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Maximum likelihood analyses of chloroplast gene *rbc*L sequences indicate relationships of *Syzygiella* (Jungermanniopsida) with Lophoziaceae rather than Plagiochilaceae

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Abstract – In recent years, *Syzygiella* has been alternatively assigned to Plagiochilaceae and Lophoziaceae. Here we use chloroplast gene *rbc*L sequences to test both hypotheses. Maximum likelihood analyses of an *rbc*L dataset including 27 species of Jungermanniopsida and *Marchantia* (Marchantiopsida, outgroup) lead to a topology with two well supported paraphyletic main clades. One main clade comprises Lejeuneaceae in a robust sister relationship with Frullaniaceae and Porellaceae. *Syzygiella anomala* and *S. perfoliata* form a well supported monophyletic lineage within the second main clade. They are placed sister to a robust clade made up of *Scapania* (Scapaniaceae), *Lophozia* and *Tritomaria* (Lophoziaceae) in an unsupported sister relationship. The *Lophozia – Tritomaria – Scapania – Syzygiella* clade is placed sister to a clade with *Jungermannia* (Jungermanniaceae), *Calypogeia* (Calypogeiaceae) and *Tylimanthus* (Acrobolbaceae). The well supported Plagiochilaceae (represented by *Chiastocaulon*, *Plagiochilion* and *Plagiochila*) form a robust sister relationship with Geocalycaceae made up of *Heteroscyphus* and *Chiloscyphus*.
The phylogentic analysis provides evidence that *Syzygiella* is loosely related to Lophoziaceae and Scapaniaceae. A closer relationship of *Syzygiella* and Plagiochilaceae is not supported by the molecular dataset.

***Syzygiella* / Scapaniaceae / Lophoziaceae / Plagiochilaceae / *rbc*L / molecular phylogeny / maximum likelihood analysis**

INTRODUCTION

Syzygiella Spruce (Jungermanniopsida) is mainly a tropical genus which includes 24 species in three subgenera (So & Grolle, 2003). The genus can easily be identified by its dioecism, an often reddish pigmentation, the succcubous, (sub-) opposite leaves with opposite bases usually connected both dorsally and ventrally (Fig. 1: 2, 3), the frequent presence of rhizoids originating from the ventral end of the leaf insertion and the ventral surface of the stem (Fig. 1: 3), as well as the strongly inflated, pluriplicate perianth with a contracted mouth (Fig. 1: 1).

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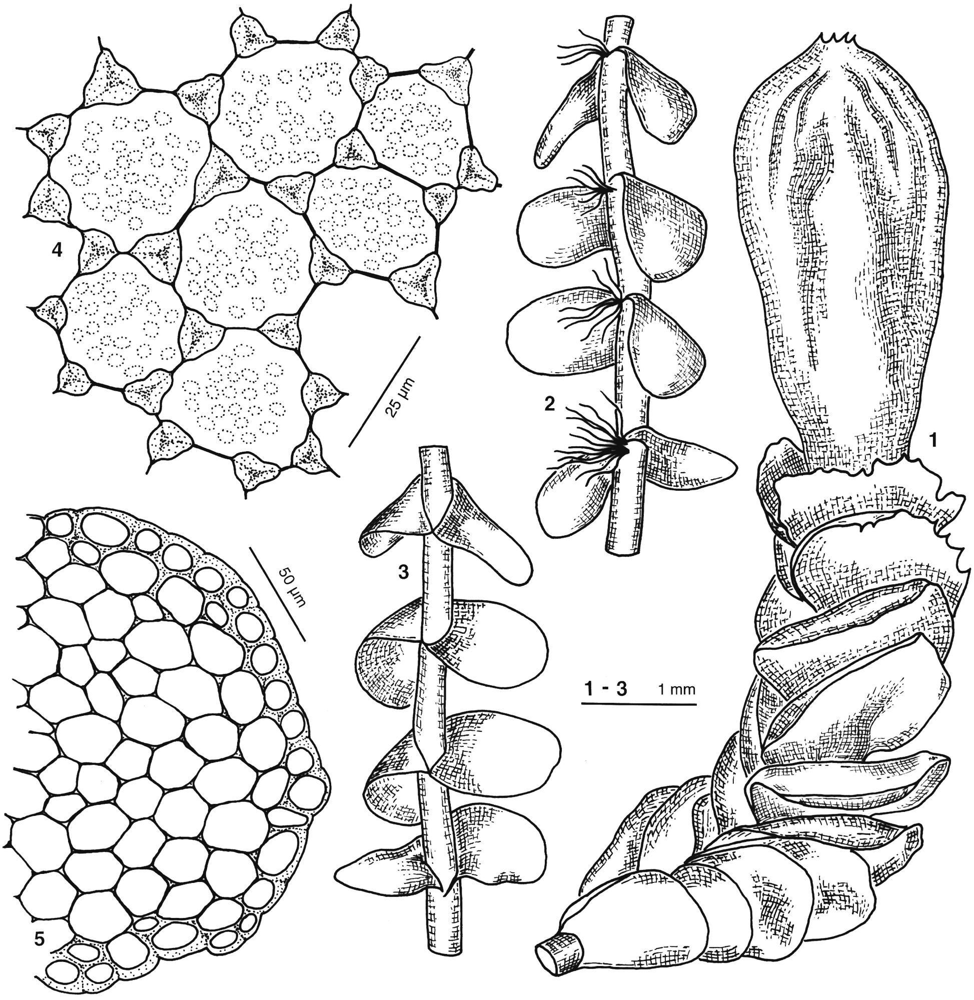

