



## Molecular, morphological, and biogeographic perspectives on the classification of Acrobolboideae (Acrobolbaceae, Marchantiophyta)

LAURA R. E. BRISCOE<sup>1,2,3</sup>, NYREE J. C. ZEREGA<sup>2,3</sup>, H. THORSTEN LUMBSCH<sup>1</sup>, MICHAEL STECH<sup>4,5</sup>, EKAPHAN KRAICHAK<sup>6</sup>, MATTHEW J. VON KONRAT<sup>1</sup>, JOHN J. ENGEL<sup>1</sup> & NORMAN J. WICKETT<sup>2,3</sup>

<sup>1</sup> Science & Education, The Field Museum, 1400 S. Lake Shore Drive, Chicago, IL 60605, USA

<sup>2</sup> Chicago Botanic Garden, 1000 Lake Cook Road, Glencoe, Illinois 60022, USA

<sup>3</sup> Northwestern University, Plant Biology and Conservation, 2205 Tech Drive, Evanston, Illinois 60208, USA

<sup>4</sup> Naturalis Biodiversity Center, PO Box 9517, 2300 RA Leiden, The Netherlands

<sup>5</sup> Leiden University, Leiden, The Netherlands

<sup>6</sup> Department of Botany, Faculty of Science, Kasetsart University, Bangkok, Thailand

Corresponding author's email: [lbriscoe@nybg.org](mailto:lbriscoe@nybg.org)

### Abstract

The liverwort subfamily Acrobolboideae has historically contained the three genera: *Acrobolbus*, *Marsupidium*, and *Tylimanthus*. Generic delimitations in this subfamily have been historically inferred from morphological characters, specifically the location of gametangia. Taxonomists have had difficulty separating the genera, with some combining *Tylimanthus* and *Acrobolbus*, whereas others merged *Marsupidium* and *Tylimanthus*. We used five chloroplast loci to reconstruct a phylogeny of the group, revealing all three genera are polyphyletic as currently described. An assessment of key morphological characters used to separate genera in the subfamily resulted in several observations: characters used to circumscribe *Acrobolbus* were homoplasious; characters used to circumscribe each genus (e.g., the placement of female reproductive organs) do not reflect phylogenetic relationships; and the evolutionary trajectories of some characters (i.e., the number of antheridia, male reproductive organs, per male bract) correspond directly with previous evolutionary hypotheses for the family, but do not follow historical taxonomic inferences. Irrespective of generic concepts, several well-supported clades within the phylogeny have a strong biogeographic structure. Using these lines of evidence, we recognize *Acrobolbus* as a single genus in Acrobolboideae.

**Keywords:** ancestral areas, *Acrobolbus*, biogeography, liverworts, molecular phylogeny, *Marsupidium*, morphology, systematics, taxonomy, *Tylimanthus*

### Introduction

The liverwort family Acrobolbaceae occurs throughout the Southern Hemisphere and has been broadly construed to include four subfamilies and eight genera: i) Acrobolboideae R.M. Schuster ex Briscoe included *Acrobolbus* Nees in Gottsche *et al.* (1844: 5), *Marsupidium* Mitten in Hooker (1867: 751) and *Tylimanthus* Mitten in Hooker (1867: 753); ii) the monogeneric Austrolophozioideae Schuster with *Austrolophozia* R.M. Schuster (1963: 282); iii) Lethocoleoideae (S. Arnell) Grolle, including *Lethocolea* Mitten in Hooker (1867: 751), *Goebelobryum* Grolle (1962: 135), and *Enigmella* G.A.M. Scott & K.G. Beckmann in Beckmann & Scott (1992: 297); and iv) the monogeneric Saccogynidioideae Crandle-Stotler, Vána & Stotler, with *Saccogynidium* Grolle (1960: 43). The family bears superficial resemblance to several other liverwort genera in the Jungermanniineae, specifically those that develop a stem-derived marsupium around the female reproductive organs and developing sporophyte (Shaw *et al.*, 2015), and can be difficult to distinguish from other liverwort families in the absence of fertile material. The lack of distinct, invariable morphological characters to circumscribe Acrobolbaceae, and specifically genera within Acrobolboideae, led to its description as “one of the most intriguing, indeed baffling, of families of leafy Hepaticae” (Schuster, 2001, p. 97), suggesting that robust molecular, morphological, and biogeographical evidence are needed to resolve the evolutionary relationships of species within Acrobolboideae.

The three genera of Acrobolboideae share a suite of characters: 1) lack of a perianth, whose function is replaced by a pendent marsupium of stem tissue, 2) a sporophyte with a conspicuously pointed or beaked capsule, 3) distinctly

granular and brown oil bodies, 4) leaf surfaces usually armed with papillae, and 5) leaves often distinctly yellow-green or even slightly glaucous (Engel & Grolle, 1971; Schuster, 2001). Despite these characters that unite the genera, different taxonomists historically placed each of these genera in separate liverwort families, often on the basis of a geographically biased understanding of global liverwort diversity (Table 1).

**TABLE 1.** Historic placement of Acrobolboideae genera within families, as recognized by different bryologists and publications through time.

Reference	<i>Acrobolbus</i>	<i>Marsupidium</i>	<i>Tylimanthus</i>
Hodgson, 1946	Jungermanniaceae		
Müller, 1951–58	Lophoziaceae	Odontoschismaceae	Plagiochilaceae
Hodgson, 1958		Cephaloziaceae	Plagiochilaceae
Hodgson, 1962	Acrobolbaceae	Acrobolbaceae	Acrobolbaceae
Schuster, 1963		Marsupidiaceae (invalid)	

The circumscription of genera in Acrobolboideae originally relied on growth habit and reproductive morphology. *Acrobolbus* was separated from *Marsupidium* and *Tylimanthus* by its creeping, prostrate growth and lack of a stolon system differentiated from leafy shoots, and by having androecia which contain only a single antheridium per male bract (Engel & Grolle, 1971; Schuster, 2001; Engel & Glenny, 2008a). *Marsupidium* and *Tylimanthus* were distinguished by the placement and morphology of reproductive branches (gametangia): *Tylimanthus* having terminal gametangia on normal leafy shoots, whereas the gametangia of *Marsupidium* develop on reduced lateral branches near the base of shoots and have reduced, echlorophyllose bracts (Engel & Grolle, 1971; Schuster, 2001; Engel & Glenny, 2008b). Some of these characters are variable. For example, the type specimen of *Marsupidium flavicans* J.J. Engel & Grolle (= *T. flavicans* (J.J.Engel & Grolle 1971: 438) Hässel & Solari 1972: 579) exhibited gynoeceia on both basal and terminal leafy shoots (Engel & Grolle, 1971). Because of this plasticity, Hässel & Solari (1972) expressed doubt in the taxonomic use of this character and synonymized all *Marsupidium* species under *Tylimanthus*. Similarly, Hodgson (1962) and Schuster (2001) questioned the separation of *Tylimanthus* and *Acrobolbus*.

The difficulty of circumscribing genera within the subfamily led Schuster to suggest that “the three genera of Acrobolboideae could reasonably be regarded as mere subgenera of a single genus; further study may dictate that this course must be adopted” (Schuster 2001, p. 98). This was supported by the first phylogenetic study on Acrobolbaceae (Stech *et al.*, 2006) inferred from the *trnL* intron and *trnL*-F intergenic spacer. In that study, all three genera were resolved as polyphyletic, but gene and taxon sampling was limited, specifically lacking the type species of each genus.

