Testing hypotheses on suprageneric relationships and morphological evolution in the Leucobryaceae (Bryophyta)

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Abstract We studied suprageneric relationships and patterns of morphological character evolution within the moss family Leucobryaceae, based on phylogenetic analyses of markers from all three genomes and representatives from 11 out of 14 genera. Three main lineages are strongly supported within the Leucobryaceae, which display different morphologies of the leaf costa, two dicranoid clades (*Campylopus* clade and *Dicranodontium* clade) and a leucobryoid clade. Character evolution analyses resolved the leucobryoid morphology as a synapomorphy for the leucobryoid clade and provided data to evaluate generic circumscriptions within the family. Patterns of molecular variation, species richness, geographical distribution, ecological amplitude and variation in costa structure seem to covary: The two lineages with the most modified morphologies (deviating from the basic dicranoid type; *Campylopus* and leucobryoid clades) are also the most molecularly variable, species diverse, and occupy the broadest distributions and widest variety of habitats. Most sampled genera have clear morpho-molecular circumscriptions, except *Campylopodiella*, *Dicranodontium* and *Leucobryum*. For the latter, two alternative topologies were resolved (*Leucobryum* monophyletic or the Asian

genera *Cladopodanthus* and *Schistomitrium* nested within *Leucobryum*).

Keywords Character evolution · Dicranoid morphology · Haplolepideous mosses · Leucobryoid morphology · SH test · Systematics

Introduction

Leucobryoid mosses are haplolepideous mosses (Dicranidae) whose gametophytes exhibit a characteristic whitish green color. This color is caused by a leaf anatomy with a very wide costa composed of two to several layers of large, hyaline cells interconnected by pores (called leucocysts or hyalocysts), embedding 1–3 layers of chlorophyllose cells (chlorocysts).

Schimper (1856) recognized four leucobryoid moss genera (*Arthrocormus*, *Leucobryum*, *Leucophanes* and *Octoblepharum*) in a single family, the Leucobryaceae. The leucobryoid genera *Cladopodanthus* and *Schistomitrium* were not mentioned by Schimper (1856), and three further genera (*Cardotia*, *Exodictyon* and *Ochrobryum*) were described later during the nineteenth century. All these genera share a generally similar habit and leaf structure, but differ in their leaf anatomy (Cardot 1899; Fleischer 1904) and peristome characters (e.g., the presence of a preperistome, thickening and ornamentation of inner and outer plates). Based on these differences, Fleischer (1904) separated the leucobryoid genera into two families: the Leucobryaceae, considered more related to the Dicranaceae, and the Leucophanaceae, related to the Calymperaceae. Several other classifications for the aforementioned genera as well as further leucobryoid genera described during the twentieth century (*Carinafolium*, *Exostratum*, *Holomitriopsis* and

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Steyermarkiella) were proposed (overview in Yamaguchi 1993). The latest classification of mosses previous to the introduction of molecular phylogenetic studies (Buck and Goffinet 2000) recognized 11 leucobryoid genera, classified in the Dicranaceae (*Holomitriopsis* and *Steyermarkiella*), the Calymperaceae (*Arthrocormus*, *Exodictyon*, *Exostratum*, *Leucophanes*, and *Octoblepharum*) and the Leucobryaceae (*Cladopodanthus*, *Leucobryum*, *Ochrobryum*, and *Schistomitrium*).

That the leucobryoid genera in the Calymperaceae (and the Octoblepharaceae, cf. Bonfim Santos and Stech 2017) were not closely related to those in the Leucobryaceae, as proposed by Fleischer (1904), was confirmed by molecular data (e.g., Bonfim Santos and Stech 2017; Cox et al. 2010; Fisher et al. 2007; La Farge et al. 2000). In contrast, molecular phylogenetic evidence suggested that a number of the Dicranaceae genera belong to the Leucobryaceae (Hedderson et al. 2004; La Farge et al. 2000, 2002; Stech 1999, 2004; Tsubota et al. 2003, 2004). These genera display morphologies that are either somewhat leucobryoid (*Brothera*, *Campylopodiella*) or dicranoid (e.g., *Atractylolcarpus*, *Campylopus*, *Dicranodontium*, *Pilopogon*). The typical dicranoid costa has a median band of enlarged deuter cells surrounded by dorsal and ventral layers of stereids, and dorsal and ventral epidermal layers (Frahm 1991). This basic structure, however, varies considerably in different genera, e.g., by the absence of epidermal or stereid layers or their further subdivision into more layers. The so-called paraleucobryoid costa, named after the genus *Paraleucobryum* (Dicranaceae), is characterized by median chlorocysts (corresponding to the deuter cells) surrounded by ventral and dorsal hyalocysts, thus resembling the costa of *Leucobryum*. The term paraleucobryoid has also been applied to the costa of *Brothera* and *Campylopodiella*, which, however, differs by the presence of stereids. In contrast to the hyalocysts of the leucobryoid costa, the hyalocysts in *Paraleucobryum*, *Brothera* and *Campylopodiella* lack interconnecting pores between cells and never form multiple layers.