Fig. 1. *Syzygiella* — **1**, female plant with perianth, lateral view; **2**, part of shoot, ventral view; **3**, part of shoot, dorsal view; **4**, papillose leaf cells, surface view; **5**, cross section of stem [1 from *Syzygiella liberata* Inoue (*Costa & Gradstein 3784*, GOET), 2-5 from *S. perfoliata* (Sw.) Spruce (*Holz & Schäfer-Verwimp* CR 99-1142, GOET)].

Placement of *Syzygiella* in a family is more difficult and has been the subject of much controversy. Spruce (1884-1885: 500) pointed out similarities in habit of *Syzygiella* and some representatives of *Plagiochila* with subopposite leaves, leading to a placement within Plagiochilaceae (Buch *et al.*, 1938). Schuster (1959, 1980) accepted the position of *Syzygiella* in Plagiochilaceae because of the *Plagiochila*-like leaf insertion and leaf dentition as well as the slightly laterally compressed perianth of a few of its representatives.

However, the monographer of *Syzygiella*, Inoue (1966), argued against the above hypothesis and characterized the *Syzygiella* perianth as being of the

Lophozia-type. He stated that Plagiochilaceae "should include such genera as *Plagiochila*, *Plagiochilion*, *Chiastocaulon*, *Acrochila*, *Pedinophyllum*, *Xenochila*, and *Plagiochilidium*" which have a "uniformly laterally compressed perianth". Accordingly, he transferred *Syzygiella* to Lophoziaceae. The latter position was accepted by Crandall-Stotler & Stotler (2000), who however included Lophoziaceae in Jungermanniaceae.

In the framework of a molecular phylogenetic study of Plagiochilaceae (e.g. Groth & Heinrichs, 2003; Groth *et al.*, 2004) we tested the above hypotheses utilizing chloroplast gene *rbc*L sequences.

MATERIALS AND METHODS

DNA – extraction — Upper parts of a few shoots were isolated from herbarium specimens and extracted with Invisorb Spin Plant Mini Kit (Invitek).

PCR – amplification — The 5' – primer *rbc*L-1-F (5' - ATG TCA CCA CAA ACA GAA ACT AAA GCA AGT - 3') and the 3' – primer M*rbc*L-1390-R (5' - CTT TCC A(AT)A (CT)TT C(AG)C AAG CAG C(AG)G – 3') (http://bioweb.usu.edu/wolf/rbcL%20primer%20map. htm) were used to amplify the first 1390 bp of the cp-*rbc*L gene. Polymerase chain reaction (Saiki *et al.,* 1988) was performed in a total volume of 50 µl, containing one unit Taq-DNA-polymerase (SilverStar, EuroGenTech), 5 µl Taq-Polymerase reaction buffer (EuroGenTech), 2 µl $MgCl_2$ (50 mM), 1 µl dNTP-mix (10 mM, MBI Fermentas), 2 µl dimethylsulphoxide, 1 µl of both forward and reverse primer (10 mM), and 1 µl template. The PCR was carried out using the following program: 120 s initial denaturation at 92 °C, followed by 30 cycles of 60 s denaturation at 92 °C, 50 s annealing at 51 °C, and 90 s elongation at 72 °C. Final elongation was carried out in one step (10 min 72 °C). Sequencing was carried out on an ABI 3100 capillary sequencer, using the BigDye^{TM} Terminator CycleSequencing kit v2.0 (PE Biosystems, Forster City, CA, USA) with the new internal primer *rbc*L-170-F (5'-GAA GAA GCA GGA GCA GC(AG) GTA GC -3'), and the primers *rbc*L-680-F (5'- GC(CT) GAA ACT GGT GAA ATT AAA G -3'), *rbc*L-700-R (5'- GTC CTT TAA TTT CAC CAG TTT C -3'), and *rbc*L-1200-R (5'- TG(CT) CC(CT) AAA GTT CCA CCA CC -3') (Wilson *et al.*, 2004).