In addition to the challenges of generic circumscriptions within Acrobolboideae, there are also unresolved relationships at the species level. As in other groups of organisms, molecular studies of liverworts have revealed many cryptic species (Shaw, 2001; Heinrichs *et al.*, 2009). For example, a new species of *Tylimanthus*, *T. andinopatagonicus* M. Stech & W. Frey in Stech *et al.* (2006: 28) was separated from the purportedly morphologically identical *T. urvilleanus* (Montagne 1843: 247) Trevisan (1877: 423) based on molecular and ecological data (Stech *et al.*, 2006). Conversely in some taxa, such as the *Tylimanthus laxus* (Lehmann & Lindenberg 1840: 68) Spruce (1855: 502) complex (Burghardt & Gradstein, 2008), species delimited on the basis of overlapping morphological variation were interpreted as representing a single variable species; a hypothesis which has only partly been tested with molecular data (Stech *et al.*, 2006).

While the morphological characters are extremely variable within the subfamily, species show strong geographical patterns with almost all species restricted to the Southern Hemisphere. Exceptions include the type species of *Acrobolbus*, *A. wilsonii* Nees in Gottsche *et al.* (1884: 5), which occurs in the U.K., *A. ciliatus* (Mitten 1861: 100) Schiffner (1893: 86), with a disjunct distribution in North America and Asia, *Marsupidium knightii* Mitten in Hooker (1867: 753), recorded throughout the South Pacific extending to Japan, and members of the *Tylimanthus laxus* complex in the tropics, extending north to the Macaronesian islands. Biogeographical patterns in Acrobolboideae are not well understood, but the subfamily has a center of diversity in New Zealand, which was interpreted as supporting a Gondwanan origin (Schuster, 1980). Alternatively, Stech *et al.* (2006) suggested southern South America as the center of origin for Acrobolboideae. So far, this strong biogeographical pattern has not been used in previous systematic classifications and can potentially shed some light on generic delimitation of this difficult subfamily.

The goals for this study were to increase the molecular sampling to include more loci and more taxa (including the generic types for each of the three genera) for phylogenetic reconstruction and subsequent analyses of character evolution and biogeographical patterns. Specifically we addressed these questions: 1) are the currently recognized

genera in Acrobolboideae monophyletic?, 2) is the distribution of phenotypical character states correlated with clades inferred from the phylogenetic analysis?, and 3) do geographic patterns reflect phylogenetic structure, and do they suggest an ancestral area of the subfamily?

## Materials and Methods

**Specimen and taxon sampling:**—Material from the herbaria of B, DUKE, H, E, EGR, F, JE, L, LISU, NSW, STU was used for this study. A total of 82 exemplars were sampled representing 30 species (including the generic type of each genus), with a minimum of six species for each genus to encompass broad taxonomic, morphological and geographic diversity. Voucher information, including GenBank accession numbers, is included in Supplementary Table 1.

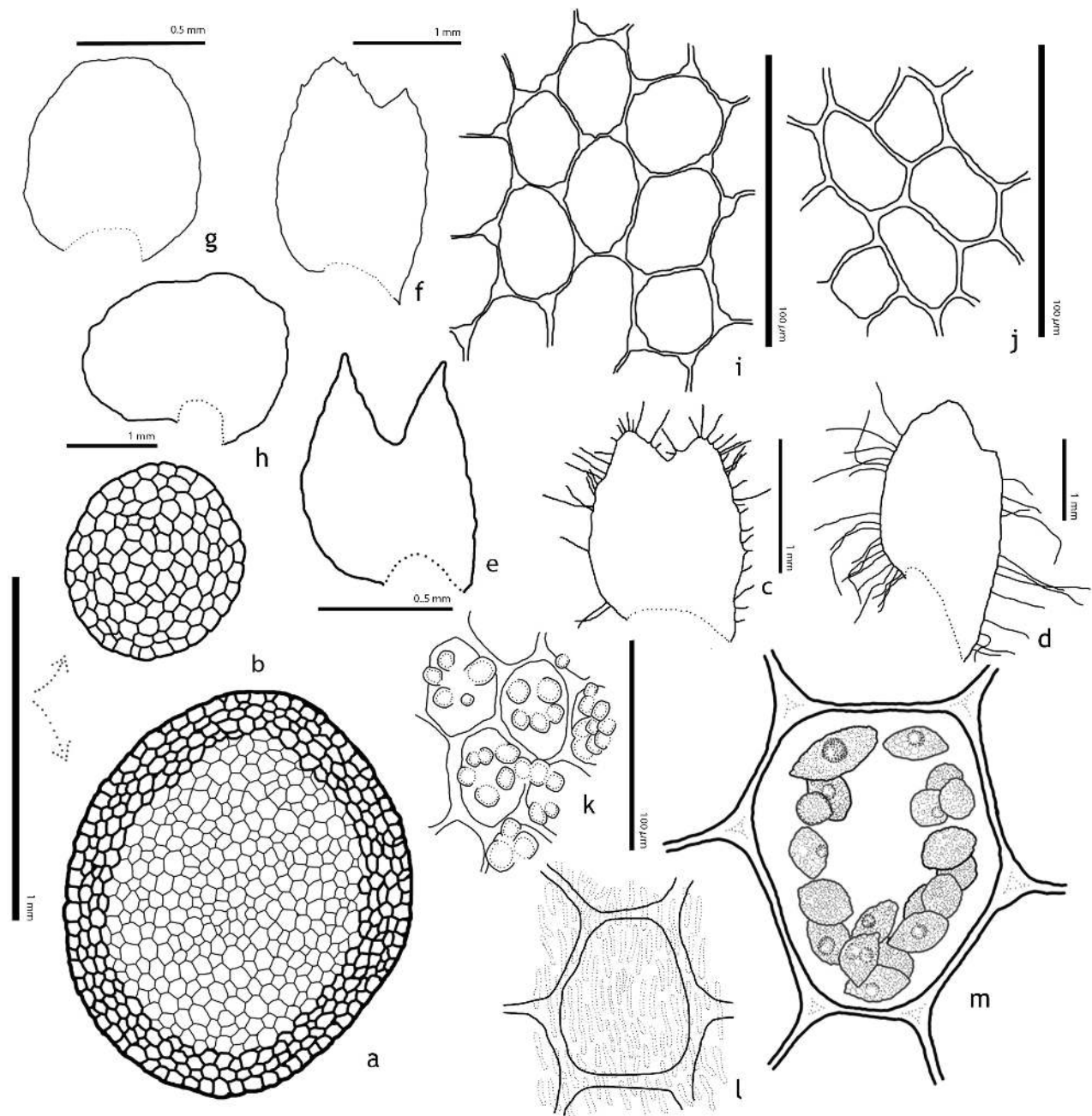
**DNA extraction, amplification and sequencing:**—DNA of 55 newly analysed specimens was extracted using both a modified CTAB method (Doyle & Doyle 1987), DNeasy plant mini kits (Qiagen Corporation) with a modification of an overnight incubation with proteinaseK and 2-mercaptoethanol, and the Invisorb Spin Plant Mini Kit (Stratec GmbH). In addition, DNA extracts from Stech *et al.* (2006) of 27 further specimens were used.