In the classification of mosses by Goffinet and Buck (2004) and Goffinet et al. (2009), seven dicranoid genera (*Atractylolcarpus*, *Brothera*, *Bryohumbertia*, *Campylopodiella*, *Campylopus*, *Dicranodontium* and *Microcampylopus*) were included in the Leucobryaceae. The classification in Frey and Stech (2009) differed from Goffinet and Buck (2004) and Goffinet et al. (2009) by placing *Microcampylopus* in the Dicranellaceae, adding *Holomitriopsis*, *Mitrobryum*, *Sphaerothercium* and *Steyermarkiella* (Dicranaceae in Goffinet et al. 2009) to the Leucobryaceae, and incorporating nomenclatural changes at genus level, synonymizing *Bryohumbertia* with *Campylopus* (Stech 2004) and mistakenly adopting the use of *Atractylolcarpus* for the *Campylopodiella* species and of *Metzleria* for

the *Atractylolcarpus* species (a misinterpretation of the nomenclatural proposal by Frahm and Isoviita 1988). The placement of *Microcampylopus* in the Dicranellaceae and of *Holomitriopsis* in the Leucobryaceae was supported by molecular data (Bonfim Santos and Stech 2017; Cox et al. 2010; Stech 2004; Stech et al. 2012). *Mitrobryum*, *Sphaerothercium* and *Steyermarkiella* have not yet been included in molecular phylogenetic studies.

While the Leucobryaceae are molecularly well circumscribed, molecular data to assess generic delimitations and relationships within the family are still limited. Cox et al. (2010) covered 10 out of 14 genera of the Leucobryaceae sensu Frey and Stech (2009), which were resolved in three lineages: a dicranoid lineage comprising *Campylopus* and *Pilopogon* (the sequences named as *Microcampylopus leucogaster* in the same clade originate from a collection that actually belongs to *Campylopus*); another dicranoid lineage comprising *Brothera*, *Campylopodiella* and *Dicranodontium*; and a leucobryoid lineage with *Ochrobryum*, *Holomitriopsis*, *Leucobryum*, *Cladopodanthus* and *Schistomitrium*. A similar topology including representatives of eight genera was resolved in Stech et al. (2012). However, relationships between the major lineages within the Leucobryaceae were contradictory between these and other molecular studies, and the taxon sampling was too limited to infer generic circumscriptions.

Furthermore, uniting gametophytically heterogeneous genera in the Leucobryaceae considerably obscured the family's morphological circumscription. The evolution of morphological traits within the Leucobryaceae and the suitability of morphological characters to assess generic delimitations and relationships remain insufficiently known, since all revisional work concerning these genera predates molecular phylogenetic studies.

Consequently, the present study aims to (1) test hypotheses of suprageneric relationships and genus circumscriptions in the Leucobryaceae based on phylogenetic analyses of a comprehensive taxon and molecular marker sampling and (2) infer the evolution of morphological characters within the family based on ancestral state reconstructions.

Materials and methods

Taxon sampling, DNA extraction and sequencing

The taxon sampling comprised 63 Leucobryaceae specimens (representing 11 out of 14 genera and 45 species) as well as *Archidium alternifolium* (Archidiaceae), *Eustichia longirostris* (Eustichiaceae) and *Micromitrium tenerum* (Micromitriaceae) as outgroup representatives following earlier phylogenetic reconstructions (Fedosov et al. 2016; Goffinet et al. 2011; Stech et al. 2012). Molecular markers from all

three genomes were sequenced: mitochondrial (mt) *nad5* G1 intron, chloroplast (cp) *trnS-trnF* region and *atpB-rbcL* spacer and nuclear (nr) ribosomal ITS1-5.8S-ITS2 (ITS) region. Sequences were obtained from GenBank and newly sequenced specimens from herbaria L, MO, SING and UB. Voucher information and GenBank accession numbers are listed in Online Resource 1.

Procedures for DNA extraction, amplification and sequencing followed Bonfim Santos and Stech (2017). Sequences were manually aligned in Geneious® v8.0.5 (Biomatters Ltd.), using the alignment from Stech et al. (2012) as a starting point.

Phylogenetic reconstructions

Three alignments were analyzed. The first alignment comprised the combined mitochondrial and chloroplast markers for all Leucobryaceae specimens, with *Archidium alternifolium*, *Eustichia longirostris* and *Micromitrium tenerum* as outgroup representatives. The second and third alignments represented two major clades within the Leucobryaceae, the *Dicranodontium* and the leucobryoid clades, respectively, and included additionally ITS, which was in parts unalignable across the whole family due to high sequence variability. Two samples of *Ochrobryum gardneri* were used as outgroup representatives, based on the analyses of the first alignment and alignability of the ITS sequences. The third major lineage within the Leucobryaceae, the *Campylopus* clade, has been extensively studied elsewhere (Stech 2004; Stech and Dohrmann 2004; Stech and Wagner 2005; Stech et al. 2010) and therefore was not studied in detail in this work. Alignment lengths as well as numbers of variable and parsimony-informative positions are provided in Online Resource 2.

Phylogenetic reconstructions were performed under maximum likelihood (ML) using RAxML v.8 (Stamatakis 2014) and Bayesian inference (BI) using MrBayes v.3.2.6 (Ronquist et al. 2012), both on the CIPRES Science Gateway v.3.3 (Miller et al. 2010). Analyses were run for the markers separated per genome (mt, cp, nr) and for the complete alignments (mt + cp or mt + cp + nr, respectively), to check for incongruence and to infer how each genome contributed to the resolution and clade support. Gaps were treated as missing data. Selection of partitioning schemes and evolutionary model testing were performed in Partition-Finder v1.1.1 (Lanfear et al. 2012) for the models that can be implemented in RAxML (GTR) and MrBayes (GTR and several of its nested models), respectively, with or without a gamma-distributed rate variation among sites (Γ) and a proportion of invariable sites (*I*). Model parameters were estimated independently for each partition. The resulting best partitioning schemes and evolutionary models according to

the Akaike information criterion (AIC) were implemented in the ML and BI analyses (Online Resource 3). In RAxML, a single type of rate heterogeneity pattern (either + Γ , + *I* or + Γ + *I*) can be applied for all partitions per analysis; thus, we implemented GTR + Γ in all ML analyses. For all maximum likelihood analyses, rapid bootstrapping with 1000 iterations was performed. For Bayesian inference, four runs with four chains (5×10^6 generations each) were run simultaneously, with the temperature of the single heated chain set to 0.4. Chains were sampled every 1000 generations, and the respective trees were written to tree files. After verifying the convergence of runs in Tracer v1.6 (Rambaut et al. 2014), fifty percent majority rule consensus trees and PP of clades were calculated, discarding the burn-in phase (25%).

