (B = 1) groups 17 species; 12 pseudobulbous *Tillandsia*, including *T. intermedia*, are mixed here with five other non-pseudobulbous species. However, there is no monophyletic group that includes only the pseudobulbous *Tillandsia* species. Relationships within Clade 1 reveal five well supported species pairs: i) *T. diguetti* and *T. intermedia*, ii) *T. paucifolia* and *T. caput-medusae*, iii) *T. pseudobaileyi* and *T. achyrostachys*, iv) *T. pruinosa* and *T. streptophylla*, v) *T. ionantha* and *T. bulbosa*. Two of these pairs (iii and v) consist of one species with pseudobulbs and one without them. Clade 2, sister to Clade 1, is composed of five species: *T. botteri*, *T. flabellata*, *T. magnusiana*, *T. punctulata*, and *T. schiedeana*.

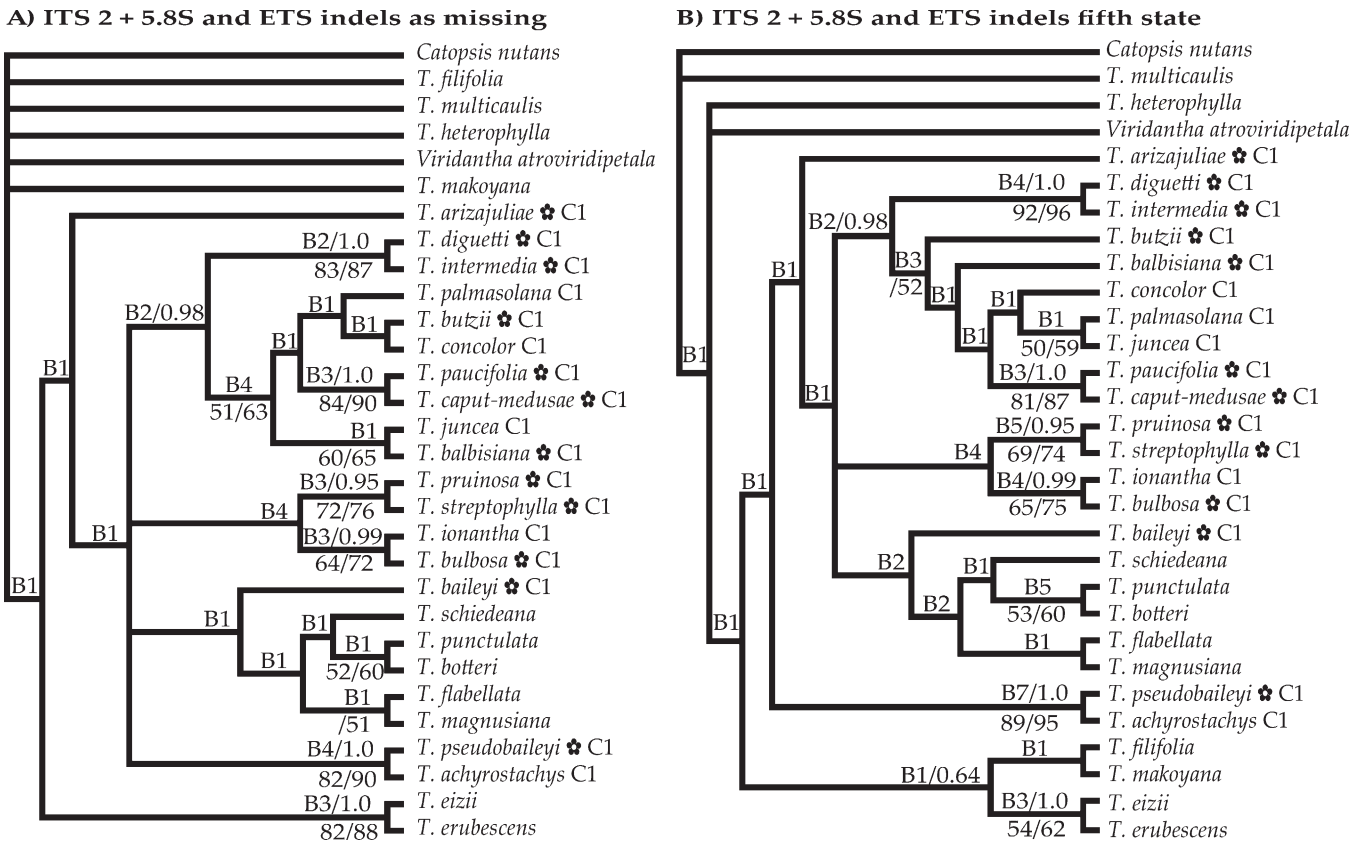

FIG. 2. Strict consensus trees from analyses of combined sequences of ITS 2 + 5.8S and ETS, with two different indel coding methods (Analyses 3 and 4). A. Strict consensus of eight equally most parsimonious trees (687 steps; CI = 0.532; RI = 0.495) when indels are treated as missing data (Analysis 3). B. Strict consensus of five equally most parsimonious trees (972 steps; CI = 0.553; RI = 0.448) treating indels as fifth states (Analysis 4). Symbols and labels are as in Fig. 1. Numbers above branches are estimated values of the Bremer support index/Bayesian values. Numbers below branches are BS/JK ≥ 50%; in some clades there is no BS value but there is JK support, in these cases the value below branches appears as "/xx".

Support for this clade is a B = 3 and JK = 51%. Clade 3, sister to Clade 1 + 2, groups four species, *T. eizii*, *T. erubescens*, *T. filifolia*, and *T. makoyana*. Clade 3 has a B = 2 and a Bayesian probability of 0.64.

Tracking the Evolution of the Pseudobulbous Habit—Optimization using the most parsimonious tree from analysis of data set 5 revealed four steps, one from absent to present and three reversals. The same result is obtained by congruence when the pseudobulbous habit is included, coded as presence or absence, in an analysis with matrix 5. The pseudobulbous habit is a synapomorphy for Clade 1. The absence of a pseudobulbous habit in five species is explained by three reversals within Clade 1, one in *T. achyrostachys*, another in *T. ionantha*, and one more on the terminal clade composed of *T. concolor*, *T. juncea*, and *T. palmasolana* (Fig. 3).