Five plastid genome regions were amplified: *rbcL* gene, *atpB-rbcL* intergenic spacer, *psbA-trnH* intergenic spacer, the *psbT-H* region (*psbT-psbN-psbH*; Stech & Quandt, 2010), and the *trnL-F* region. Each PCR reaction contained 12 µl deionized (DI) water, 3 µl dNTPs, 1 µl MgCl<sub>2</sub> (50mM), 1 µl of each primer, 1 µl BSA (20mg/mL), 0.5 µl Taq polymerase, and 1.5 µl template DNA. The PCR conditions for *rbcL* were: initial step at 94°C for 5 minutes followed by 30 cycles of 94°C for 1 minute, 52°C for 50 seconds, and 72°C for 90 seconds with a final extension period of 10 minutes at 72°C; for *atpB-rbcL*: 94°C for 4 minutes, followed by 30 cycles of 94°C for 90 seconds, 52°C for 30 seconds, and 72°C for 90 seconds with a final extension period of 10 minutes at 72°C; for *psbA-trnH*: 94°C for 2 minutes, then 30 cycles of 94°C for 1 minute, 57°C for 50 seconds, 72°C for 90 seconds followed by a final extension of 5 minutes at 72°C; for *psbT-H*: 95°C for five minutes, 35 cycles of 95°C for 1 minute, 52°C for 1 minute, 72°C for 90 seconds, with a final extension for 10 minutes at 72°C; for *trnL-F*: 94°C for 4 minutes, 32 cycles of 94°C for 45 seconds, 52°C for 30 seconds 72°C for 90 seconds, followed by a final extension of 7 minutes at 72°C. Previously published primers were used (Borsch & Quandt, 2009; Chiang *et al.*, 1998; Gradstein *et al.*, 2006; Stech *et al.*, 2003; Vanderpoorten & Long, 2006). PCR products were cleaned by EtOH precipitation and suspended in 20 µl of DI water. Samples were cycle sequenced using BigDye Terminator 3.1 (Applied Biosystems) and were sequenced on an ABI 3730 DNA Analyzer (Foster City, California, U.S.A.). Sequences were assembled and edited using Codon Code Aligner (CodonCode Corporation), and submitted to GenBank (Supplementary Table 1).

**Phylogenetic analyses:**—Sequences were aligned manually using MacClade v.4.07 (Maddison & Maddison 2005) for individual loci. Outgroups were chosen from Balantiopsaceae, *Saccogynidium* (Acrobolbaceae, subfamily Saccogynidioideae), and *Lethocolea* (Acrobolbaceae, subfamily Lethocoleoideae) based on results from the Liverwort Tree of Life Project (Shaw *et al.* 2015). Outgroup sequences were obtained from GenBank. Maximum likelihood (ML) analyses were conducted with RAxML v.7.3.2 (Stamatakis, 2006). Analyses used the GTR+Γ model as inferred by jModelTest (Guindon & Gascuel, 2003) as the best-fit model, with 1000 rapid bootstrap replicates. ML bootstrap analyses were performed on the individual data sets, and consensus trees were examined for conflict, i.e., incongruences with at least 75% bootstrap support (Lutzoni *et al.* 2004). The five loci were concatenated into a super matrix including all vouchers. To assess the effect of missing data on clade support and topology, a second supermatrix was used for ML rapid bootstrapping that was limited to vouchers with sequences from at least four loci. The latter supermatrix, consisting of 46 vouchers representing 22 species, was used for all subsequent analyses. Phylogenetic reconstructions using maximum parsimony (MP) were performed using PAUP\* (Swofford, 2006). Parameters were set to conduct tree-bisection-reconstruction (TBR) branch swapping, and a heuristic search of 100 replicates, with starting trees generated by stepwise taxon addition with random addition sequences. Up to 100 trees were retained per replicate. An output of 10,000 trees was converted into a consensus tree using SumTrees (Sukumaran & Holder, 2010). Bootstrap support for clades under MP was obtained with 1000 replicates. The Bayesian (B/MCMC) analysis was conducted using MrBAYES 3.1.2 (Huelsenbeck & Ronquist 2001), with the same substitution model as in the ML analysis. Two parallel runs with 20,000,000 generations each, starting with a random tree and employing four simultaneous chains, were executed. No molecular clock was assumed. Heating of chains was set to 0.2. Posterior probabilities were approximated by sampling trees using a variant of Markov Chain Monte Carlo (MCMC) method. To avoid autocorrelation, trees were sampled every 1,000<sup>th</sup> generation. The first 5,000 trees were discarded as burn in. We used AWTY (Nylander *et al.* 2007) to compare splits frequencies in the different runs and to plot cumulative split frequencies to ensure that stationarity was

reached. A majority-rule consensus tree with average branch lengths was calculated from the sampled trees using the sumt option of MrBAYES.

A second round of ML rapid bootstrapping was performed by partitioning the supermatrix. In this analysis, the GTR+ $\Gamma$  model was applied to each partitioned gene separately, to accommodate variance of evolution between various gene regions.



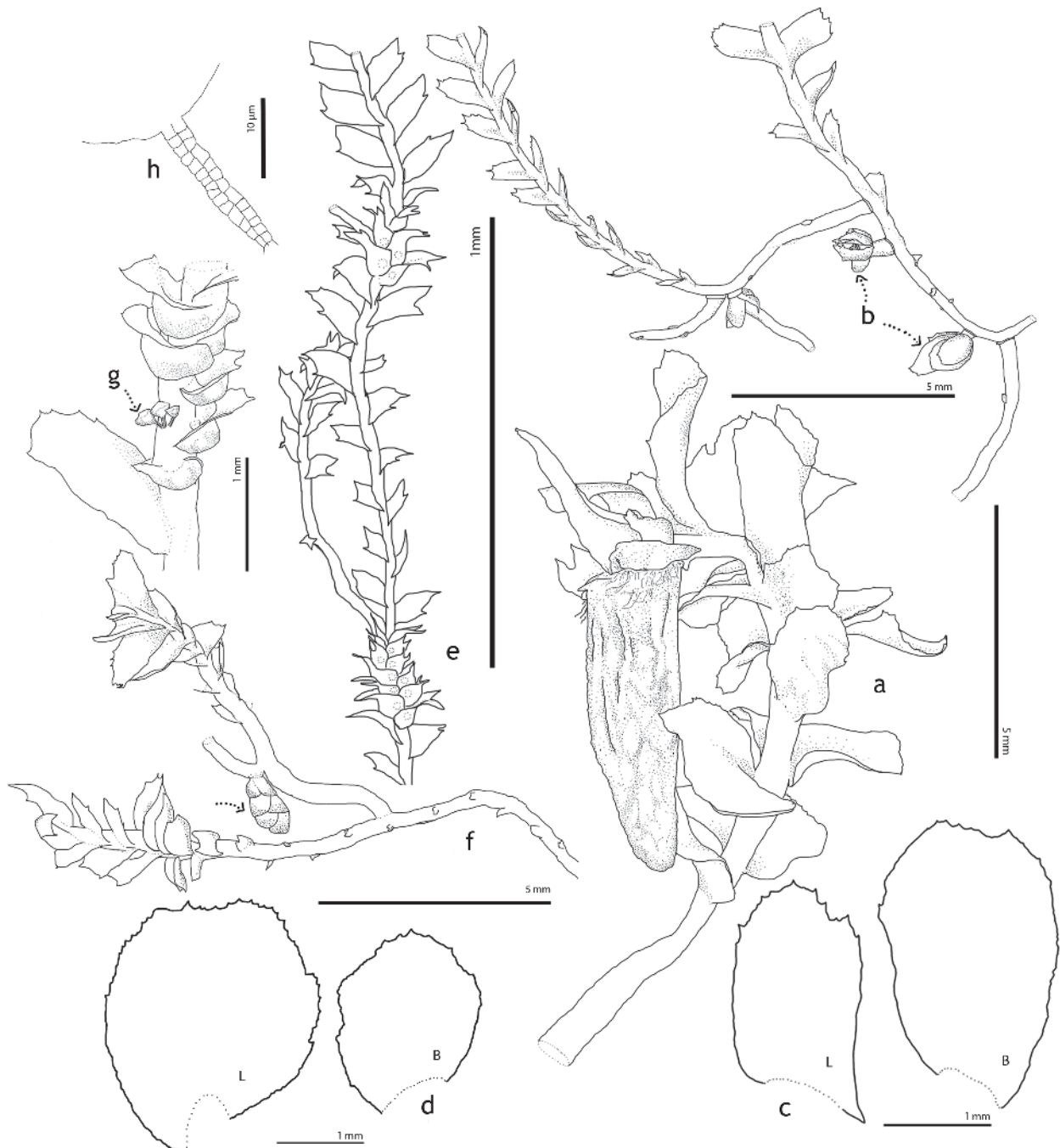
**FIGURE 1.** Sterile gametophyte characters. **a.** differentiated cortex, large stem drawn from *Tylimanthus saccatus* Engel 28427 (LB185); **b.** undifferentiated cortex, small stem drawn from *Marsupidium* sp. Renner s.n. (LB203); **c–d.** leaf rhizoids: **c.** drawn from *A. ciliatus* Long 34895 (LB198); **d.** drawn from *T. laxus* Holz CR00-197 (LB162); **e–h.** leaf shapes: **e.** symmetrically bifid leaf drawn from *A. wilsonii* Long 39345 (LB144); **f.** asymmetrically bifid leaf drawn from *T. flavicans* Frey & Schaumann 01-372c; **g.** orbicular leaf drawn from *Acrobolbus concinnus* Renner 5251 (LB197); **h.** reniform leaf drawn from *M. surculosum* Engel 21271 (LB183); **i.** cell walls with trigones drawn from *T. madeirensis* Stech 04-509 (LB169); **j.** cell walls lacking trigones drawn from *T. saccatus* Engel 18427 (LB185); **k–l.** leaf surface papillae: **k.** welt-papillae drawn from *A. ochrophyllus* Engel 26633 (LB191); **l.** striate papillae drawn from *T. urvilleanus* Briscoe 1100; **m.** oil bodies drawn from *T. urvilleanus* Briscoe 1191.