The Shimodaira–Hasegawa (SH) test (Goldman et al. 2000; Shimodaira and Hasegawa 1999) was applied to compare selected alternative phylogenetic hypotheses for the Leucobryaceae and the *Dicranodontium* clade. For the Leucobryaceae, the ML tree (topology as in Fig. 1) was compared with the hypotheses of (1) the sister group relationship of the *Campylopus* clade and the *Dicranodontium* clade (as resolved in Stech 2004), (2) the sister group relationship of the *Campylopus* clade and the leucobryoid clade (Tsubota et al. 2004), (3) a monophyletic *Leucobryum* (as resolved in the analyses of the leucobryoid clade alignment) and (4) a clade formed by *Cladopodanthus*, *Holomitriopsis*, *Ochrobryum* and *Schistomitrium*, a relationship suggested by Eddy (1990) and Robinson (1990). For the *Dicranodontium* clade, the ML tree (topology as in Fig. 2) was compared with the hypotheses of (1) a monophyletic *Campylopodiella*, (2) a monophyletic *Dicranodontium*, (3) a monophyletic *Dicranodontium* including *D. subporodictyon*, (4) a monophyletic *Dicranodontium* including *Atractylocarpus intermedius* and (5) a monophyletic *Dicranodontium* including both *D. subporodictyon* and *A. intermedius*, all hypotheses based on the taxonomic literature (Allen 1992a; Allen and Ireland 2002; Frahm 1997; Müller and Frahm 1987). Constraint trees were used as an input to ML with RAxML. The resulting trees with branch length values and corresponding alignment were loaded into PAUP* v.4.0b10 (Swofford 2002), where these trees were compared with the respective unconstrained topologies using the SH test with 10,000 bootstrap replicates and the resampling estimated log-likelihood method (RELL).

The NeighborNet algorithm (Bryant and Moulton 2004) implemented in SplitsTree4 (Huson and Bryant 2006) was applied to the Leucobryaceae alignment for visualization of the data in a phylogenetic network. Missing data obscured the network patterns; thus, all specimens with missing data for an entire molecular marker were removed from the alignment.

Fig. 1 Bayesian inference consensus tree of 63 Leucobryaceae representatives based on mitochondrial and chloroplast DNA sequences (*nad5*, *trnS-trnF* region and *atpB-rbcL*). *Archidium alternifolium* (Archidiaceae), *Eustichia longirostris* (Eustichiaceae) and *Micromitrium tenerum* (Micromitriaceae) were used as outgroup representa-

tives. Branch support is indicated for Bayesian inference (BI) and maximum likelihood (ML) analyses of the same alignment. Bold branches represent posterior probabilities (PP) ≥ 0.95 . Actual bootstrap (BS) values are shown if $\geq 70\%$

Fig. 2 Bayesian inference consensus tree of 13 representatives of the *Dicranodontium* clade of the Leucobryaceae based on nuclear, mitochondrial and chloroplast DNA sequences (ITS, *nad5*, *trnS-trnF* region and *atpB-rbcL*). Two samples of *Ochrobryum gardneri* (Leucobryaceae) were used as outgroup representatives. Branch support is

indicated for Bayesian inference (BI) and maximum likelihood (ML) analyses of the same alignment. Bold branches represent posterior probabilities (PP) ≥ 0.95 . Actual bootstrap (BS) values are shown if $\geq 70\%$

Ancestral state reconstructions

Maximum likelihood ancestral state reconstructions were performed with Mesquite v.3.2 (Maddison and Maddison 2017) under the Markov k-state model (Lewis 2001). The analyzed characters for the Leucobryaceae were leucobryoid morphology (0 absent, 1 present), seta orientation when young or moist (0 straight, 1 twisted, 2 cygneous), capsule orientation (0 orthotropous, 1 homotropous) and calyptra shape (0 cucullate, 1 mitrate, 2 reduced). The analyzed characters for the *Dicranodontium* clade were the ventral costa layer (0 differentiated in ventral epidermis and stereid band below, 1 stereid band, 2 hyalocysts), dorsal costal stereids (0 in groups, 1 in a continuous band, 2 absent), cell type in the dorsal epidermal layer of costa (0 chlorocysts, 1 stereids, 2 hyalocysts), occurrence of pitted basal lamina cells (0 absent, 1 present) and seta orientation when young or moist (0 straight, 1 twisted, 2 cygneous). For both alignments, the character evolution analyses were performed on the ML tree and on the constrained ML trees representing plausible alternative hypotheses according to the SH test results (Leucobryaceae: sister group relationship of the *Campylopus* clade and the *Dicranodontium* clade, sister group relationship of the *Campylopus* clade and the leucobryoid clade, and *Leucobryum* monophyletic; *Dicranodontium* clade: *Campylopodiella* and *Dicranodontium* reciprocally monophyletic).

