Phylogeny of haplolepideous mosses — challenges and perspectives

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The haplolepideous mosses (Dicranidae) form the second largest group of mosses and are morphologically and ecologically highly diverse. This review summarizes the current state and addresses the most urgent remaining problems in unravelling systematic relationships in the haplolepideous mosses. The main results of early molecular phylogenetic reconstructions based on few chloroplast markers are compared with recent approaches based on markers from different genomes as well as with a new phylogeny based on a novel combination of non-coding plastid markers (*rps*4-*trn*F region and *atp*B-*rbc*L spacer). According to the available molecular data, three major groups are provisionally distinguished within Dicranidae. The first group comprises morphologically diverse species from different families (Bryoxiphiaceae, Catoscopiaceae, Distichiaceae, Ditrichaceae *p.p.*, Drummondiaceae, Pottiaceae *p.p.*, Rhabdoweisiaceae *p.p.*, and Scouleriaceae *p.p.*), which form grades branching off first in the phylogenetic reconstructions. The second group, which appears as a grade or unsupported clade, includes Grimmiales, Leucobryaceae, Archidiaceae, Eustichiaceae, and *Saelania glaucescens* (Ditrichaceae). The third group comprises the largest portion of the haplolepideous mosses, namely most families of Dicranales as well as the most speciose Pottiales; the respective clades receive significant statistical support in part of the analyses. The position of *Amphidium* in between the second and third group remains ambiguous. It is concluded that further phylogenetic analyses based on new combinations of markers are necessary at different taxonomic levels, especially to resolve the backbone of the Dicranidae phylogeny, but also to tackle large and taxonomically complex genera that are severely understudied. Implications of the molecular phylogenetic reconstructions for morphological character evolution are exemplarily discussed for the different types of haplolepideous peristomes. Furthermore, genetic and genomic research using haplolepideous taxa is briefly reviewed.

Keywords: Dicranidae, Haplolepideous mosses, Non-coding plastid markers, Phylogeny, Review

Introduction

With about 4000 species in 232 genera and 30 families (Frey & Stech, 2009), the haplolepideous mosses or haplolepids (Dicranidae) form the second largest subclass of mosses (Bryophyta). Dicranidae are morphologically and ecologically highly diverse and occur in almost all terrestrial ecosystems. They are characterized by the arthrodontous–haplolepideous (*Dicranum*-type) peristome, which usually consists of a single row of teeth around the capsule mouth. All molecular phylogenetic reconstructions so far support the monophyly of Dicranidae and a position nested within the arthrodontous–diplolepideous mosses, as sister to the largest subclass of mosses, the diplolepideous-alternate Bryidae (reviewed in Stech & Frey, 2008 and Cox *et al.*, 2010). The molecular data therefore indicate that the haplolepideous peristome evolved from a diplolepideous ancestor.

In the first half of the last decade, a booming period of phylogenetic analyses of the major moss lineages, several analyses were published from which ordinal and family-level relationships within the Dicranidae could be inferred. These studies either explicitly focussed on the haplolepideous mosses (Stech, 1999a,b; La Farge *et al.*, 2000, 2002; Tsubota *et al.*, 2003; Hedderson *et al.*, 2004) or included a considerable number of haplolepideous taxa in analyses of a broader range of mosses (Goffinet *et al.*, 2001;

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Tsubota *et al.*, 2004). They were all based on one or more of three plastid DNA regions (*trn*L-F, *rps*4, and *rbc*L) that are still among the most commonly used standard markers in moss phylogenetics (*cf.* Stech & Quandt, 2010). Thereafter only few new phylogenies of mosses or land plants in general were published (e.g. Qiu *et al.*, 2006; Stech & Quandt, 2006; Stech & Frey, 2008), which did not contribute much to resolving higher-level relationships within Dicranidae. However, recent approaches based on novel mitochondrial loci (Wahrmund *et al.*, 2009, 2010) as well as the genus-level phylogeny of mosses by Cox *et al.* (2010) and phylogenetic inference in Dicranidae by Goffinet *et al.* (2011), both based on markers from all three genomes (*rps*4, mitochondrial *nad*5, and nuclear ribosomal 26S), could mark the beginning of a new period of tackling the remaining problems of Dicranidae phylogeny.

This paper reviews the current state of knowledge on phylogenetic relationships at ordinal to (supra-) generic levels within Dicranidae and discusses future challenges, in particular which strategy should be followed to resolve the remaining ambiguous backbone relationships. The earlier approaches are compared with a novel phylogenetic reconstruction of a 50+ taxon set based on non-coding plastid markers, the *rps*4-*trn*T-*trn*L-*trn*F region (Hernández-Maqueda *et al.*, 2008b) and *atp*B-*rbc*L spacer. Implications of the available molecular data for morphological character evolution are exemplarily discussed for the different types of haplolepideous peristomes. Furthermore, genetic and genomic research using haplolepideous model taxa is briefly reviewed.

Materials and Methods

Plant material and compilation of sequence data

The present taxon sampling comprised 54 species of Dicranidae as well as *Timmia austriaca* Hedw. (Timmiidae) and *Encalypta streptocarpa* Hedw. (Encalyptidae) as outgroup representatives. Specimens from Stech (1999a, 2004), Stech *et al.* (2006) and Stech & Frey (2008) as well as additional herbarium collections from L were used (Appendix). In addition to available *trn*L-F and *atp*B-*rbc*L sequences from the above mentioned studies, *rps*4-*trn*L sequences and, as far as possible, missing sequences of *trn*L-F and *atp*B-*rbc*L were newly generated for the present study. In some cases, the dataset was completed with sequences from GenBank (Appendix). Classification of Bryophyta follows Frey & Stech (2009).