**TABLE 2.** Matrix of characters scored directly from genetic vouchers and supplemented by additional specimens and literature reports. Characters: 1 stem cortex, 2 stem size, 3 leaf rhizoids, 4 stolon system, 5 growth habit, 6 leaf shape, 7 trigones, 8 surface papillae, 9 oil bodies, 10 gynoeical placement, 11 chlorophyll in gametangia, 12 female bract size, 13 male bract size, 14 number of antheridia.

Voucher	1	2	3	4	5	6	7	8	9	10	11	12	13	14
105 <i>Marsupidium</i> sp.	0	0	0	1	1	0	0	1	?	01	1	?	0	?
106 <i>Tylimanthus flavicans</i>	1	0	0	1	1	1	0	2	0	01	1	01	0	1
107 <i>Marsupidium renifolium</i>	1	1	0	1	1	0	0	0	?	01	1	1	0	?
108 <i>Marsupidium renifolium</i>	1	1	0	1	1	0	0	0	?	01	1	1	0	?
110 <i>Tylimanthus urvilleanus</i>	1	1	0	1	1	1	1	2	1	0	0	0	0	?
114 <i>Tylimanthus urvilleanus</i>	1	1	0	1	1	1	0	2	1	0	0	0	0	?
118 <i>Tylimanthus urvilleanus</i>	1	1	0	1	1	1	1	2	1	0	0	0	0	?
138 <i>Tylimanthus saccatus</i>	0	1	0	1	1	0	0	0	1	1	1	1	1	2
140 <i>Tylimanthus madeirensis</i>	1	0	1	1	1	1	1	1	?	1	1	1	1	?
141 <i>Tylimanthus laxus</i>	1	1	1	1	1	1	1	2	?	1	1	1	1	1
144 <i>Acrobolbus wilsonii</i>	0	0	0	0	1	2	1	0	0	1	1	1	0	0
145 <i>Tylimanthus pseudosaccatus</i>	0	1	0	1	1	0	1	2	?	1	1	1	1	2
151 <i>Tylimanthus madeirensis</i>	1	0	1	1	1	1	1	2	1	1	1	1	1	?
155 <i>Tylimanthus madeirensis</i>	1	0	1	1	1	1	0	1	1	1	1	1	1	?
156 <i>Tylimanthus azoricus</i>	1	0	1	1	1	1	0	1	1	1	1	1	1	?
158 <i>Marsupidium renifolium</i>	1	1	0	1	1	0	1	0	1	01	1	1	0	?
159 <i>Tylimanthus andinopatigonicus</i>	1	1	0	1	1	1	1	0	1	0	0	0	0	?
160 <i>Tylimanthus kunkelii</i>	1	1	0	1	1	1	0	1	?	1	1	1	1	1
162 <i>Tylimanthus laxus</i>	1	1	1	1	1	1	1	1	1	1	1	1	1	2
166 <i>Tylimanthus andinopatigonicus</i>	1	1	0	1	1	0	1	2	1	0	0	0	0	?
169 <i>Tylimanthus madeirensis</i>	1	1	1	1	1	1	1	1	1	1	1	1	1	?
171 <i>Tylimanthus andinopatigonicus</i>	1	1	0	1	1	1	1	0	1	1	0	0	0	?
172 <i>Tylimanthus ruwenzorensis</i>	1	0	1	1	1	1	1	2	1	1	1	1	1	?
173 <i>Tylimanthus ruwenzorensis</i>	1	0	1	1	1	1	1	2	1	1	1	1	1	?
174 <i>Tylimanthus ruwenzorensis</i>	1	0	1	1	1	1	1	2	1	1	1	1	1	?
177 <i>Tylimanthus ruwenzorensis</i>	1	0	1	1	1	1	1	2	1	1	1	1	1	?
178 <i>Tylimanthus saccatus</i>	1	1	0	1	1	01	0	0	1	1	1	1	1	2
179 <i>Marsupidium renifolium</i>	0	1	0	1	1	0	0	0	1	01	0	1	0	?
180 <i>Tylimanthus urvilleanus</i>	1	1	0	1	1	01	1	2	1	0	0	0	0	?
181 <i>Tylimanthus viridis</i>	1	0	0	1	1	1	1	2	?	?	?	?	?	?
183 <i>Marsupidium surculosum</i>	1	1	0	1	1	0	1	0	0	?	?	?	?	?
184 <i>Marsupidium surculosum</i>	1	1	0	1	1	0	1	0	0	?	?	?	?	?
185 <i>Tylimanthus saccatus</i>	1	1	0	1	1	1	0	0	1	1	1	1	1	2
186 <i>Tylimanthus saccatus</i>	1	1	0	1	1	1	0	0	1	1	1	1	1	2
188 <i>Marsupidium epiphytum</i>	1	1	0	1	1	1	1	2	1	0	0	0	0	1
189 <i>Marsupidium</i> sp.	1	1	0	1	1	1	1	0	?	1	1	1	1	1
191 <i>Acrobolbus ochrophyllus</i>	1	0	0	0	0	2	1	3	1	1	1	1	0	?
192 <i>Acrobolbus spinifolius</i>	1	0	0	0	0	1	1	1	1	?	1	?	0	0
197 <i>Acrobolbus concinnus</i>	0	0	1	1	0	0	1	0	0	1	1	1	0	0
198 <i>Acrobolbus ciliatus</i>	0	0	1	0	0	2	1	2	0	1	1	1	1	0
201 <i>Marsupidium knightii</i>	0	0	0	1	1	1	1	0	1	0	0	0	0	1
202 <i>Marsupidium knightii</i>	0	0	0	1	1	1	1	2	1	0	0	0	0	1
203 <i>Marsupidium</i> sp.	0	0	0	1	0	1	1	2	1	?	?	?	?	?
205 <i>Acrobolbus ochrophyllus</i>	1	0	1	1	1	2	0	3	1	1	1	1	0	?
206 <i>Acrobolbus ochrophyllus</i>	0	0	0	1	1	2	0	3	1	1	1	1	0	?
207 <i>Marsupidium</i> sp.	0	0	0	1	1	1	1	1	1	?	?	?	?	?