Results

Phylogenetic reconstructions

Figures 1, 2 and 3 show consensus trees from Bayesian inference of the concatenated mitochondrial and chloroplast

markers for the Leucobryaceae, all concatenated markers for the *Dicranodontium* clade and all concatenated markers for the leucobryoid clade, respectively. Branch support values are indicated for Bayesian inference (posterior probabilities, PP) and maximum likelihood analyses (bootstrap support, BS). Trees resulting from analyses of subsets of data are shown in Online Resource 4a–p, and relevant aspects of their topologies and branch support patterns are mentioned below. The trees obtained from analyses of markers from each genome separately differed in resolution and branch support, but did not reveal statistically supported incongruence (i.e., no PP ≥ 0.95 or BS $\geq 70\%$ for the conflicting branches in both incongruent topologies). They were also congruent with the trees resulting from the combined analyses, except for the BI analyses of the leucobryoid clade (see below).

The Leucobryaceae genera included in this study were split in three well-supported clades (Fig. 1), the *Campylopus* clade (PP 1, BS 100%), which comprised the dicranoid genera *Campylopus* (PP 1, BS 100%) and *Pilopogon* (PP 1, BS 98%), the *Dicranodontium* clade (PP 1, BS 100%), which comprised the remaining dicranoid genera (*Atractyllocarpus*, *Brothera*, *Campylopodiella*, *Dicranodontium*) and the leucobryoid clade (PP 1, BS 99%), which comprised the leucobryoid genera (*Cladopodanthus*, *Holomitriopsis*, *Leucobryum*, *Ochrobryum*, *Schistomitrium*). Within the leucobryoid clade, *Cladopodanthus* and *Schistomitrium* were both monophyletic and formed sister clades with maximum support (PP 1, BS 100%). They appeared in these analyses as sister to *Leucobryum sanctum* (PP 1, BS 82%). The *Leucobryum/Cladopodanthus/Schistomitrium* clade was monophyletic (PP 1, BS 100%) and sister to *Holomitriopsis* (PP 1, BS 95%). *Ochrobryum* was monophyletic (PP 1, BS 100%) and sister to the clade

Fig. 3 Bayesian inference consensus tree of 36 representatives of the leucobryoid clade of the Leucobryaceae based on nuclear, mitochondrial and chloroplast DNA sequences (ITS, *nad5*, *trnS-trnF* region and *atpB-rbcL*). Two samples of *Ochrobryum gardneri* (Leucobryaceae) were used as outgroup representatives. Branch support is

indicated for Bayesian inference (BI) and maximum likelihood (ML) analyses of the same alignment. Bold branches represent posterior probabilities (PP) ≥ 0.95. Actual bootstrap (BS) values are shown if ≥ 70%

formed by all other leucobryoid genera. The sister group relationship of the *Dicranodontium* clade and the leucobryoid clade was not significantly supported by BI, but well supported by ML (BS 92%), while in the analyses

of the chloroplast markers separately it was supported by both methods (PP 0.99, BS 91%). Analyses of the chloroplast markers resulted in trees with the same topology and similar branch support as the analyses of all markers

combined, whereas in analyses of *nad5* alone relationships within the main Leucobryaceae clades were unresolved or weakly supported (Online Resource 4a–d).

Within the *Dicranodontium* clade (Fig. 2), *Atractylocarpus* was monophyletic (PP 0.97, BS 82%) and the two *Brothera leana* samples formed a clade with maximum support (PP 1, BS 100%), whereas *Campylopodiella* was not monophyletic, with a well-supported sister group relationship between *Brothera leana* and *Campylopodiella flagellacea* (PP 1, BS 95%). *Dicranodontium* was not monophyletic either, since the *D. pulchroalare*/*D. porodictyon* clade (PP 1, BS 100%) was sister to the weakly supported *Brothera*/*Campylopodiella* clade (PP 0.96). *Dicranodontium subporodictyon* Broth. was well supported within this clade as sister to the *Atractylocarpus* clade (PP 0.97, BS 80%). Resolution and branch support were highest in the separate ITS analyses, followed by the cp markers, and *nad5* provided the least resolution (Online Resource 4e–j).

For the leucobryoid clade, the results shown in Fig. 3 mostly agreed with those in Fig. 1, except for the relationships between the *Cladopodanthus*/*Schistomitrium* clade and *Leucobryum* species. In the analyses of the leucobryoid clade (Fig. 3), the *Cladopodanthus*/*Schistomitrium* clade (PP 1, BS 100%) and the monophyletic *Leucobryum* (PP 0.99) were sister groups (PP 1, BS 95%), while as shown in Fig. 1, *Cladopodanthus* and *Schistomitrium* were nested within *Leucobryum*, causing *Leucobryum* to be paraphyletic. Bayesian inference analyses of the leucobryoid clade for each genome did not resolve the same relationships as all markers combined, but repeated the topology as seen in Fig. 1. Maximum likelihood analyses for all markers combined recovered a monophyletic *Leucobryum* as well, but with low support, and thus did not represent an incongruence in relation to the analyses per genome. Chloroplast markers were the most informative for the relationships within the leucobryoid clade, followed by *nad5*, and ITS provided the least resolution (Online Resource 4k–p).

The SH test applied to the Leucobryaceae alignment (Table 1) did not reject the two alternative hypotheses of sister group relationships between the three main Leucobryaceae clades, nor the hypothesis of *Leucobryum* being monophyletic (as in Fig. 3) as significantly less likely than the unconstrained ML topology (as in Fig. 1). The alternative topology with *Cladopodanthus*, *Holomitriopsis*, *Ochrobryum* and *Schistomitrium* forming one clade was rejected. The SH test applied to the *Dicranodontium* clade alignment (Table 1) did not reject the hypotheses of the monophyly of *Campylopodiella* and of *Dicranodontium*, but the hypotheses of a broader circumscription of *Dicranodontium*, including *D. subporodictyon* and/or *Atractylocarpus intermedius*, were rejected.