Morphologically it may be possible to distinguish two types of pseudobulb in the species of *Tillandsia* studied here. The first type consists of an almost circular leaf sheath that is constricted abruptly at the union with the linear blade as in *T. arizajuliae*, *T. baileyi*, *T. bulbosa*, *T. butzii*, and *T. seleriana*. In the second type, the leaf sheath is broader than the blade but the constriction is gradual; the blade is laminar and involuted. This second type is present in *T. balbisiana*, *T. caput-medusae*, *T. diguetti*, *T. paucifolia*, *T. pruinosa*, *T. pseudobaileyi*, and *T. streptophylla*. Additionally, this pseudobulb type 2 is also known in *T. intermedia*, a species which has never been classified with the pseudobulbous *Tillandsia*. Therefore we also coded the pseudobulb in three states (absence, type 1, and type 2) for optimization on the same tree. There are eight steps from absence to the presence of any of the two types including several reversals. Considering the three character states, optimization of change for Clade 1 is ambiguously optimized.

DISCUSSION

Implications of Indels as Phylogenetic Information—Including indels in the analyses increases the phylogenetic information (from 14% of informative characters for indels as missing data to 19% for indels as fifth state or under the simple indel coding method) (Table 1). The three tree topologies found with and without indel information are congruent. Differences among the three combined analyses seems to be more related to resolution and number of most parsimonious trees; the topology from the simple indel coding analysis is the most resolved with a single tree (Figs. 2 and 3; Table 1).

Support levels seem not to be affected by the inclusion of indel information. Similar patterns of support are obtained on the three topologies from analyses 3, 4, and 5, corresponding to indels as missing data, indels as fifth states, and coded with the simple indel coding method respectively. For BS and JK analyses, ten clades are supported on topologies from indels as missing and under the simple indel coding method; meanwhile nine clades are supported for indels as fifth states. The topology that has more clades with Bremer support is that obtained by the simple indel coding method and the same happened with Bayesian posterior probabilities (Figs. 2 and 3).