**Morphological assessment:**—Morphological characters from 39 molecular vouchers (representing 22 species) were scored in a binary (0/1) or multistate (0/1/2/3) matrix (Table 2), supplemented by additional specimens and literature reports to accommodate for plasticity within species. The following fourteen characters and states were recorded: **1. Stem anatomy:** cortex of differentiated cells (smaller and with thicker walls) present = 1 (Fig. 1a); cortex absent = 0 (Fig. 1b). **2. Stem cell diameter:** stems in cross section 15+ cells in diameter = 1; stems in cross section <15 cells in diameter = 0. **3. Leaf rhizoids:** leaves with marginal rhizoids (Fig. 1c–d) = 1; rhizoids absent = 0. **4. Stolon system:** system of stoloniform branches giving rise to leafy shoots present = 1; stolon system absent = 0. **5. Growth habit:** plants growing erect = 1; plants creeping = 0. **6. Leaf shape** was scored as a multistate character with three states: leaves symmetrically bifid (Fig. 1e) = 2; leaves asymmetrically bifid (Fig. 1f) = 1; leaves orbicular to reniform (Fig. 1g–h) = 0. **7. Trigones:** Presence of intracellular trigones (Fig. 1i) = 1; trigones absent (Fig. 1j) = 0. **8.**

**Leaf surface** was scored as a multistate character with four states: papillae large, welt-like projections (Fig. 1k) = 3; papillae long-striate (Fig. 1l) = 2; papillae minute, round = 1; papillae absent = 0. **9. Oil-body count:** Intracellular oil-bodies numbering (occasionally 5) 9 to >15 (Fig. 1m) = 1; oil-bodies numbering < 5 = 0. **10. Gynoecial placement:** Gynoecia developing on terminal leafy shoots (Fig. 2a) = 1; gynoecia developing on short basal branches (Fig. 2b) = 0. **11. Chlorophyll in gametangia:** gametangia chlorophyllose = 1; gametangia echlorophyllose = 0. **12. Female bract size:** bracts of similar size or much larger than leaves (Fig. 2c) = 1; bracts reduced, much smaller than leaves (Fig. 2d) = 0. **13. Male bract size:** bracts of similar size or larger than leaves (Fig. 2e) = 1; bracts reduced, much smaller than leaves (Fig. 2f) = 0. **14. Antheridia** number was scored as a multistate character with three states: Antheridia 5+ (Fig. 2g) = 2; antheridia 2–3 = 1; antheridia solitary = 0.



**FIGURE 2.** Reproductive characters. **a.** Marsupium terminal on leafy shoot drawn from *T. saccatus* Shaw 6318 (LB178); **b.** basal gynoecia on abbreviated branches (at arrow) drawn from *M. epiphytum* Engelm 21928; **c.** leaf (=L) with large bract (=B) drawn from *T. sylvaticus* Shaw 4109; **d.** leaf (=L) with reduced bract (=B) drawn from *T. turvilleanus* von Konrat 6505. **e.** intercalary androecia with large bracts drawn from *Acrobolbus spinifolius* Engelm 28447 (LB192); **f.** basal androecia with small, spicate bracts (at arrow) drawn from *Marsupidium knightii* Engelm 26663; **g.** dissected male bract showing placement of three antheridia (at arrow) drawn from *Tylimanthus laxus* Schäfer-Verwimp 22378; **h.** typical long, biserial antheridial stalk drawn from *T. urvilleanus* Engelm 26269.

Images were captured on an Olympus SZX-12 dissecting microscope and Olympus BH-2 compound microscope using a ProgRes camera and software.

**Morphological character evolution:**—In order to trace the evolution of the morphological characters, we mapped the binary character states (present or absent) onto the resulting ML phylogram and performed maximum parsimony reconstruction of ancestral states, using the MPR function within the R-package ape (Paradis *et al.* 2004).

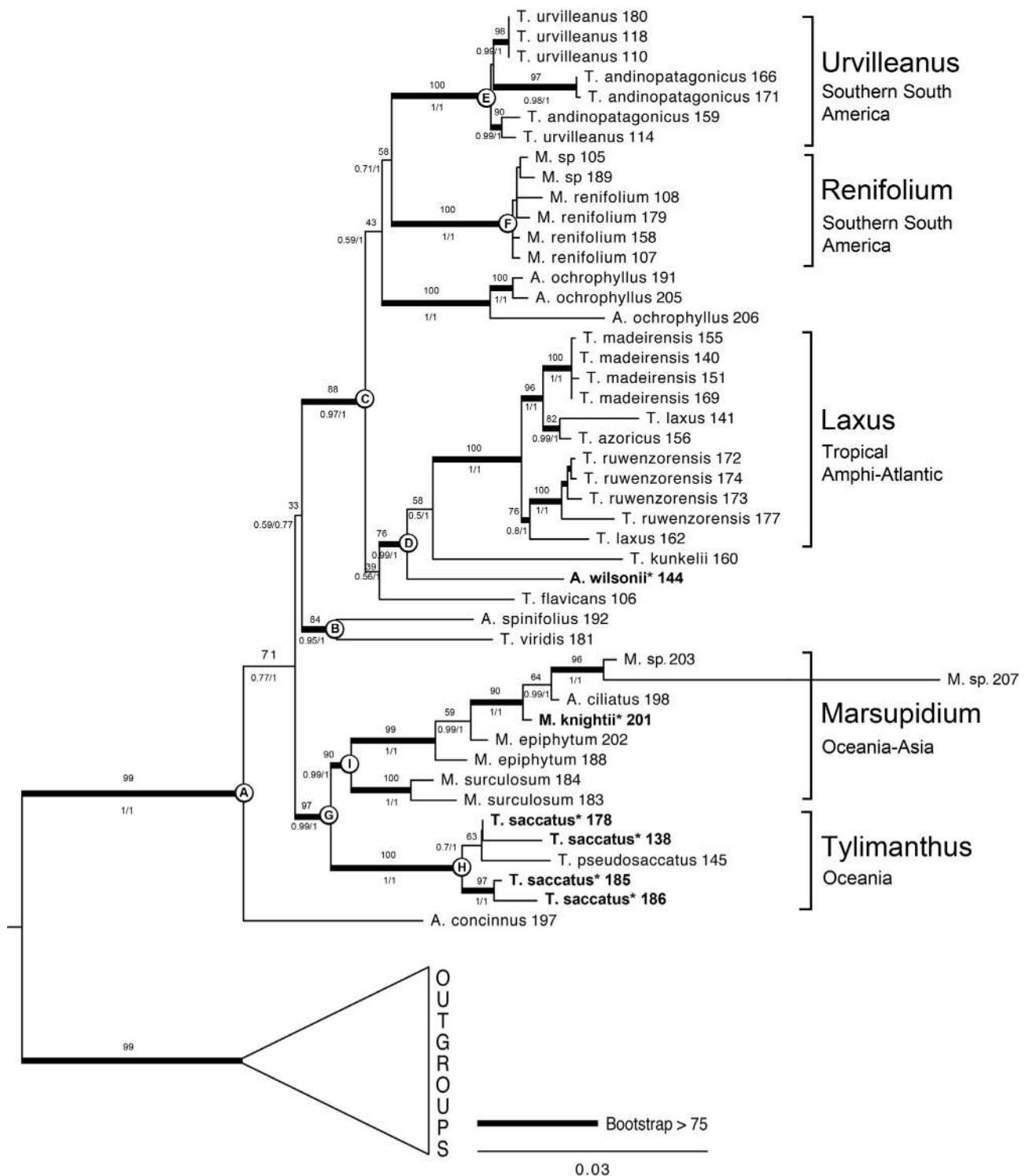
**Ancestral area reconstructions:**—The likelihood-based approach of ancestral area reconstruction (AAR) developed by Ree and colleagues (Ree *et al.* 2005, Ree & Smith 2008) was used to estimate the ancestral area of Acrobolboideae, to explore the ancestral ranges of the major clades within the group, and to test the hypothesis that the family originated in the paleoaustral region and subsequently spread north into South America and Africa (Stech *et al.* 2006). Analyses were conducted on an ultrametric tree estimated using the lognormal relaxed clock model (Drummond *et al.* 2006) implemented in an MCMC framework in the program BEAST v1.8.0 (Drummond & Rambaut 2007), using the ML tree as the start tree and a GTR+I+ $\Gamma$  model (chosen as optimal model) of nucleotide substitution, with a total run of 10 million generations. This tree was imported into LaGrange 2.0 (<http://lagrange.googlecode.com>) using the LaGrange configuration module (<http://www.reelab.net/lagrange/configurator/index>). Presence in eight different areas (southern South America, Neotropics, Atlantic islands, Africa, Europe, Oceania, Asia, and North America) was coded for all species with no restrictions on the number of allowed areas in which ancestral species may have been present (Sanmartin & Ronquist 2004).