DNA extraction, PCR, and sequencing

Distal parts of shoots were thoroughly cleaned with distilled water. Total genomic DNA was extracted using the DNeasy® Plant Kit (Qiagen, Hilden, Germany). The *rps*4-*trn*F region was amplified and sequenced in two parts, *rps*4-*trn*L and *trn*L-F. The first part comprised the 3′ end of the *rps*4 gene, the *rps*4-*trn*T_{UGU} and *trn*T_{UGU}-*trn*L_{UAA} intergenic spacers, the *trn*T_{UGU} gene as well as the *trn*L_{UAA} 5′ exon and 5′ end of the *trn*L_{UAA} intron, while the second part spanned the complete *trn*L_{UAA} intron, *trn*L_{UAA} 3′ exon, and *trn*L_{UAA}-*trn*F_{GAA} spacer. PCR protocols and primers used were as described in previous studies: *rps*4-*trn*L (Hernández-Maqueda *et al.*, 2008b; primers rps4-166F and P6/7), *trn*L-F (Hernández-Maqueda *et al.*, 2008b; primer C_M by Frey *et al.*, 1999; primer F by Taberlet *et al.*, 1991), and *atp*B-*rbc*L (Stech, 2004; primers atpB-1 and rbcL-1 by Chiang *et al.*, 1998). In cases of difficulties with obtaining PCR products, the *rps*4-*trn*L part was split into two halves, which were amplified and sequenced separately with primers rps4-166F/A-Rbryo and A-Fbryo/P6/7, respectively (Hernández-Maqueda *et al.*, 2008b). PCR products were purified using the Wizard® DNA Clean-up kit (Promega, Madison, WI, USA) or by Macrogen Inc. (www.macrogen.com), where the automated sequencing was performed as well. Sequencing primers were those used for PCR. GenBank accession numbers for all sequences used in this study are given in the Appendix. The *rps*4-*trn*F region is comprised in one accession per specimen; if earlier accession numbers were already available for parts of the region (e.g. *trn*L-F), these were updated.

Alignment, sequence analysis, and phylogenetic reconstructions

DNA sequences were manually aligned in PhyDE® v0.995 (Müller *et al.*, 2006). Phylogenetic reconstructions according to the maximum parsimony (MP) optimality criterion were performed using PAUP 4.0b10 (Swofford, 2002). Heuristic searches under parsimony were implemented using random sequence addition with 1000 replicates and tree bisection-reconnection branch swapping. Gaps were either treated as missing data or coded as informative by a simple indel coding (SIC) strategy (Simmons & Ochoterena, 2000) as implemented in SeqState (Müller, 2004a). To search the tree space for islands of more parsimonious trees, parsimony ratchet analyses were performed with PRAP2 (Müller, 2004b) in combination with PAUP, employing the default options (200 iterations, 25% of randomly chosen positions up-weighted to 2) and superimposed 10 random addition cycles. Heuristic bootstrap searches under parsimony were performed with 1000 replicates and 10 random addition cycles per bootstrap pseudoreplicate with the same options in effect.

A maximum likelihood (ML) analysis was also performed with PAUP. Bayesian posterior probabilities (PP) were calculated based on the Metropolis-coupled Markov chain Monte Carlo method, using MrBayes v3.1 (Huelsenbeck & Ronquist, 2001). Prior to these model-based analyses, model testing was

performed in Modeltest 3.7 (Posada & Crandall, 1998) employing MrMTgui (Nuin, 2005). Both the hierarchical likelihood ratio test and the AIC criterion indicated GTR+I+G as best-fit model. Consequently, the settings Basefreq=(0.4531 0.0722 0.0845), Nst=6, Rmat=(0.7917 2.9656 0.1421 1.5770 2.9656), Rates=gamma, Shape=1.3539 and Pinvar =0.2714 were used for ML analysis and Nst=6 and Rates=invgamma for the Bayesian analysis. In a second Bayesian analysis, the indels coded by SIC were included, with sequence and indel data treated as separate and unlinked partitions, employing the restriction site model ('F81') for the indel matrix. The *a priori* probabilities supplied were those specified in the default settings of the program. Four runs with four chains (10^6 generations each) were run simultaneously, with the temperature of the single heated chain set to 0.2. Chains were sampled every 1000 generations and the respective trees were written to a tree file. Fifty percent majority rule consensus trees and PP of clades were calculated by combining the four runs and using the trees sampled after the chains converged. Trace plots generated in Tracer v1.5 (Rambaut & Drummond, 2007) were used to check for convergence of the runs (plateaus of all runs at comparable likelihoods) and to infer the 'burnin', which approximately ranged between the first 100 000 and 120 000 generations (first 100–120 sampled trees). Consequently, the first 150 trees (15%) were deleted to ensure that only trees of the stationary phase were included.