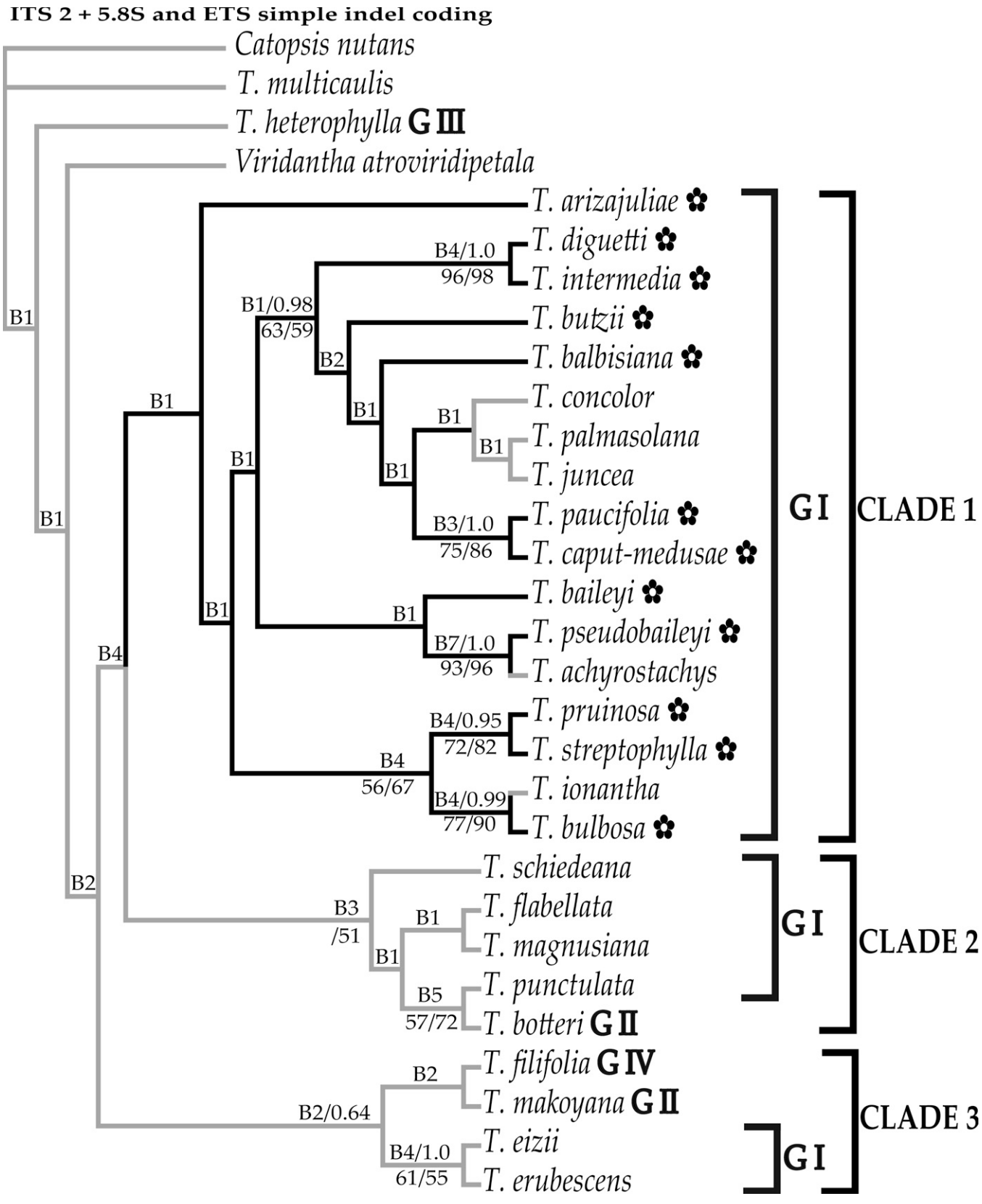

FIG. 3. Phylogenetic hypothesis of the relationships of pseudobulbous *Tillandsia* and evolution of the pseudobulbous habit. The most parsimonious tree (952 steps; CI = 0.502; RI = 0.423) using the simple indel coding method (Analysis 5). This tree summarizes taxonomic implications of the current paper. Clade 1, Clade 2, and Clade 3 group exemplars of subgenus *Tillandsia*. Pseudobulbous *Tillandsia* species (rosette symbol) are within Clade 1. Species marked as GI, GII, GIII, GIV, and GV indicate their placement in Groups I-V in the classification of Gardner (1986). The pseudobulbous habit was coded as "presence" or "absence" of this character. Black branches indicate "presence", grey branches as "absence". Numbers above and below branches are as in Fig. 2.