## Results

**Phylogenetic analysis:**—The aligned 5-locus matrix contained 2639 unambiguously aligned nucleotide position characters, 597 in *rbcL*, 605 in *trnL-F*, 561 in *psbT-H*, 287 in *psbA-trnH* and 759 in *atpB-rbcL*, with a total of 827 variable characters, of which 504 were parsimony-informative sites. Single-locus analyses resulted in no topological conflict between loci, so a concatenated matrix was used for all inferences. There was also no topological conflict between partitioned and unpartitioned analyses of the concatenated data. The most-likely phylogram obtained from the ML analysis is shown in Fig. 3 with nodes in bold indicating strong support in Bayesian analyses (i.e., PP  $\geq$  0.95); nodes with MP and ML bootstrap values equal or above 75% are annotated directly on the tree. Our analyses recovered Acrobolboideae as a strongly supported monophyletic group. All genera were polyphyletic. However several highly supported clades were found, including the Urvilleanus Group with *Tylimanthus urvilleanus* and *T. andinopatagonicus*, the Renifolium Group including *Marsupidium renifolium* and an undescribed species, the Laxus Group, consisting of a complex of morphologically similar tropical species, the Marsupidium Group, containing the generic type, *Marsupidium knightii*, and the Tylimanthus Group, containing the generic type *Tylimanthus saccatus*.

**Morphological character evolution:**—The results of the maximum parsimony reconstruction of ancestral states inferred two main patterns of homoplasy: an ancestral state that was lost multiple times throughout the tree (differentiation of stem cortex, development of a stolon system, presence of trigones, loss of leaf surface papillae, change in number of oil bodies, loss of chlorophyll in gametangia, reduction of female bracts), or a character that underwent multiple switches from the ancestral state (marginal leaf rhizoids, erect growth habit, reduction of gynoecia, reduction of male bracts, number of antheridia per bract). We show the results of two characters, the presence of the stem cortex and gynoecial position that illustrate these patterns. The presence of a differentiated stem cortex and terminal gynoecia were both inferred as ancestral for the subfamily (Figs. 4 and 5). The loss of a differentiated stem cortex occurred multiple times, even within clades. Similarly, there were at least two switches from terminal gynoecia to basal gynoecia at the *T. urvilleanus/andinopatagonicus* clade and *M. epiphytum/M. sp.* (Fiji).

**Ancestral area reconstructions:**—Results of the ancestral range reconstructions are summarized in Table 3, with nodes marked on the tree in Fig. 3. The ancestral range reconstructions for six of the nodes revealed only a single most likely ancestral range within the confidence window of two log-likelihood units (Edwards 1972). Southern South America was reconstructed as the ancestral range for nodes **B**, **F** (Renifolium Group), and **E** (Urvilleanus Group), while the ancestral range for nodes **G**, **H** (Tylimanthus Group), and **I** (Marsupidium Group) was reconstructed as being Oceania. At node **D** (Laxus Group) the analysis suggests the potential of a vicariance event of a clade evolving in Europe and the sister occurring in the Neotropics, the Atlantic islands, Africa, and Oceania. For nodes **A** and **C** different ancestral ranges are statistically plausible, indicating localized uncertainty (Table 3).



**FIGURE 3.** Maximum likelihood tree (1000 rapid bootstrap replicates) of a concatenated supermatrix consisting of taxa with at least four sequences from five targeted gene regions: *rbcl*, *trnL-F*, *psbT-H*, *psbA* and the *atpB-rbcL* spacer. Thick branches represent ML bootstrap proportions >75. ML bootstrap values are listed above branches, with Bayesian posterior probabilities and maximum parsimony bootstrap proportions listed below branches. Generic types are marked with a \*. Informal clade names and biogeographic information are listed to the right of the tree. Selected nodes (A-I) referenced for ancestral area reconstruction (see Table 3).



**TABLE 3.** Inferences about the ancestral area and range evolution parameters of the genus *Acrobolbus* at selected nodes as indicated in Fig. 3. Only inferences inside the confidence window of two log-likelihood units (Edwards 1972) listed. Relative probability (Rel. Prob.) of the global likelihood for the optimal optimization is given (in bold) and compared with the alternative(s). The first of the two distributions for each node leads to the upper daughter branch and the second to the lower daughter branch in Figure 3. Global ML at root node:  $-\ln L=92.32$  (dispersal = 0.2039, extinction <0.001). Ancestral range patterns were inferred using the following geographic areas: (A) southern South America, (B) Neotropics, (C) Atlantic islands, (D) Africa, (E) Europe, (F) Oceania, (G) Asia, and (H) North America.