Results

In the present dataset, length ranges of the sequenced non-coding markers within Dicranidae were 261–331 nucleotides (nt) for *rps*4-*trn*T spacer, 252–328 nt for *trn*T-L spacer, 243–325 nt for *trn*L intron, 59–78 nt for *trn*L-F spacer, and 445–567 nt for *atp*B-*rbc*L spacer. No length variation was observed in the sequenced coding regions except for one additional nt in the *trn*L 3′ exon in *Catoscopium nigritum* (Hedw.) Brid. The combined alignment comprised 3079 positions. Of these, 1176 (38.2%) were variable, and 719 (23.4 or 61.1% of the variable positions) were parsimony-informative. Inclusion of indel characters by SIC yielded another 467 parsimony-informative characters, resulting in 1186 parsimony-informative characters in total.

MP analyses without and with indels included by SIC retained eight or two most parsimonious trees, respectively [without indels: lengths 3325, CI 0.523, RI 0.623, RC 0.326; with SIC: lengths 4924, CI 0.548, RI 0.620, RC 0.452]. PRAP searches recovered trees of the same lengths but did not find shorter trees. In the ML analysis, a single optimal tree was found (ln L=−20030.388180), which is shown in Figure 1, with bootstrap support and PP from the respective MP and Bayesian analysis without indels indicated. One of the two most parsimonious trees including indels is depicted in Figure 2, showing BS and PP values of the respective analyses with SIC. The second most parsimonious tree differs only within the Leucobryaceae, showing *Atractylocarpus* and *Campylopodiella* on separate branches, but without significant support.

In all phylogenetic reconstructions, the species *Catoscopium nigritum*, *Hymenoloma crispulum* (Hedw.) Ochyra, *Ditrichum flexicaule* (Schwägr.) Hampe, and *Drummondia prorepens* (Hedw.) E.Britton were placed sister to the rest of the Dicranidae in the phylogenetic reconstructions, either as grades or as an unsupported clade (Figures 1 and 2). *Bryoxiphium norvegicum* (Brid.) Mitt. branched off next (84% BS in the MP SIC analysis, PP 1.00) and was sister to a clade of the remaining species, which was statistically supported only by the Bayesian analyses (PP 1.00). Within the latter, three main clades were resolved as a polytomy in the analyses without indels (Figure 1), the first comprising Grimmiales (91% BS, PP 1.00), the second Leucobryaceae as well as *Archidium alternifolium* (Hedw.) Mitt. and *Eustichia longirostris* (Brid.) Brid. (PP 1.00), and the third the remaining included taxa of Dicranales and Pottiales (PP 1.00). In the analysis with SIC (Figure 2), the first two of these clades formed one unsupported clade, and the third clade of the remaining taxa received BS (89%) in addition to a PP of 1.00. Within this latter clade, all families except Ditrichaceae were monophyletic with significant support. A close relationship of Dicranaceae *s.str.*, Hypodontiaceae, and Calymperaceae was resolved with moderate to high support (BS 78–80%, PP 1.00). Other family relationships remained unsupported in the MP analyses, and for some clades also in the Bayesian analyses, or were contradictory. For example, *Amphidium* was placed sister to the other taxa based on substitutions only (Figure 1) and in the Bayesian analysis with SIC (tree not shown), but nested inside the clade as sister to *Fissidens* in the MP SIC analysis (Figure 2), albeit without support.

Discussion

Molecular phylogeny of Dicranidae — the first decade (1999–2009)

From the first molecular phylogenetic reconstructions of Dicranidae (Stech, 1999a,b; La Farge *et al.*, 2000, 2002; Goffinet *et al.*, 2001; Tsubota *et al.*, 2003, 2004; Hedderson *et al.*, 2004), four main results emerged. First, Dicranidae were resolved as monophyletic, including a number of families with either reduced or peculiar double peristomes formerly considered as diplolepideous, whose systematic position had long been debated (Archidiaceae, Amphidiaceae, Catoscopiaceae, Drummondiaceae, Ephemeraceae, Erpodiaceae,