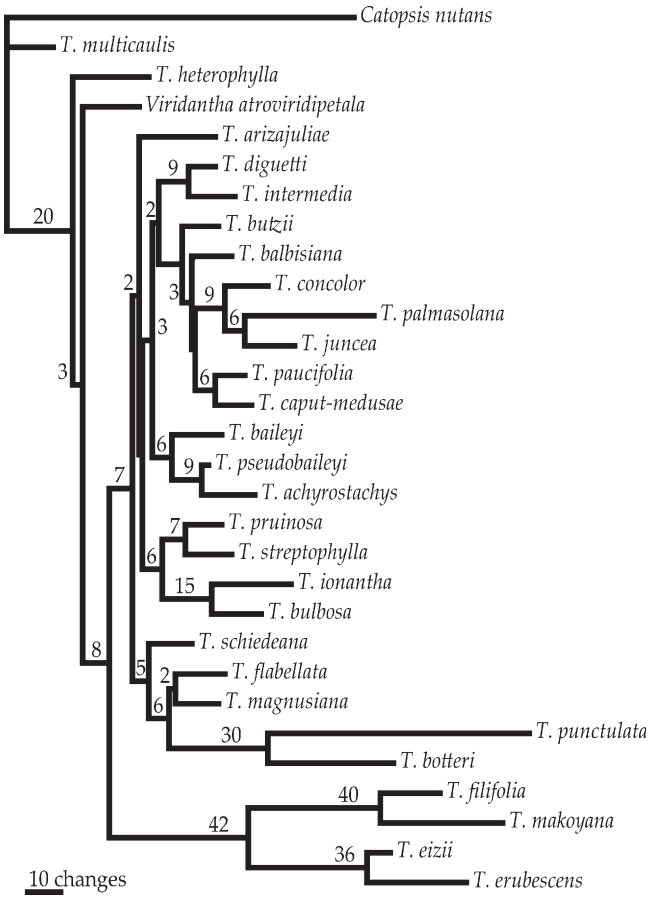

FIG. 4. Phylogram of the most parsimonious tree using the simple indel coding method (Analysis 5). Branch length (ACCTRAN optimization) is given above the branches.

Homoplasy levels in our three combined analyses are also not affected by the inclusion of indels as characters. Our levels of homoplasy are within the ranges found for 30–35 terminals in other studies; in fact they are close to the mean. They range between 0.3 and 0.7 (mean CI = 0.502; Sanderson and Donoghue 1989; Hauser and Boyajian 1997).

In general, the analyses with or without indels are not incompatible; the inclusion of indels as information on the cladistic analyses allowed us to better resolve the phylogenetic relationships of pseudobulb *Tillandsia* without affecting levels of homoplasy or including a stronger signal than expected.

Phylogeny of the Pseudobulbous Tillandsia—The pseudobulbous *Tillandsia,* as circumscribed by Gardner (1986), are not a natural group and do not merit taxonomic recognition. Eleven out of 12 pseudobulbous *Tillandsia* recognized by Gardner (1986) were represented in our analyses. The one excluded species was *Tillandsia seleriana,* for which we did not obtain sequences data for the ITS region. The 11 species with complete sequences for ITS 2 + 5.8S and ETS are grouped in Clade 1 (Fig. 3). Analysis of ETS sequences alone recovers *T. seleriana* in a group with nine other species (Fig. 1B) placed in Clade 1 in our combined analyses. Additionally, *T. intermedia*, a species also known as pseudobulbous but not classified by Gardner (1986), is unambiguously recovered in all analyses within Clade 1, sister to *T. diguetti*. The five non-pseudobulbous species included in Clade 1 are: *T. achyrostachys*, *T. concolor*, *T. ionantha*, *T. juncea*, and *T. palmasolana* (Fig. 3). This phylogenetic hypothesis for the existence of Clade 1 is stable in all the five analyses and it is weakly supported in combined analyses (B = 1; Fig. 3).