As a graphic representation of the distances between the aligned sequences, the phylogenetic network for the Leucobryaceae alignment (Fig. 6) shows alternative relationships between Leucobryaceae representatives are possible than those resolved by our analyses. By far, most of the sequence divergence represented in the graph is found after the early splits between the three main Leucobryaceae clades, while distances in this initial evolution of the family are quite small and allow for the three alternative topologies of sister group relationships. Additionally, the network also shows the uncertainty regarding the relationships between representatives of *Leucobryum* and the genera *Cladopodanthus* and *Schistomitrium*.

Ancestral state reconstructions

Ancestral state reconstructions of the Leucobryaceae resolved the leucobryoid morphology as a derived character which originated in the most recent common ancestor (MRCA) of the leucobryoid clade (Fig. 4a). The cygneous seta originated at least twice, in the MRCA of the genus *Campylopus* and within the *Dicranodontium* clade (Fig. 4b). According to this analysis, the twisted seta originated in

Table 1 Results from the SH tests applied to the Leucobryaceae alignment and to the *Dicranodontium* clade alignment

Constrained topology	Diff lnL	P
Leucobryaceae alignment		
<i>Campylopus</i> clade and <i>Dicranodontium</i> clade monophyletic	0.81090	0.7011
<i>Campylopus</i> clade and leucobryoid clade monophyletic	0.81090	0.7010
<i>Leucobryum</i> monophyletic	6.91759	0.5329
<i>Cladopodanthus</i> , <i>Holomitriopsis</i> , <i>Ochrobryum</i> and <i>Schistomitrium</i> monophyletic	61.38622	0.0010*
<i>Dicranodontium</i> clade alignment		
<i>Campylopodiella</i> monophyletic	14.32059	0.3515
<i>Dicranodontium</i> monophyletic	3.65772	0.7721
<i>Dicranodontium</i> monophyletic including <i>D. subporodictyon</i>	57.95291	0.0014*
<i>Dicranodontium</i> monophyletic including <i>Atractylocarpus intermedius</i>	30.23904	0.0499*
<i>Dicranodontium</i> monophyletic including <i>D. subporodictyon</i> and <i>A. intermedius</i>	53.40964	0.0037*

*Statistically worse trees at $P < 0.05$

Fig. 4 Maximum likelihood character evolution analyses for the occurrence of leucobryoid morphology (a), seta orientation (b), capsule orientation (c) and calyptra shape (d) for the Leucobryaceae alignment, under the phylogenetic hypothesis represented by the unconstrained maximum likelihood tree

the MRCA of the *Dicranodontium* clade. However, due to the low resolution of the relationships within the *Dicranodontium* clade in the analyses for the entire family (Fig. 1), character evolution in this clade is better interpreted in the

analyses for the *Dicranodontium* clade (Figs. 2, 5). Homotropous capsules originated twice, in the MRCAs of *Campylopus* and *Leucobryum*, and reversed to the plesiomorphic state in the MRCA of *Cladopodanthus* and *Schistomitrium*

(Fig. 4c). The mitrate calyptra originated twice, in the MRCAs of *Ochrobryum* and *Cladopodanthus/Schistomitrium* (Fig. 4d). The reconstructions under the alternative hypotheses differed only for the capsule orientation under the hypothesis of a monophyletic *Leucobryum*, which would have originated twice, in the MRCAs of *Campylopus* and *Leucobryum*, without reversals to the plesiomorphic state (Online Resource 5).

For the *Dicranodontium* clade, the analysis based on the ML tree (Fig. 5a–e, left) resolved the ventral costa layers forming a stereid band as originating independently within *A. intermedius* and in the ancestors of *C. flagellacea* and *C. stenocarpa*. The hyalocyst layer in the ventral costa originated in the MRCA of *Brothera* (Fig. 5a). The isolated stereid groups originated either twice, in the MRCA of *Brothera/Campylopodiella flagellacea* and in the ancestral of *C. stenocarpa*, or less likely only once, in the previous node (Fig. 5b). The stereids forming the dorsal epidermis originated in the MRCA of *Atractylorcarpus/D. subporodictyon*, while hyalocysts originated either twice, in the MRCA of *Brothera/Campylopodiella flagellacea* and in the ancestor of *C. stenocarpa*, or less likely only once in the previous node (Fig. 5c). Pitted basal lamina cells most likely originated in the MRCA of *Atractylorcarpus/D. subporodictyon* (Fig. 5d). The cygneous seta originated in the MRCA of the entire *Dicranodontium* clade and was modified to an erect and twisted seta in the *Campylopodiella/Brothera* clade and in the MRCA of *A. longisetus/A. alticaulis* (Fig. 5e). The analyses based on the hypothesis of both *Campylopodiella* and *Dicranodontium* monophyletic (Fig. 5f–j, right) differed in that the dorsal stereids in isolated groups would have originated in the MRCA of *Brothera/Campylopodiella* (Fig. 5g), as the hyalocysts in the dorsal epidermis (Fig. 5h) and the twisted seta (Fig. 5j).