Phylogenetic analyses — Fifteen new *rbc*L sequences as well as 13 sequences from Ahonen *et al.* (2003), He-Nygrén & Piippo (2003), Lewis *et al.* (1997) and Manhart (1994) were used for the analyses (Table 1). The ingroup included the type of *Syzygiella*, *S. perfoliata* (Sw.) Spruce, and *Syzygiella anomala* (Lindenb. & Gottsche) Steph. as well as representatives of Plagiochilaceae, Lophoziaceae and several other families of Jungermanniopsida. *Marchantia polymorpha* L. (Marchantiopsida) was chosen as outgroup, according to the results of Lewis *et al.* (1997).

The sequences were aligned manually in BioEdit version 5.0.9 (Hall, 1999). Regions of incomplete data were identified and excluded from subsequent analyses, resulting in a dataset including 973 homologous sites (alignment available upon request).

Phylogenetic trees were inferred using maximum likelihood (ML) criteria as implemented in PAUP* version 4.0b10 (Swofford, 2000). To choose the nucleotide substitution model with the smallest number of parameters that best fits the data, the program Modeltest 3.06 (Posada & Crandall, 1998) was used that employs two statistics: the likelihood ratio test (LRT) and the Akaike information

Table 1. Geographic origins, voucher numbers, and GenBank accession numbers of the investigated taxa. Accession numbers of new sequences in bold.

Taxon	*Country*	*Voucher*	*Accession No:*
Bazzania tricrenata (Wahlenb.) Trevis.	Austria	Heinrichs 4388 (GOET)	**AY699990**
Bazzania trilobata (L.) Gray	?	?	L11056
Calypogeia muelleriana (Schiffner) K.Müller	U.S.A.	Crandall-Stotler s.n. (?)	U87065
Chiastocaulon dendroides (Nees) Carl	Japan	Kurita 84 (HIRO)	**AY699991**
Chiloscyphus cuspidatus (Nees) J.J.Engel & R.M.Schust. [*Lophocolea cuspidata* (Nees) Limpr.]	China	Koponen *et al.* 48430 (H)	AY149845
Chiloscyphus latifolius (Nees) J.J.Engel & R.M.Schust. [*Lophocolea bidentata* (L.) Dumort.]	Poland	Jedrzejko & Stenel W58 (H)	AY149842
Chiloscyphus pallescens (Ehrh. ex Hoffm.) Dumort.	Germany	Renker & Beyer 409 (GOET)	**AY699992**
Drepanolejeunea erecta (Stephani) Mizut.	China	Koponen *et al.* 50605 (H)	AY125940
Frullania dilatata (L.) Dumort.	Finland	Ahonen & Engblom 15 (H)	AY125929
Herbertus pensilis (Taylor) Spruce	Puerto Rico	Mishler 3780 (DUKE)	U87073
Herbertus sendtneri (Nees) Lindb.	Bolivia	Groth s.n. (GOET)	**AY699993**
Heteroscyphus coalitus (Hook.) Schiffner	Nepal	Long 30316 (H)	AY149844
Lejeunea cavifolia (Ehrh.) Lindenb.	Finland	Ahonen s.n. (H)	AY125945
Lophozia ventricosa (Dicks.) Dumort.	Belgium	Heinrichs 3826 (GOET)	**AY699994**
Marchantia polymorpha L.	U.S.A.	Mishler 3783 (DUKE)	U87079
Plagiochila alternans Lindenb. & Gottsche	Bolivia	Heinrichs *et al.* 4178 (GOET)	**AY699995**
Plagiochila asplenioides (L.) Dumort.	Germany	Heinrichs & Groth 4339 (GOET)	**AY699996**
Plagiochila ovata (L.) Dumort.	Costa Rica	Heinrichs *et al.* 4158 (GOET)	**AY699997**
Plagiochila porelloides (Torr. ex Nees) Lindenb.	Germany	Heinrichs & Groth 4340 (GOET)	**AY699998**
Plagiochilion mayebarae S.Hatt	Japan	Onishi 5588 (HIRO)	**AY699999**
Plagiochilion oppositum (Reinw., Blume & Nees) S.Hatt.	Bali	Schäfer-Verwimp 20935 (GOET)	**AY700000**
Porella pinnata L.	U.S.A.	De Luna & Hopple 3773 (DUKE)	U87088
Scapania undulata (L.) Dumort.	Finland	He-Nygren & Piippo 1468 (H)	AY149840
Syzygiella anomala (Lindenb. & Gottsche) Steph.	Costa Rica	Gradstein & Mues 9657 (GOET)	**AY700001**
Syzygiella perfoliata (Sw.) Spruce	Brazil	Gradstein 9827 (GOET)	**AY700002**
Tritomaria quinquedentata (Huds.) H.Buch	Germany	Heinrichs 2978 (GOET)	**AY700003**
Tylimanthus laxus (Lehm. & Lindenb.) Spruce	Costa Rica	Holz CR-00-0197 (GOET)	**AY700004**