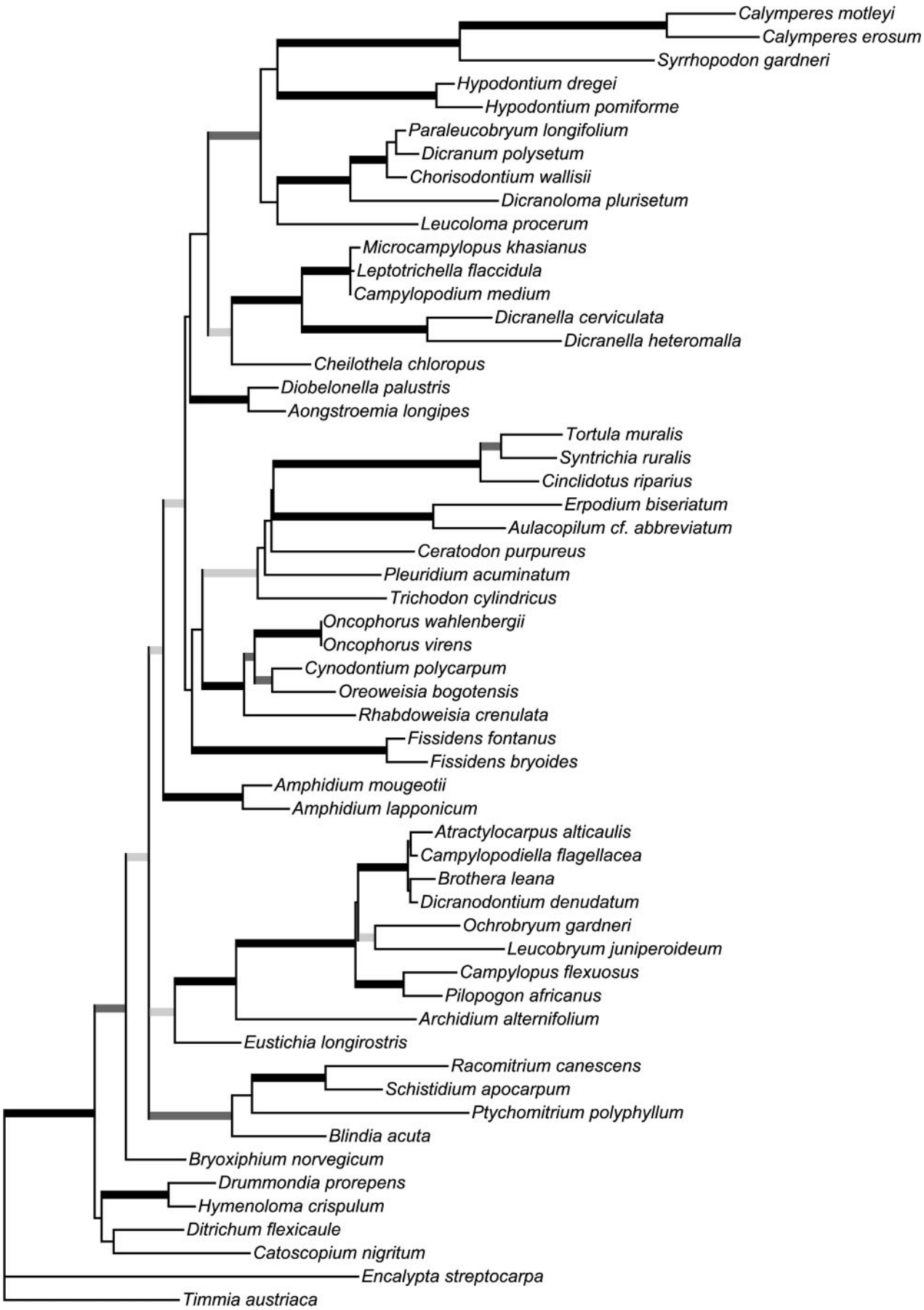

Figure 1 Single optimal maximum likelihood tree of 54 representatives of haplolepideous mosses (Dicranidae) based on chloroplast DNA sequences (*rps*4-*trn*F region, *atp*B-*rbc*L spacer). *Timmia austriaca* (Timmiidae) and *Encalypta streptocarpa* (Encalyptidae) were used as outgroup representatives. Thick lines indicate bootstrap support (BS) values from a respective maximum parsimony analysis and significant posterior probabilities (PP) from a respective Bayesian analysis: BS >90%/PP >95 (black), BS >70%/PP >95 (dark grey), PP>95 (light grey).

Mitteniaceae, Rhachitheciaceae, Splachnobryaceae, and Wardiaceae). Second, some of the more speciose families, such as the Calymperaceae, Fissidentaceae, Grimmiaceae, and Pottiaceae, were monophyletic almost in their traditional circumscription or with certain changes in their generic composition (*Indusiella* and *Jaffueliobryum* removed from Grimmiaceae; Hernández-Maqueda *et al.*, 2008b). Other families, in contrast, were resolved as polyphyletic, especially Dicranaceae and Ditrichaceae. Third, the backbone of the phylogeny was rather weakly supported and the ordinal classification of Dicranidae was not recognizable in the phylogeny. Fourth, a morphologically diverse assembly of species from different families, called 'protohaplolepideous' taxa (Hedderson *et al.*, 2004), branched off first in the phylogeny (see enumeration below). Subsequently, a number of systematic rearrangements were made and incorporated in the two main recent synopses of classification of Bryophyta (Frey & Stech, 2009; Goffinet *et al.*, 2009). Hypodontiaceae were segregated from the Pottiaceae as a new family and Oncophoraceae (Rhabdoweisiaceae) separated from the Dicranaceae *s.l.* Furthermore, Dicnemonaceae were reincluded in the Dicranaceae *s.str.*, Cinclidotaceae and Ephemeraceae were included in the Pottiaceae, and Leucobryaceae were expanded by the former subfamilies Campylopoideae and Paraleucobryoideae *p.p.* of the Dicranaceae, which resulted in a more heterogeneous circumscription of the Leucobryaceae comprising both 'leucobryoid' and 'dicranoid' genera (*cf.* Frey & Stech, 2009). Further segregates of Dicranaceae *s.l.* were placed into the resurrected or newly described families Amphidiaceae, Aongstroemiaceae, and Dicranellaceae (Stech & Frey, 2008), which were incorporated in Frey & Stech (2009), but not in Goffinet *et al.* (2009).