Discussion

In line with earlier phylogenetic studies (Cox et al. 2010; Fedosov et al. 2015, 2016; Hedderson et al. 2004; La Farge et al. 2000, 2002; Stech 2004; Tsubota et al. 2003, 2004), the analyses of the present dataset resolved three well-supported lineages within the Leucobryaceae: the dicranoid *Campylopus* clade, the dicranoid *Dicranodontium* clade and the leucobryoid clade. The latter is characterized by the leucobryoid costa as synapomorphic character (Fig. 4a). The twisted seta could represent a synapomorphy for the *Dicranodontium* clade in the ancestral state reconstruction of the Leucobryaceae (Fig. 4b). However, *Atractylorcarpus intermedius* and *Dicranodontium* present a cygneous seta, which more likely represents the ancestral character state in the analyses of the *Dicranodontium* clade separately (Fig. 5e, j). The evolution of a cygneous seta occurred

twice in the Leucobryaceae, possibly from different ancestral states, namely from a twisted seta in *Dicranodontium* and from a straight seta in *Campylopus* (Fig. 4b). The other morphological characters analyzed here (capsule orientation and calyptra shape) changed character states within the *Campylopus* and/or leucobryoid clades, and thus do not represent synapomorphies for either clade. Contrary to what was suggested by Robinson (1990), *Leucobryum* is a derived genus and its homotropous capsules are not a plesiomorphic trait shared with *Campylopus*, but evolved independently in both genera (Fig. 4c). Thus, the orthotropous capsules of the remaining leucobryoid genera do not represent evidence of shared ancestry, and neither does the mitrate calyptra found in *Ochrobryum* and in the *Cladopodanthus/Schistomitrium* clade (Fig. 4d).

Earlier phylogenetic analyses could not resolve the relationships between the three major Leucobryaceae clades (Hedderson et al. 2004) or supported different sister group relationships: *Campylopus* clade and leucobryoid clade (Tsubota et al. 2004), *Campylopus* clade and *Dicranodontium* clade (one tree in Stech 2004) and, most frequently, *Dicranodontium* clade and leucobryoid clade (Cox et al. 2010; Fedosov et al. 2015, 2016; La Farge et al. 2000, 2002; Stech et al. 2012; Tsubota et al. 2003, 2004; and another tree in Stech 2004). Three studies (Cox et al. 2010; La Farge et al. 2002; Tsubota et al. 2003, 2004) recovered the latter topology with $\geq 70\%$ bootstrap support or ≥ 0.95 Bayesian posterior probability. Our results corroborate these studies, since our analyses also recovered the sister group relationship of the *Dicranodontium* and leucobryoid clades, supported in ML analyses for the total alignment (Fig. 1) and in both BI and ML analyses for the chloroplast markers (Online Resource 4c, d). However, the three possible alternative topologies of sister group relationships could not be rejected based on the SH test performed on our data (Table 1, Leucobryaceae alignment). Relationships between the major lineages within the Leucobryaceae thus remain somewhat uncertain even with a larger taxon and marker sampling than in previous studies. The patterns observed in the phylogenetic network (Fig. 6) indicate that the early evolution of the Leucobryaceae may have been an event of rapid radiation, with the least sequence divergence occurring until the split of the three main Leucobryaceae lineages. The shorter the branches, e.g., the least substitutions the branches represent, the harder it is to reconstruct the relationships associated with them, and this phylogenetic network puts in evidence this challenge in the phylogenetic reconstruction for the early evolution of the Leucobryaceae.

We found the *Dicranodontium* clade to have the lowest sequence variability for the molecular markers applied in this study (except for ITS), resulting in short branch lengths in the phylogenetic reconstructions (Figs. 1, 3). This clade is also the least species rich (ca. 30 species, Frey and Stech

Fig. 5 Maximum likelihood character evolution analyses for the ventral costa layer (a, f), dorsal stereids (b, g), cell type in the dorsal costa epidermis (c, h), occurrence of pitted basal lamina cells (d, i) and seta orientation (e, j) for the *Dicranodontium* clade alignment, under two different phylogenetic hypotheses: the unconstrained maximum likelihood tree (a–e, left) and the constrained maximum likelihood tree with *Campylopodiella* and *Dicranodontium* monophyletic (f–j, right)

2009). Its species have the narrowest habitat range: temperate to subtropical and montane/alpine tropical regions, mostly in moist forest habitats (except some *Atractylolcarpus* only found above the tree line) and absent from tropical lowlands (Frahm 1991; Padberg and Frahm 1985), and the least variation in costa structure. The latter ranges from a typically dicranoid costa in *Dicranodontium* to variations with reduced numbers of cell types or modifications on the ventral side (hyalocysts in *Brothera*, stereids in *Campylopodiella*, Fig. 5a, f) and the dorsal side (stereids in *Atractylolcarpus*, hyalocysts in *Brothera/Campylopodiella*, Fig. 5c, h). Dorsal stereids remain present, albeit partly in reduced numbers, in all genera, either in groups or as a continuous stereid band (Fig. 5b, g).

The *Campylopus* and leucobryoid clades are similar in containing more molecular variation than the *Dicranodontium* clade, indicated by their long branch lengths (Figs. 1, 3). The largest number of Leucobryaceae species (ca. 160, Frey and Stech 2009), the broadest distribution and habitat range (from latitude 70°N to 65°S, from sea level to 4800 m a.s.l.; Frahm 1991) and the greatest variety of modifications of the dicranoid costa structure are found in the *Campylopus* clade. *Campylopus* and *Pilopogon* species may either have the basic dicranoid costa structure, or be modified in various ways. Part of the species have a ventral epidermis of chlorocysts or stereids, with the dorsal epidermis ribbed (with protruding cells) or forming lamellae up to seven cells high (e.g., Gama et al. 2016), or with all costa layers but the deuter cells reduced to stereids. Other *Campylopus* species have ventral hyalocysts that may cover more than half of the costa section, with the dorsal epidermis consisting of smaller cells (smooth, ribbed or forming lamellae) or also of hyalocysts (Frahm 1983, 1991). The leucobryoid clade is also species rich (ca. 110 species, Frey and Stech 2009). Its species are concentrated in the tropics (with the exception of some *Leucobryum* species), with maximum diversity in tropical and subtropical rainforests (Eddy 1990). The costa in this clade is highly modified, although rather invariable when compared to the *Campylopus* clade. Its leucobryoid pattern varies solely in the number of ventral and dorsal hyalocyst layers.