criterion. Based on the results of the tests, the model selected by the hierarchical LRT was the TrN model (Tamura & Nei, 1993) with gamma shape parameter (G) for among site variation calculated from the data set (TrN + G). A ML-analysis (with the TrN + G model) was implemented as a heuristic search with 10 random-addition sequence replicates. The confidence of branching was assessed with 200 bootstrap resamplings in ML-analysis (Felsenstein, 1985; Hillis & Bull, 1993) and using Bayesian inference as implemented in the program MrBayes (version 3.0b4; Huelsenbeck & Ronquist, 2001). The Modeltest 3.06 output values were applied to the command block of MrBayes. Five hundred thousand generations were calculated of which every hundredth was saved, resulting in 5,000 saved trees. Trees collected before the likelihood score had stabilized were deleted. The remaining 4,000 trees with a stable likelihood score were condensed to a majority rule consensus tree. Branch support of the consensus tree, referred to as posterior probabilities, was regarded as significant when exceeding 0.95 (Larget & Simon, 1999).

RESULTS

The maximum likelihood analysis resulted in a single optimal topology (– ln = 5820.8293) which is presented in Figure 2. The 27 ingroup species are placed in two robust paraphyletic main clades. One main clade includes Lejeuneaceae in a well supported sister relationship to Frullaniaceae and Porellaceae. The second main clade includes the remainder of the ingroup. Only a few deeper nodes of this main clade have good bootstrap or Bayesian support. *Plagiochila alternans* Lindenb. & Gottsche and *P. ovata* Lindenb. & Gottsche of *P.* sect. *Alternantes* Carl are placed in a robust sister relationship with *P. asplenioides* (L.) Dumort. and *P. porelloides* (Nees) Lindenb. of *P.* sect. *Plagiochila*. The *Plagiochila* clade is placed sister to a well supported clade with *Chiastocaulon dendroides* (Nees) Carl as well as *Plagiochilion mayebarae* S.Hatt. and *Plagiochilion oppositus* (Reinw., Blume & Nees) S.Hatt. Together these genera form the robust Plagiochilaceae clade. Plagiochilaceae are placed sister to the well supported Geocalycaceae [represented by *Heteroscyphus* Schiffn. and *Chiloscyphus* Corda s.l. (including *Lophocolea* (Dumort.) Dumort., He-Nygrén & Piippo, 2003)]. The sister relationship of Plagiochilaceae and Geocalycaceae achieves a bootstrap support of 86 and a posterior probability of 1.00. Geocalycaceae / Plagiochilaceae are placed sister to *Bazzania* Gray (Lepidoziaceae) in an unsupported sister relationship. *Herbertus* Gray (Herbertaceae) is revealed as the sister of Geocalycaceae, Lepidoziaceae, and Plagiochilaceae but the position is not supported.