Evolution of the Pseudobulbous Habit—The hollow pseudobulb in *Tillandsia* is formed by overlapping, coriaceous, and involuted leaves. Leaf sheaths are broad, inflated, and constricted at the union with the blade (Gardner 1982, 1986). It is a pseudobulb because a true bulb is a modified stem where the upper part and the apical meristem are covered by several concentric layers of thick scales. Among the 269 species included in subgenus *Tillandsia* by Gardner (1986) this morphological habit is known in only 13 species. Our analyses show that the pseudobulb habit is a synapomorphy for Clade 1 (Fig. 3). The pseudobulb syndrome may be an adaptation of this group of plants to life in xeric environments. A pseudobulb is also present in two species of *Tillandsia* subgenus *Allardtia* (*T. disticha* Kunth. and *T. ehlersiana* Rauh). Barfuss et al. (2005) sampled *T. disticha* but it did not group with their 12 exemplars of subgenus *Tillandsia* (clade K), which included one pseudobulbous species (*T. caput-medusae*). They concluded "the position of *T. disticha* is very unstable in different analyses and currently not interpretable" (p. 343 in Barfuss et al. 2005). This seems to indicate that the same syndrome originated independently in pseudobulbous species of *Tillandsia* subgenus *Allardtia*. Future research on the *Tillandsia* phylogeny could include these two pseudobulb *Tillandsia* species to investigate their relationships to the pseudobulb group and the evolutionary origins of this habit.

Most of pseudobulbous species of *Tillandsia* are also similar in rooting into arboreal ant nests. They are named myrmecophytic Tillandsias because they form "ant gardens," deriving nutritional benefit from the ants and helping to stabilize the nest. They seem to house ants in their leaf axils, which form the pseudobulb. Unlike many bromeliads whose leaves channel water towards a central reservoir, myrmecophytic *Tillandsia* species have pointed leaf tips that shed rain and keep the ant colony dry. Trichomes on the leaves, which typically absorb moisture and windborn minerals, probably serve to absorb minerals from the ants' waste (Huxley 1980). Myrmecophytic species with pseudobulbs include *Tillandsia bulbosa*, *T. butzii*, *T. caput-medusae,* and *T. pseudobaileyi* (Benzing 1970; Davidson and Epstein 1989). During fieldwork in Chiapas, Mexico, we were able to document the association of *T. streptophylla* with ants. Given that all of the pseudobulbous *Tillandsia* inhabit similar ecological habitats, such as the branches of trees in deciduous tropical forests, it is possible that the other pseudobulbous species might be myrmecophytic, but has not yet been documented.

Relationships within Subgenus Tillandsia (Clade K sensu Barfuss et al. 2005)—The five groups within *Tillandsia* subgenus *Tillandsia* proposed by Gardner (1986) were not recovered in the present analyses. Barfuss et al. (2005) sampled only 12 of 269 species in the subgenus and they recovered it as monophyletic (their clade K) based on analyses of chloroplast markers. We expanded sampling to 31 exemplar species for ITS 2 + 5.8S and ETS sequences. Although this is still limited compared with the large number of species in the subgenus, our results also recovered the same monophyletic group (B = 1), except *T. heterophylla*, which was resolved outside this clade. This result is not well supported and it remains to be corroborated particularly because the clade appears on the trees from the three combined analyses (Analyses 3–5). Given that our

exemplar species were selected from the five Groups (Gardner 1986), it is also significant that well resolved Clades (1–3) in our phylogenetic hypothesis do not correspond with any of the five Groups within the subgenus proposed by Gardner (1986). For example, the 24 species we selected from her Group I are distributed in all three of our Clades (GI; Fig. 3). Our current analyses suggest that the classification into five groups within subgenus *Tillandsia* is no longer tenable and needs further phylogenetic investigation.