Node*	Area(s) inferred	$-\ln L$	Rel. Prob.
<b>A</b>	<b>AF-F</b>	<b>92.96</b>	<b>0.5261</b>
	F-F	93.09	0.4607
<b>B</b>	<b>A-F</b>	<b>92.32</b>	<b>1.0</b>
<b>C</b>	<b>A-A</b>	<b>92.42</b>	<b>0.9025</b>
	A-AF	95.78	0.0314
	AC-A	95.9	0.0276
<b>D</b>	<b>A-DE</b>	<b>93.54</b>	<b>0.294</b>
	A-CE	93.54	0.2929
	A-D	94.05	0.177
	A-C	94.06	0.1754
	A-CD	95.61	0.03706
<b>E</b>	<b>A-A</b>	<b>92.32</b>	<b>1.0</b>
<b>F</b>	<b>E-BCDF</b>	<b>92.32</b>	<b>1.0</b>
<b>G</b>	<b>F-F</b>	<b>92.32</b>	<b>1.0</b>
<b>H</b>	<b>F-F</b>	<b>92.32</b>	<b>1.0</b>
<b>I</b>	<b>F-F</b>	<b>92.32</b>	<b>1.0</b>

\* See Figure 3 for details of the taxa included in each node.

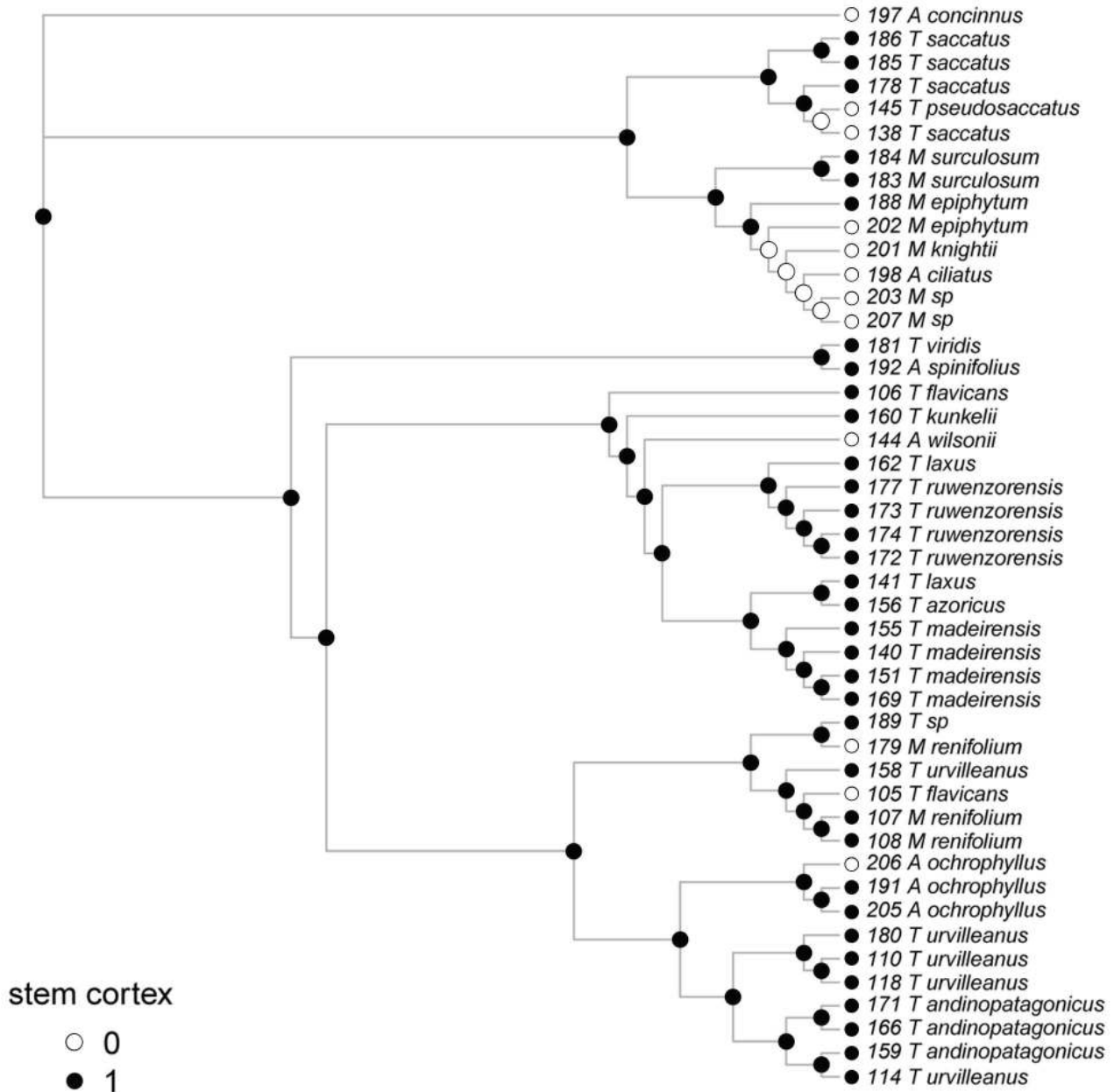
## Discussion

**Phylogenetic analysis:**—Acrobolboideae is recovered as a monophyletic group, but all three constituent genera, *Acrobolbus*, *Marsupidium*, and *Tylimanthus*, are polyphyletic. Based on the polyphyly, and the difficulty circumscribing any of the lineages within the family using morphological characters, we support the recognition of a single genus, a broadly circumscribed *Acrobobus* (Briscoe *et al.* 2015). This is consistent with previous suggestions from Schuster (2001) and Stech *et al.* (2006), who both recommended that all species within Acrobolboideae could be accommodated within one genus.

It is worth noting that the geographic biases of historic generic concepts are reflected in the strong geographic structure of the phylogeny. Hodgson's (1946, 1958, 1962) concepts were largely based on Australasian material, only recognizing *Marsupidium* as a valid genus, but observing no difference at the generic level between taxa of *Acrobolbus* and *Tylimanthus*. The phylogeny of Australasian taxa shows that in that region, those two groups are indeed represented as separate clades. In southern South America, Hässel & Solari (1972) could not determine a difference between *Tylimanthus* and *Marsupidium*, again reflected by the paraphyly of these two genera in the South American clades of the tree. This underscores the critical need for generic circumscription within a global context in this subfamily.

**Morphological analysis:**—Morphological characters historically considered taxonomically informative do not represent synapomorphies at the genus level in Acrobolboideae and are more variable than previously thought. The presence or absence of a distinct stem cortex has been used to distinguish among the three genera (e.g., Schuster, 2001; Engel & Glenny, 2008a; Gradstein, Churchill, & Salazar Allen, 2001). However, we inferred the loss of a differentiated stem cortex happened several times throughout the phylogeny, and both states can be found in species of all three genera (Fig. 4). When studying all taxa of Acrobolboideae together, presence of a differentiated stem cortex is probably a function of plant size rather than indicative of evolutionary relationships. The main character, which has been used to distinguish between *Tylimanthus* and *Marsupidium*, has been the position of gynoecia. Mitten (in Hooker, 1867) originally described the two genera, distinguished from each other by *Marsupidium* bearing gynoecia on short, basal branches and *Tylimanthus* bearing gynoecia on leading, leafy shoots. This character, mapped onto the phylogenetic tree (Fig. 5), supports the view of Schuster (2001) who interpreted gynoecia on terminal leafy shoots as a plesiomorphic trait. Exceptionally, the gynoecia of *Acrobolbus concinnus* are borne on specialized, reduced branches at the apex of a shoot (Schuster, 2001). Within Australasian species, this character can separate the Marsupidium Group from the

Tylimanthus Group. However, the position of gynoecia exhibits no geographic or phylogenetic structure. Our results support the observations of Hässel & Solari (1972), who were the first to suggest that this character might be more plastic than usually described (e.g., Engel & Grolle, 1971). This character shows a wide degree of variability, as evidenced from the type specimen of *T. flavicans* having both terminal and basal marsupia (Engel & Grolle, 1971).