The findings discussed above indicate that patterns of molecular variation, species richness, geographical distribution, ecological amplitude and of variation in costa structure covary in the three main lineages of the Leucobryaceae. The

two lineages with the most modified morphologies are also the most molecularly variable, species diverse, and occupy the broadest distributions and widest variety of habitats. Thus, it can be hypothesized that costa structure modifications, by allowing an improved exploitation of the available ecological niches and environment resources, could have triggered higher phylogenetic diversity in the *Campylopus* and leucobryoid clades. Within the *Campylopus* clade, the variety of modified costa forms may be a response to (or perhaps the cause of) the broad ecological spectrum of the genus and may represent different optimization strategies for photosynthesis, water uptake, water storage and mechanical fixation (Frahm 1985). The rather invariable leucobryoid costa, in contrast, seems to be most successful in quite distinct environments, possibly representing a strategy to optimize gas exchange and water balance in the damp habitat of tropical forests (Robinson 1985, 1990). It is not, however, restricted to the Leucobryaceae, but appears as a derived state in at least two other families of haplolepideous mosses, the Calymperaceae (genera *Arthrocnemum*, *Exodictyon*, *Exostratum* and *Leucophanes*) and the Octoblepharaceae (*Octoblepharum*) (Bonfim Santos and Stech 2017; Cox et al. 2010; Fisher et al. 2007; La Farge et al. 2000), which occur mainly in tropical rainforests as well.

An evolutionary history with species-rich long branch clades and short branch clades with much lower species diversity was also observed in the flowering plant family Annonaceae (Richardson et al. 2004). However, later studies of the Annonaceae have shown that differences in species numbers could not be attributed to diversification rate shifts, nor could the observed rate shifts be correlated with key morphological innovations (Erkens et al. 2012). Whether this is the case also in the Leucobryaceae remains to be tested.

The presently estimated phylogenetic relationships raise doubts concerning the delimitation of some of the genera in the *Dicranodontium* clade (Fig. 2) and about the monophyly of *Leucobryum* (Figs. 1, 3). Within the *Dicranodontium* clade, the genus *Atractylolcarpus* is molecularly well supported (this study) and distinguished from the other genera by its leaves gradually tapering into a long subula, the position of rhizoid initials and pitted basal lamina cells (Frahm 1991, Padberg and Frahm 1985; Fig. 5d, i). According to our results, its circumscription should include *Dicranodontium subporodictyon*. This species received much attention due to its peculiar disjunct distribution pattern (British Columbia/Canada, Scotland/UK, Sikkim/India and Yunnan Province/China). Its systematic position remained unclear because of incompatible gametophytic characters with each of the three genera in which it was placed, i.e., *Campylopus*, *Dicranodontium* (Leucobryaceae) and *Dicranum* (Dicranaceae), aggravated by the fact that its sporophytes are still unknown (Allen and Ireland 2002; Chien and Tong 1992; Corley and

Fig. 6 NeighborNet phylogenetic network of 30 Leucobryaceae representatives based on nuclear, mitochondrial and chloroplast DNA sequences (ITS, *nad5*, *trnS-trnF* region and *atpB-rbcL*). The out-

group representatives *Archidium alternifolium* (Archidiaceae) and *Eustichia longirostris* (Eustichiaceae) are included in the graph

Wallace 1974; Frahm 1997). The present molecular data unequivocally support a placement of *D. subporodictyon* in the *Dicranodontium* clade, where it appears as sister to the *Atractyllocarpus* species included in our study. This relationship could have been predicted since the species displays the diagnostic characters of *Atractyllocarpus* (long subulate leaves, incrassate, pitted lamina cells and position of rhizoid initials), which seem to have been overlooked or misinterpreted in previous studies. Consequently, we propose a new combination here (see Taxonomic treatment). The monospecific *Brothera* can be recognized by the absence of ventral stereids and the presence of hyalocysts in its costa (Frahm 1991; Müller and Frahm 1987).

The delimitations of *Campylopodiella* and *Dicranodontium*, however, are less clear. Both genera were resolved as paraphyletic (Fig. 2), although the SH test results did not reject the hypotheses of their monophyly (see Table 1). As far as morphological characters are concerned, the cygneous seta, as discussed above, does not represent a synapomorphy for the genus *Dicranodontium*, but also occurs in *Atractyllocarpus*, while the twisted seta is shared by *Atractyllocarpus* and *Campylopodiella* (Fig. 5e, j), and elongate upper lamina cells occur in all genera of the *Dicranodontium* clade (Müller and Frahm 1987; Padberg and Frahm 1985). *Dicranodontium*, however, is the only genus of this clade

with a typical dicranoid costa. Considering the uncertainty regarding generic limits in the *Dicranodontium* clade, we do not yet propose major changes in the classification. In case future studies support the paraphyly of *Campylopodiella* and *Dicranodontium*, a broader circumscription of a morphologically variable *Dicranodontium* may be adopted, which would be separated from *Atractyllocarpus* by the characters listed above. Although *Brothera* and *Campylopodiella* have distinctive morphological characters in relation to *Dicranodontium*, those could be interpreted as “budding” diversification (Vanderpoorten and Long 2006).