Our phylogenetic hypothesis is consistent and completely resolved even when support levels are low in deep relationships. In spite of that, we think it is the best phylogenetic hypothesis at the light of these two nuclear rDNA markers, there are several synapomorphies supporting every one of the clades within the cladogram (Fig. 4).

The hypothesis proposed here for *Tillandsia* relationships could be used as the starting point for further investigation under the light of new evidence and more taxa as any other phylogenetic hypothesis (Grant and Kluge 2003).

***Utility of ITS 2 + 5.8S and ETS*—**The use of nuclear rDNA markers, including ITS and ETS, in phylogenetic studies has been recently criticized (Álvarez and Wendel 2003). These authors have argued that phylogenetic inferences derived from rDNA markers may be misleading due to potentially frequent "paralogous networks" and the possibility of high levels of homoplasy. Therefore, Álvarez and Wendel (2003) suggest the use of single or low copy nuclear genes instead of ITS or ETS. However, there is evidence that this generalization is not applicable to every case. For example, duplication events, higher frequencies of pseudogenes, incomplete homogenization, compensatory base changes, and problems in alignments due to indel accumulation are more commonly present in large-scale analyses than when dealing with relatively recent radiations (Spalik and Downie 2006; Feist and Downie 2008). These authors found that variation in ITS and ETS sequences among closely related species was easily aligned and with low levels of indels. Our studies also revealed that variation on ETS and ITS 2 + 5.8S sequences among closely related species of *Tillandsia* was sufficiently homogeneous and contain conserved regions to allow us an unambiguous alignment, despite differences in sequence length and insertion of gaps. Furthermore, Mort el al. (2007) found ITS sequences contained more informative sites than cpDNA sequences, although the number of informative indels, levels of homoplasy, phylogenetic reconstructions, and clade support levels from each marker were almost equal. Similarly, levels of homoplasy in our ETS (CI = 0.567) and ITS 2 + 5.8S (CI = 0.624) data sets alone are not unusually high for 35 or 30 terminal analyses, respectively. Ensemble consistency indexes in other studies with this number of taxa range between 0.3 and 0.7 (mean CI = 0.502; Sanderson and Donoghue 1989; Hauser and Boyajian 1997). In the combined analysis of ETS and ITS 2 + 5.8S, the homoplasy level for 30 sequences (CI = 0.502) is not higher than expected either.

Our results clearly show that these two nuclear markers, ITS 2 + 5.8S and ETS, contain enough sequence variation for resolution among closely related species within *Tillandsia*. This is significant because several markers (e.g. *trnL* and *ndhF*) already explored for Bromeliaceae have been shown to be almost invariant even among distantly related species (Terry et al. 1997a, b; Horres et al. 2000; Barfuss et al. 2005). We did restriction enzyme digestions for two noncoding chloroplast regions (*trnL–D* and *rbcL–atpB*) and two nuclear markers (ITS 2 + 5.8S and ETS) to estimate variability prior to sequencing. Following this strategy, we compared restriction enzyme digestions for six selected *Tillandsia* species. We found that the noncoding chloroplast region of the *trnL–D* and the intergenic region between *rbcL* and *atpB* were invariant, whereas the intergenic nuclear ribosomal markers (ITS 2 + 5.8S and ETS) were variable. Further sequencing of 30 *Tillandsia* species in this study allowed full resolution of phylogenetic relationships among them. Our analyses show that these two markers are the most promising sources of sequence variation when compared with any of the previously sequenced chloroplast markers for the Bromeliaceae. Although the two nuclear ribosomal markers individually did not solve relationships, in combination they provided a completely resolved tree. This is in contrast with a previous study (Barfuss et al. 2005) where it was necessary to combine seven cpDNA markers (which also included indels as information) to obtain a similar level of resolution. In that study they found better levels of support when using combined cpDNA markers but trees still lacked complete resolution. It would be interesting and probably useful to complement information from these two molecular sources to obtain support and resolution at deep and tip cladogram levels. Also, as Barfuss et al. (2005) pointed out it might be necessary to generate more extensive morphological data sets for Bromeliaceae to complement molecular information.