Of the three largest orders, namely Grimmiales, Dicranales, and Pottiales, only Grimmiales (Campylosteliaceae, Grimmiaceae, Ptychomitriaceae, and Seligeriaceae) have unequivocally been shown to represent a monophyletic group with molecularly well-resolved relationships (Tsubota *et al.*, 2003; Hernández-Maqueda *et al.*, 2008b). Dicranales were clearly not monophyletic. Despite efforts to resolve relationships within the large family Pottiaceae (see below), circumscription of Pottiales remained difficult to assess. Representatives of the monogeneric Pleurophascaceae and Serpotortellaceae were only included in Shaw *et al.* (2005), who assessed molecular diversity in mosses based on a large-scale phylogenetic reconstruction of moss genera. Inference on the systematic position of individual taxa, however, was not possible from that article as the taxon names were only given in the appendix and not indicated in the phylogenetic tree. The position of *Pleurophascum* within the diplolepideous Bryaceae in Goffinet *et al.* (2001) might be an artefact. The monotypic Mitteniaceae were either included in Pottiales (Goffinet *et al.*, 2009) or treated as a separate order Mitteniales (Shaw, 1985; Frey & Stech, 2009; *cf.* also O'Brien, 2007 and discussion in Stech & Frey, 2008).

Molecular phylogeny of Dicranidae — recent developments and current state

The most recent molecular phylogenetic reconstructions allowing further inferences of relationships within the haplolepideous mosses comprise Cox *et al.* (2010), Wahrmund *et al.* (2010), Goffinet *et al.* (2011), and the present study. The circumscription of Dicranidae has been expanded by including *Bryowijkia* (Cox *et al.*, 2010), which was already separated as family Bryowijkiaceae within the Hedwigiales by Frey & Stech (2008). One of the most surprising findings of several earlier studies, the existence of a number of morphologically diverse taxa branching off first in the Dicranidae phylogeny, seems to be real, as such a topology is also resolved in Cox *et al.* (2010), Wahrmund *et al.* (2010), and the present study (Figures 1 and 2). Although not every study included all respective taxa, they seem to comprise, in summary, Bryoxiphiaceae, Catoscopiaceae, Distichiaceae, Scouleriales (Drummondiaceae, Scouleriaceae *p.p.*: *Scouleria aquatica* Hook.) as well as *Hymenoloma crispulum* (Rhabdoweisiaceae), *Ditrichum flexicaule* (Ditrichaceae), and *Timmiella anomala* (Bruch & Schimp.) Limpr. (Pottiaceae, see below). The close relationship between *Drummondia prorepens* and *Hymenoloma crispulum* (Hedderson *et al.*, 2004; present study), the position of *Chrysoblastella chilensis* (Mont.) Reimers (Ditrichaceae) as sister to *Distichium* and *Timmiella* (Cox *et al.*, 2010), and the position of *Tridontium tasmanicum* Hook.f. (Scouleriaceae) in Pottiaceae (Cox *et al.*, 2010; Goffinet *et al.*, 2011) need further study. *Hymenoloma crispulum* was traditionally included in *Dicranoweisia*, but separated by Ochyra *et al.* (2003) based on morphological characters, a point of view supported by molecular data. *Dicranoweisia s.str.* clearly belongs to Rhabdoweisiaceae according to the position of *Dicranoweisia cirrata* (Hedw.) Lindb. ex Milde close to *Rhabdoweisia* in phylogenetic reconstructions (La Farge *et al.*, 2002; Hedderson *et al.*, 2004; Tsubota *et al.*, 2004).

The other haplolepideous taxa seem to be divided into two large groups. The first group, which appears as a grade or unsupported clade in the phylogenies, comprises Grimmiales, Archidiaceae, Leucobryaceae (Hedderson *et al.*, 2004; Stech & Frey, 2008; Cox *et al.*, 2010; Wahrmund *et al.*, 2010) as well as the recently described Micromitriaceae (Goffinet *et al.*, 2011), *Saelania glaucescens* (Hedw.) Broth. of Ditrichaceae (Cox *et al.*, 2010; Goffinet *et al.*, 2011), and Eustichiaceae (this study). The second group comprises the largest portion of the haplolepideous mosses, namely