In the leucobryoid clade, all genera but *Leucobryum* share a costa mostly formed by two hyalocyst layers at leaf base, hypocentric chlorocysts (closer to the dorsal surface in transverse section, due to a difference in the depth of the ventral and dorsal hyalocyst layers), capsules that are orthotropous and symmetrical, entire peristome teeth (except *Ochrobryum*, peristome absent) and a mitrate calyptra (except *Holomitriopsis*, cucullate) (Allen 1992b; Eddy 1990; Magill 1993; Robinson 1965). *Leucobryum*, in contrast, is characterized by irregularly subdivided hyalocysts forming three to several layers at some portions of the costa at leaf base, asymmetrical, homotropous to orthogonal, curved and gibbose capsules, peristome teeth divided to the middle and a cucullate calyptra (Yamaguchi 1993).

Leucobryum subobtusifolium (Broth.) B.H.Allen, a species which was originally placed in *Ochrobryum*, but transferred to *Leucobryum* based on the presence of apical clusters of brood leaves instead of globose propagules (its sporophyte is unknown) (Allen 1992b), indeed belongs to the latter genus according to the present molecular analyses.

The conclusion by Eddy (1990) and Robinson (1990) that *Cladopodanthus*, *Holomitriopsis*, *Ochrobryum* and *Schistomitrium* are closely related and should be separated from *Leucobryum* in the family Schistomitriaceae A. Eddy (1990), however, is rejected by the present molecular data (Figs. 1, 3, Table 1). Our results suggest that the shared character states of these genera are either convergences (as the mitrate calyptra) or retained ancestral character states (as the orthotropous capsules), while at least part of the distinguishing traits of *Leucobryum* correspond to apomorphic character states within the leucobryoid clade.

The close relationship between *Cladopodanthus*/*Schistomitrium* and *Leucobryum* supported here is in agreement with results of previous studies (Cox et al. 2010; La Farge et al. 2000; Tsubota et al. 2004). However, our study provided conflicting results regarding the relationships between these genera. While the family-level analyses with *nad5* and chloroplast markers support the sister group relationship of *Cladopodanthus*/*Schistomitrium* and the Asian species *L. sanctum*, and thus resolve *Leucobryum* as paraphyletic, the clade-level analyses (with additionally ITS) recover either this same topology (separate analyses of markers per genome, supported for mitochondrial and chloroplast markers) or the monophyly of *Leucobryum* (for all markers combined, Fig. 3, supported for BI only). The results of the SH test (Table 1) show that the available data do not support a preference for either hypothesis. Since morphology indicates that *Leucobryum* is monophyletic, the contradictory topology could be caused by plesiomorphic molecular characters shared by *Leucobryum* species (*L. bowringii*, *L. crispum*, *L. giganteum* and *L. sanctum*) as well as *Cladopodanthus* and *Schistomitrium*. On the other hand, the phylogenetic network (Fig. 6) puts in evidence the uncertainty of these relationships and may indicate the occurrence of non-tree-like patterns in the evolution of these taxa. Hybridization and introgression are phenomena shown to be related to the origin of some species and genera in bryophytes (see Natcheva and Cronberg 2004); thus, their possible role in shaping the patterns found in the Leucobryaceae cannot be disregarded.

Taxonomic treatment

Atractyllocarpus subporodictyon (Broth.) Bonfim Santos & Stech, **comb. nov.** $\equiv$ *Dicranodontium subporodictyon*

Broth., Symb. Sin. 4: 20. 1929. $\equiv$ *Campylopus subporodictyon* (Broth.) B.H.Allen & Ireland, Lindbergia 27: 76. 2002. $\equiv$ *Dicranum subporodictyon* (Broth.) C.Gao & T.Cao, Bryobrothera 1: 218. 1992.—TYPE: China, “NW-Y.: An nassen Granitfelsen der wtp. St. im birm. Mons. bei Schutsche am Dijou-djiang (e Irrawadi-Oberlauf), 27°54', 2000 m. 7. VII. 1916” *Handel-Mazzetti* 9433 (holotype: H-BR; isotype: H).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Information on Electronic Supplementary Material

Online Resource 1. Voucher information and GenBank accession numbers of the specimens analyzed in the present study.

Online Resource 2. Length, number of variable and parsimony-informative positions per alignment.

Online Resource 3. Partitioning scheme and nucleotide substitution models selected with PartitionFinder per alignment.

Online Resource 4. Trees resulting from analyses of subsets of markers for each alignment. Figures a, c, e, g, i, k, m and o are Bayesian inference consensus trees, and figures b, d, f, h, j, l, n and p are maximum likelihood trees. **a–d** Trees for the Leucobryaceae alignment with *Archidium alternifolium*, *Eustichia longirostris* and *Micromitrium tenerum* as outgroup representatives. **a, b** Mitochondrial marker *nad5*. **c, d** Chloroplast markers. **e–j** Trees for the *Dicranodontium* clade alignment with two samples of *Ochrobryum gardneri* as outgroup representatives. **e, f** Mitochondrial marker *nad5*. **g, h** Chloroplast markers. **i, j** Nuclear ITS1-5.8S-ITS2 region. **k–p** Trees for the leucobryoid clade alignment with two samples of *Ochrobryum gardneri* as outgroup representatives. **k, l** Mitochondrial marker *nad5*. **m, n** Chloroplast markers. **o, p** Nuclear ITS1-5.8S-ITS2 region.

Online Resource 5. Maximum likelihood character evolution analyses for the occurrence of leucobryoid morphology (**a**), seta orientation (**b**), capsule orientation (**c**) and calyptra shape (**d**) for the Leucobryaceae alignment, under the phylogenetic hypothesis represented by the constrained ML tree with *Leucobryum* monophyletic.

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