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Appendix 1. Voucher specimens and GenBank accession numbers of sequences generated in this study for the *Catopsis*, *Tillandsia*, and *Viridantha* species sampled. Numbers following the collector and collector number are GenBank accession numbers in this order: ETS, ITS 2 + 5.8S. Voucher specimens are deposited in XAL, except where indicated otherwise in parenthesis.

Catopsis nutans (Sw.) Griseb. —*Chew 188*; EU126827, EU126834—

Tillandsia achyrostachys E. Morren ex Baker —*A. Espejo et al. 6532* (UAMIZ); FJ666937, FJ666959. *Tillandsia arizajuliae* L. B. Smith & Jimenez. —*Koide T504* (*Bird Rock Tropicals*); FJ666939, FJ666962. *Tillandsia baileyi* Rose ex Small. —*Chew 179*; EU126835, FJ666961. *Tillandsia balbisiana* Schult. f. —*Chew 167*; EU126833, FJ666960. *Tillandsia botteri* E. Morren ex Baker —*Chew 181*; DQ870640, DQ870646. *Tillandsia bulbosa* Hook. —*Chew 126*; FJ666933, FJ666954. *Tillandsia butzii* Mez —*Chew 133*; EU126828, FJ666943. *Tillandsia caput-medusae* E. Morren —*Chew 100*; FJ666934, FJ666955. *Tillandsia concolor* L. B. Sm. —*A. Espejo et al. s. n.* (UAMIZ); EU126837, FJ666958. *Tillandsia dasyliriifolia* Baker —*E. Ucán Ek 538*; FJ666925. *Tillandsia deppeana* Steud. —*Chew 51*; FJ666926. *Tillandsia diguetti* Mez & Rol.-Goss. ex Mez —*A. R. López-Ferrari et al. 2972* (UAMIZ); FJ666923, FJ666942. *Tillandsia eizii* L. B. Sm. —*Ceja et al. 1374* (MEXU); EU126830, FJ666947. *Tillandsia erubescens* Schltdl. —*A. Espejo 6548* (UAMIZ); EU126831, FJ666953. *Tillandsia filifolia* Schltdl. & Cham. —*Chew 95*; FJ666924, FJ666944. *Tillandsia flabellata* Baker —*A. Espejo et al. 6419* (UAMIZ); FJ666928, FJ666949. *Tillandsia heterophylla* E. Morren —*Chew 52*; FJ666927, FJ666946. *Tillandsia ionantha* Planch. —*Chew 38*; FJ666931, FJ666951. *Tillandsia intermedia* Mez —*Chew 189;* FJ666935, FJ666956. *Tillandsia juncea* (Ruiz & Pav.) Poir. —*Chew 57*; EU126832, EU126836. *Tillandsia magnusiana* Wittm. —*Chew 130*; FJ666941, FJ666963. *Tillandsia makoyana* Baker —*Chew 135*; FJ666936, FJ666957. *Tillandsia multicaulis* Steud. —*Chew 47*; EU126829, FJ666945. *Tillandsia palmasolana* Matuda —*Chew 182*; DQ870636, DQ870649. *Tillandsia paucifolia* Baker —*Chew 183*; DQ870637, DQ870648. *Tillandsia pruinosa* Sw. —*Chew 123*; EU126838, FJ666948. *Tillandsia pseudobaileyi* C. S. Gardner —*Chew 120*; DQ870638, DQ870651. *Tillandsia punctulata* Schltdl. & Cham. —*Chew 49*; FJ666930, FJ666950. *Tillandsia schiedeana* Steud. —*Chew 40*; DQ870635, DQ870647. *Tillandsia seleriana* Mez —*Chew 121*; FJ666929. *Tillandsia streptophylla* Scheidw. ex E. Morren —*Chew 143*; DQ870639, DQ870650. *Tillandsia usneoides* (L.) L. —*Chew 50*; FJ666938. *Tillandsia utriculata* L. —*Chew 143*; FJ666940.

Viridantha atroviridipetala (Matuda) Espejo —*Chew 89*; FJ666932, FJ666952.