The type species of *Syzygiella*, *S. perfoliata* forms a robust monophyletic lineage with *Syzygiella anomala* of *S.* subgen. *Pseudoplagiochila* Inoue. *Syzygiella* is placed sister to a well supported clade made up of *Lophozia* (Dumort.) Dumort. and *Tritomaria* Loeske (Lophoziaceae) as well as *Scapania* (Dumort.) Dumort. (Scapaniaceae). The sister relationship of *Syzygiella* and *Lophozia* / *Scapania* / *Tritomaria* achieves a posterior probability of 0.94 but no bootstrap support.

Lophoziaceae are paraphyletic since *Scapania* is placed sister to *Tritomaria*. The clade with *Syzygiella*, *Scapania*, *Lophozia* and *Tritomaria* is placed sister to a moderately (bootstrap) to well (Bayesian inferrence) supported clade with Jungermanniaceae, Calypogeiaceae and Acrobolbaceae. This sister relationship is unsupported.

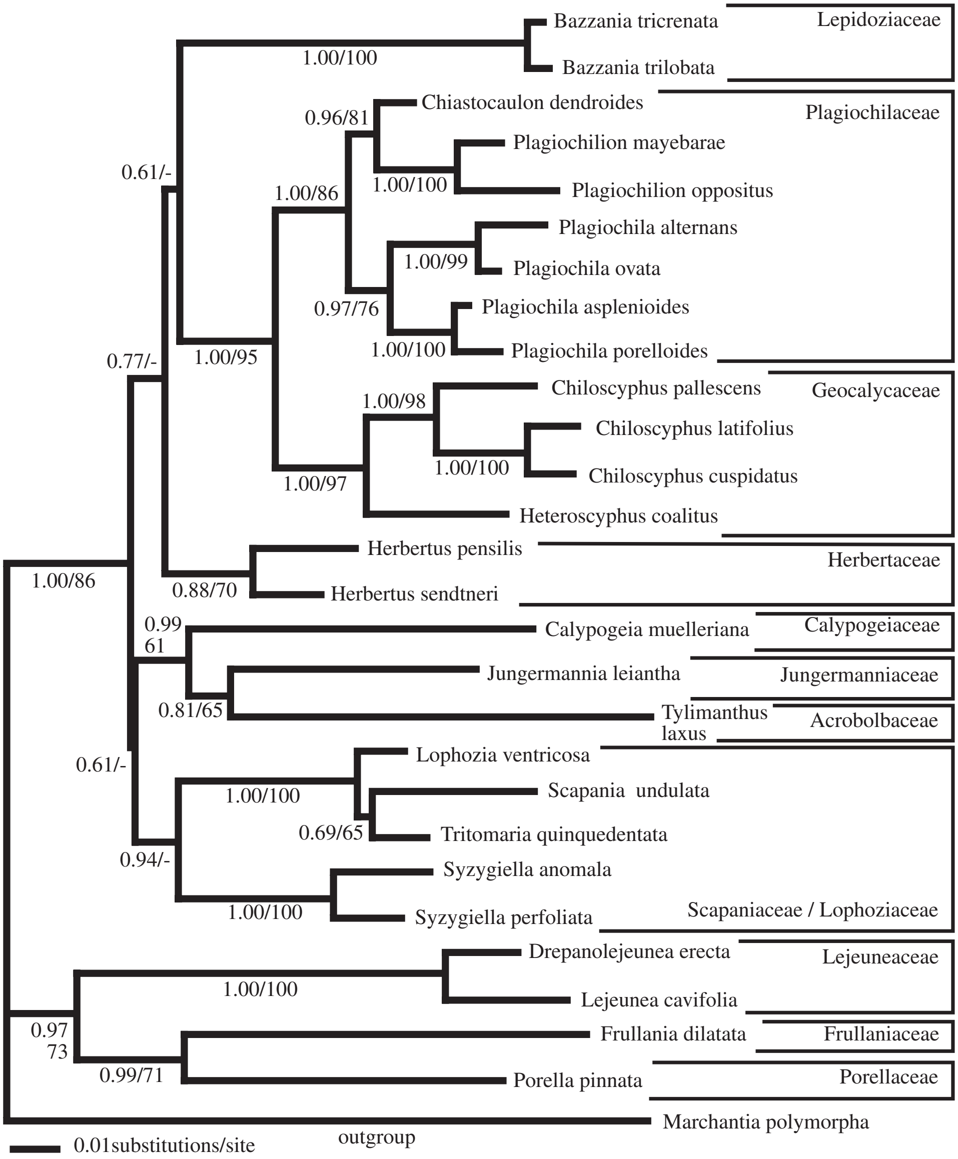

Fig. 2. Molecular phylogeny of Jungermanniopsida species based on *rbc*L sequence comparisions using 973 aligned positions. The rooted tree shown resulted from a maximum likelihood analysis of 28 sequences using the model of Tamura & Nei (1993) with estimated gamma shape (G = 0,249), calculated as the best model by Modeltest 3.06 (Posada & Crandall, 1998). Bayesian support (not bold) and bootstrap percentage values (>50 %, bold) at branches. *Syzygiella* is not closely related to Plagiochilaceae but shows relationships to Scapaniaceae/Lophoziaceae.

DISCUSSION

*Rbc*L gene sequences have proven to be a powerful source of phylogenetic signal above the genus level and have been utilized to analyze the major bryophyte lineages (Lewis *et al.*, 1997) as well as larger groups of mosses (e.g. De Luna *et al.*, 1999; Tsubota *et al.*, 2002) or liverworts (e.g. Wilson *et al.*, 2004).

Here they allow us to reject the hypothesis of Schuster (1959, 1980) that *Syzygiella* is a member of Plagiochilaceae. The exclusion of *Syzygiella* from Plagiochilaceae sensu Schuster (1980) leads to a circumscription of the family as proposed by Inoue (1966) and allows a more precise morphological circumscription of the group: Plagiochilaceae are characterized by the uniformly laterally compressed perianth. According to current knowledge they include *Acrochila* R.M.Schust., *Chiastocaulon*, *Pedinophyllum* (Lindb.) Lindb., *Plagiochila*, *Plagiochilidium* Herzog, *Plagiochilion* S.Hatt., and *Xenochila* R.M.Schust. (Crandall-Stotler & Stotler, 2000; Groth & Heinrichs, 2003). *Szweykowskia* Gradst. & M.Reiner and *Steereochila* Inoue (Heinrichs, 2002) as well as *Rhodoplagiochila* R.M.Schust. (Heinrichs *et al.