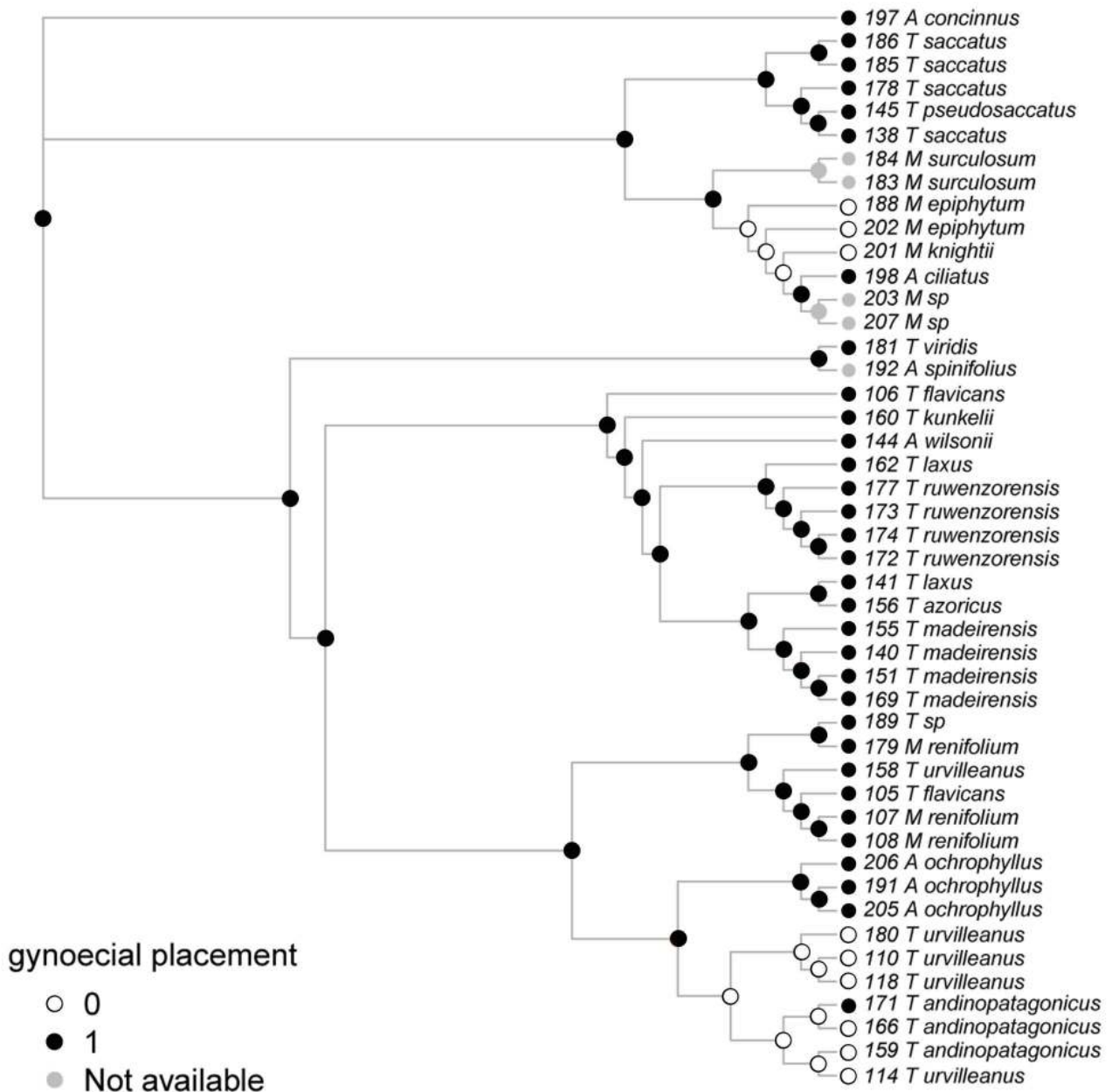


**FIGURE 4.** Maximum parsimony reconstruction of ancestral states of stem morphology mapped onto clades, (0 = cortex absent, 1 = stem cortex present).

At the species level, there is lack of support for *Tylimanthus andinopatagonicus* as a separate species from *T. urvilleanus*, which are both paraphyletic within the Urvilleanus Group clade. Conversely, several species that had previously been synonymized under *T. laxus* have been recovered with support here, including *T. madeirensis*, *T. azoricus*, and *T. ruwenzorensis* with evidence indicating they represent distinct lineages. It is worth noting the strongly supported sister relationship of a *T. laxus* voucher from Ecuador and *T. azoricus*, suggesting perhaps that *T. azoricus* could have a wider distribution and may not necessarily be a Macaronesian endemic. More work is necessary to find morphological evidence to segregate or combine these similar species.

**Historical biogeography:**—Subfamily Acrobolboideae comprises ca. 40 species, the majority with a southern hemisphere distribution. Our studies suggest that the ancestor of Acrobolboideae was present either in South America, or in Oceania, or it was widespread in both regions, since all reconstruction of ancestral nodes (A, B, C, G) suggest

presence in either of those regions. In the absence of a time-calibrated phylogeny we are unable to distinguish between vicariance and long-distance dispersal, but our results clearly indicate that the diversification of each clade happened independently in each geographical region. The reconstruction of nodes D and F as Europe indicates the likelihood of long-distance dispersal to northern temperate areas from the southern Hemisphere. Long-distance dispersal events are often interpreted as the likely explanation for liverwort diversification (Heinrichs *et al.* 2009) and wide disjunctions in distributional ranges can be attributed to long-distance dispersal (e.g. Shaw *et al.* 2003, Heinrichs *et al.* 2005, Feldberg *et al.* 2007). Our results suggest that long-distance dispersal has played a role in shaping the distributional ranges of *Acrobolbus* species but we are unable to rule out the impact of vicariance. The inclusion of North-American-Asian disjunct *Acrobolbus ciliatus* within the Marsupidium clade warrants more study of the relationships between Asian and Pacific species and species complexes. With such dynamics, the historical classifications that rely on geographically biased sampling should be revised.



**FIGURE 5.** Maximum parsimony reconstruction of ancestral states of gynoecial placement mapped onto clades, (0 = gynoecia basal, 1 = gynoecia terminal, ? = information not available).

## Conclusion

The close phylogenetic relationship between all taxa of this subfamily and the lack of reliable morphological characters to separate species into natural groups support the transfer of all species of *Tylimanthus* and *Marsupidium* into *Acrobolbus*. We recognize the close relationship between taxa in Acrobolboideae and find that the morphological characters historically used to delineate the three genera does not describe clades, but rather shows the range of variability of morphological traits throughout the subfamily.

Molecular and morphological data support species status for *T. azoricus*, *T. madeirensis*, and *T. ruwenzorensis*, given their recovery in monophyletic clades as well as their restricted distribution areas. The synonymy of *T. andinopatagonicus* under *T. urvilleanus*, was also supported as additional molecular markers recovered individuals of both taxa within a single monophyletic group. Nomenclatural novelties reflecting the evidence presented here were published in Briscoe *et al.* (2015).

The results here suggest that a similar approach, including increased sampling and a more rigorous morphological analysis including more characters is necessary to fully understand the systematic position of taxa within Acrobolboideae, the relationship between all subfamilies within Acrobolbaceae, as well as to ascertain the identities of the potentially undescribed species in Chile and Fiji.

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