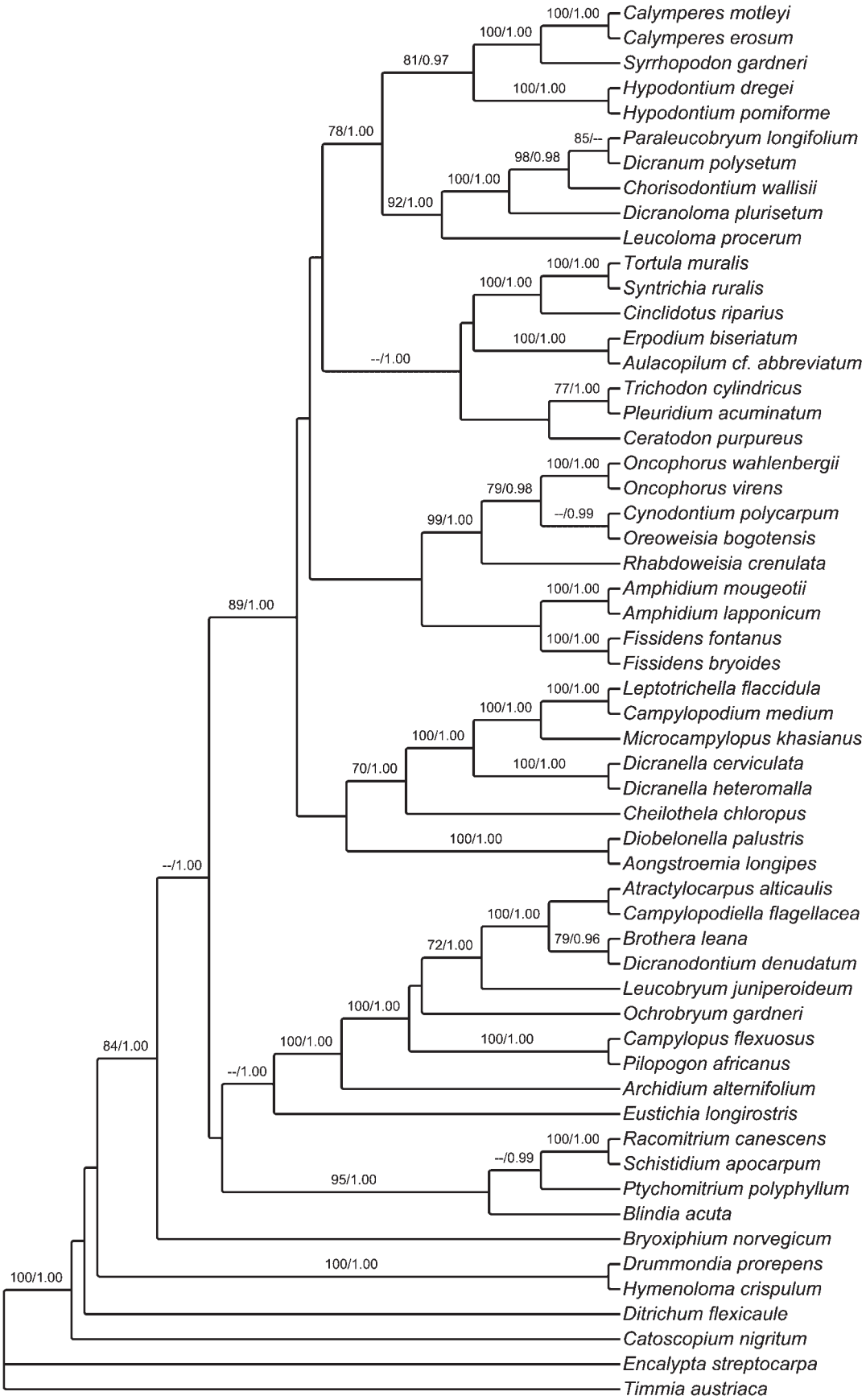

Figure 2 One out of two most parsimonious reconstructions (shown as phylogram) of 54 representatives of haplolepideous mosses (Dicranidae) based on chloroplast DNA sequences (*rps*4-*trn*F region, *atp*B-*rbc*L spacer), with indel characters coded by simple indel coding (SIC) included. *Timmia austriaca* (Timmiidae) and *Encalypta streptocarpa* (Encalyptidae) were used as outgroup representatives. Bootstrap support values >70% and significant posterior probabilities >95 from a respective Bayesian analysis with indels included are given at the branches.

most families of Dicranales *sensu* Frey & Stech (2009) (Aongstroemiaceae, Bruchiaceae, Calymperaceae, Dicranaceae, Dicranellaceae, Ditrichaceae *p.p.*, Erpodiaceae, Fissidentaceae, Hypodontiaceae, Oncophoraceae, Rhachitheciaceae, and Schistostegaceae) plus Bryowijkiaceae as well as the most speciose Pottiales. The respective clade of this group receives significant statistical support in Wahrmund *et al.* (2010), in the present analysis with indels (Figure 2) and, excluding *Amphidium* (Amphidiaceae), in Cox *et al.* (2010) and Goffinet *et al.* (2011). The position of *Amphidium* remains ambiguous in the present study as well (Figure 1 versus Figure 2).

Except for *Chrysoblastella chilensis*, *Ditrichum flexicaule*, and *Saelania glaucescens*, representatives of Ditrichaceae analysed so far seem to cluster into two groups in the molecular phylogenetic reconstructions. *Eccremidium* and *Garckea* form a well-supported clade with *Aongstroemia* (Aongstroemiaceae) and *Cladophascum* (Bruchiaceae) (Cox *et al.*, 2010; Goffinet *et al.*, 2011). In contrast, a close relationship is indicated between *Astomiopsis*, *Ceratodon*, *Ditrichum pallidum* (Hedw.) Hampe/*D. heteromallum* (Hedw.) E.Britton, *Pleuridium*, *Pseudephemerum*, and *Trichodon* (La Farge *et al.*, 2002; Tsubota *et al.*, 2003, 2004; Hedderson *et al.*, 2004; Cox *et al.*, 2010; Goffinet *et al.*, 2011; present study), although statistical support is lacking. This latter group might represent Ditrichaceae *s.str. Cheilothela* should be close to *Ceratodon* as well (McDaniel, 2005); its position in the trees of the present study (Figures 1 and 2) needs to be confirmed by further material.