*, 2004) have recently been placed in the synonymy of *Plagiochila*. The placement of *Pedinophyllopsis* R.M.Schust. & Inoue in Plagiochilaceae (He-Nygrén & Piippo, 2003) needs further study.

Schuster & Engel (1982) pointed out the similar morphology of Plagiochilaceae and representatives of Geocalyceaceae with a bilabiate perianth and postulated a close relationship of both families. This view is strongly supported by the molecular topology with a robust sister relationship of Plagiochilaceae and Geocalycaceae.

Syzygiella has its center of diversity in the Neotropics (Gradstein *et al.*, 2001; So & Grolle, 2003). The results of this study indicate exclusion of *Syzygiella* from Plagiochilaceae. This has important consequences for the understanding of the biogeography of this huge Jungermanniopsida family. Until recently, the Neotropics have been regarded as a center of diversity of Plagiochilaceae (Gradstein & Reiner-Drehwald, 1995). Molecular data however indicate that Plagiochilaceae in the Neotropics are represented solely by *Plagiochila* and that the phylogenetic center of diversity is actually located in tropical Asia and Australasia (Groth & Heinrichs, 2003; Groth *et al.*, 2004; Heinrichs *et al.*, 2004).

The molecular data provide some evidence for a placement of *Syzygiella* in Lophoziaceae as proposed by Inoue (1966). However, the position of *Syzygiella* within Lophoziaceae lacks statistical support. Hence, the position may change when larger datasets become available. Interestingly, *Scapania* (Scapaniaceae) is nested within Lophoziaceae. This topology indicates a close relationship of both families. However, an emended morphological and molecular circumscription of Scapaniaceae/Lophoziaceae should await the study of a larger taxon sampling. Crandall-Stotler & Stotler (2000) included Lophoziaceae in Jungermanniaceae. According to our molecular topology, the inclusion of Lophoziaceae in Jungermanniaceae also requires the inclusion of Acrobolbaceae and Calypogeiaceae. Therefore we think that for the time being Lophoziaceae and Jungermanniaceae would best be kept as separate units.

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