The available phylogenetic reconstructions and the systematic rearrangements inferred from them represent an important step towards a classification of haplolepideous mosses that better reflects phylogenetic relationships. However, further molecular analyses based on increased taxon and marker sampling are necessary to clarify still ambiguous relationships within the Dicranidae at different taxonomic levels. At the highest level, this concerns the preliminary distinction of three major groups versus an ordinal classification. Whether the distinction of formal orders within the Dicranidae will remain useful or should be replaced by informal node-based names to characterize major lineages above the family level, as has been done, e.g. by Bell *et al.* (2007) for the main pleurocarpous lineages, needs to be discussed based on such extended phylogenies. Besides, circumscriptions and relationships of families such as Aongstroemiaceae, Bruchiaceae, Dicranellaceae, Oncophoraceae, and especially Ditrichaceae, remain preliminary and need further study. Although in some organism lineages higher level relationships might not be completely solved with tree-based approaches (e.g. Hallström & Janke, 2010), the recent developments of phylogenetic research in the Dicranidae indicate that considerable progress can still be made based on phylogenetic reconstructions of extended data sets.

Molecular marker sampling

A general problem of many phylogenetic analyses in bryophytes has been the focus on only few molecular markers (*cf.* Stech & Quandt, 2010). Concerning the Dicranidae, all initial higher-level phylogenetic analyses were based on three widely used plastid DNA regions, *trn*L-F, *rps*4, and *rbc*L. Aside from problems with single-marker analyses (e.g. Slowinski & Page, 1999; Gontcharov *et al.*, 2004; Bell & Hyvönen, 2010), all initial analyses of Dicranidae thus only reflect evolutionary patterns of the plastid genome. Fortunately, recent approaches to improve phylogenetic reconstructions of mosses (or all land plants) have evaluated new markers, especially from the mitochondrial genome (Wahrmund *et al.*, 2009, 2010), and used combined markers from two or even all three different plant genomes (Qiu *et al.*, 2006; Quandt *et al.*, 2007; Cox *et al.*, 2010; Wahrmund *et al.*, 2010; Goffinet *et al.*, 2011). Another strategy was followed by Stech & Frey (2008), who evaluated the suitability of combined non-coding plastid markers for phylogeny reconstruction of mosses, which is continued and extended in the present study for the Dicranidae.

Which strategy of marker selection should be followed to resolve the remaining uncertainties in Dicranidae phylogeny? As Stech & Quandt (2010) have recently discussed for bryophytes in general, the trend of using multiple markers and comparing markers from different genomes should be continued. But at the same time new markers must be identified that provide sufficient variability and phylogenetic structure at the respective taxonomic level under study. For example, the coding markers used to infer land plant relationships by Qiu *et al.* (2006), which show slow to moderate evolutionary rates, did not resolve relationships within Dicranidae and are thus not useful to employ with a larger taxon sampling. Non-coding markers, as tested in Stech & Frey (2008) and especially in the present study, are very well suited to resolve and support the different families of Dicranidae, whereas their relationships remain largely unsupported, at least in the MP analyses. Compared to Stech & Frey (2008), the present marker combination provides more phylogenetic information, as the very short *psb*A-*trn*H spacer was replaced by spacers of the *rps*4-*trn*L region, which were already employed successfully at family level in haplolepideous mosses (Stech, 2004 [only *trn*T-L spacer]; Hernández-Maqueda *et al.*, 2008b). One problem of resolving the backbone phylogeny of Dicranidae with non-coding markers might be the

considerable amount of homoplasy, as can be inferred, e.g. from the low RC values of the present most parsimonious reconstructions (*cf.* results). The amount of homoplasy seems to be even higher in the substitutions than in the indels coded by SIC. Although it might be considered critical to use indel characters at higher taxonomic levels given the high length variability of non-coding DNA regions, these characters are generally congruent with the substitution data, and even provide higher support for some clades, in the present study.

As Stech & Quandt (2010) further discussed, one perhaps has to combine several suboptimal markers to collect the small amount of synapomorphic sites in each of them (thereby also considering indel characters) until well-resolved phylogenetic trees can be produced. To do so, further plastid markers such as group 2 introns (*trn*G, *trn*V, *rpl*16, and *trn*K introns) and fast-evolving genes such as *mat*K or *ndh*B, should be tested. The most suitable plastid markers should be combined with mitochondrial markers and newly developed single- or low-copy nuclear markers, taking into account potentially different evolutionary patterns between the organelle and nuclear markers.

Examples of single- or low-copy nuclear markers already utilized at lower taxonomic level in Dicranidae are *adk* and *phy*2 (McDaniel & Shaw, 2005) as well as *gpd* (Wall, 2002, 2005). Nuclear introns are essential for evolutionary genetic analyses at this scale for two reasons. First, nuclear introns are often sufficiently variable to distinguish closely related species or populations; and second, multiple, independent loci are critical for distinguishing between incomplete lineage sorting and hybridization (including polyploidy) as explanations for close relationships among species. Because introns diverge much faster than the coding portions of duplicate genes, it is relatively straightforward to design primers to amplify a single paralog of a multi-copy gene family. Nuclear genes are likely to be equally important for deep phylogenetics, particularly for thorny problems like resolving the backbone of the Dicranidae. For this level of analysis, however, the choice of loci is more challenging. Analyses of loci that contain few variable sites, either because they are small genes or because they are under rigid functional constraints, may be misleading because the few sites that can change have already experienced multiple changes across the phylogeny. However, markers that are too freely evolving, such as nrITS, may contain insertions, deletions, micro-inversions, and gene duplications that dramatically increase the complexity of the analysis. A concerted effort to identify a set of markers with appropriate characteristics based on a comparison of available genomic data (see below), using approaches like those outlined in Tekle *et al.* (2010), provides a way forward. Preliminary analyses (McDaniel, unpublished data) suggest that the phytochrome gene family is a strong candidate, but the rate of diversification at the base of the Dicranidae indicates that additional loci may be required.

Implications for character evolution

The available molecular data allow preliminary inferences of the evolution of key morphological characters, as exemplarily discussed for the haplolepideous peristome below. More precise insights into character evolution should be based on cladistic analyses of morphological characters as well as ancestral state reconstructions. These, in turn, need to be based on expanded and better supported phylogenies and will probably also need further morphological–anatomical analyses for homology assessment and character coding. All three major groups of haplolepideous mosses distinguished here comprise taxa with very different morphologies that will pose challenges for the interpretation of character evolution in Dicranidae. For example, the morphological diversity of the first diverging taxa is already considerable, including peristome reductions (in *Bryoxiphium*, *Catoscopium*, *Drummondia*, and *Scouleria p.p.*) and similar morphologies with other haplolepids, such as the *Fissidens*-like leaf architecture in *Bryoxiphium* and the pottiaceous morphology of *Timmiella* (see below). A striking example of parallel gametophyte reduction is displayed by *Ephemerum* and *Micromitrium* of the former Ephemeraceae, which are molecularly unrelated (Goffinet *et al.*, 2011), in addition to the long known differences in chromosome numbers and sporophyte characters between both genera (Bryan & Anderson, 1957).

The early stages of peristome development, up to the point where the amphithecium is differentiated into three layers, namely the outer (OPL), primary (PPL), and inner peristomial layer (IPL), are virtually identical between the haplolepideous peristome and the other major peristome types (Shaw *et al.*, 1989). Thereafter, a unique sequence of cell divisions leading to a PPL:IPL arrangement of 2:3 cells for a two-cell segment of the PPL (one-eighth of the peristome) characterizes the haplolepideous peristome developmentally (Shaw *et al.*, 1989). The formula OPL:**PPL**:IPL 4:**2**:3 (or 0:**2**:3 as the OPL/outer PPL walls have disappeared in the mature peristome), however, is modified in several haplolepideous taxa due to the formation of a second row of teeth or reduction to a 2:2 pattern. Resulting formulas are, e.g. 4:**2**:2(–3) in Seligeriaceae, (4:)**2**:2(–3) in Calymperaceae, (8:4:)**2**:2(–3) in Hypodontiaceae (Edwards, 1979), and a final pattern of 8:4:**2** in *Glyphomitrium humillimum* (Estébanez *et al.*, 2006). Double haplolepideous

peristomes mostly result from preperistome formation on the OPL side, which is usually restricted to the base of the teeth, or rarely (*Mittenia*) by involving the inner periclinal walls of the IPL and adjacent cell walls of the outermost endothecial layer (Shaw, 1985). Morphological variation of the haplolepideous peristome is furthermore considerable with respect to the shape, degree of incision, and ornamentation of inner and outer surfaces of the peristome teeth. Peristome reductions obviously occurred several times independently across the whole Dicranidae.

Nevertheless, haplolepideous peristomes can be grouped into four main types, namely the dicranoid, seligerioid, syrrhopodontoid, and pottioid type (*cf.* Frey & Stech, 2009). The syrrhopodontoid and pottioid types seem to be synapomorphic for the monophyletic and well-supported Calymperaceae (except *Octoblepharum*) and Pottiaceae (but see discussion on *Timmiella* below), respectively. The peristome of *Octoblepharum* is reduced and consists of 8 or 16 entire teeth, with the formula **2(−3)**:2 referring to a single tooth, which makes inferences about relationships difficult (Edwards, 1979). In molecular phylogenies, *Octoblepharum* was either resolved as sister to the remaining (well-supported) Calymperaceae with low support (Tsubota *et al.*, 2003; Hedderson *et al.*, 2004) or separated from them (Tsubota *et al.*, 2004). These results indicate that *Octoblepharum* might be better placed in its own family Octoblepharaceae (e.g. Eddy, 1990; Ellis, 2007).

The expression of the pottioid peristome displayed by *Timmiella*, with 32 filamentous, spiculose, twisted teeth arising from a basal membrane, seemed to have evolved several times in different genera of Pottiaceae as well as in the molecularly distant *Timmiella* (molecular dataset by Werner *et al.*, 2004; re-analysed by Zander, 2006). However, Zander (2006) argued that the twisted peristome, similar to other morphological traits of *Timmiella*, is plesiomorphic and represents an example of homoiology, i.e. a gene cluster determining the existence of major organs that is highly adaptive and, once evolved, can be silenced and re-activated later in another phylogenetic lineage. In this interpretation, the twisted peristome of *Timmiella* and (other) Pottiaceae resulted from a 'deep' developmental homology (a shared deep ancestor with a twisted peristome), not on independent parallel evolution. Whether the development of the *Timmiella* peristome is in fact developmentally homologous to the twisted peristomes in (other) Pottiaceae remains to be investigated.

Taxa with seligerioid peristomes occur in Scouleriales, Grimmiales, Rhachitheciaceae, and Oncophoraceae (*Glyphomitrium*), which belong to different haplolepideous lineages (Cox *et al.*, 2010; Goffinet *et al.*, 2011). Similarly, taxa with dicranoid peristomes are found in several different families such as Ditrichaceae *p.p.*, Leucobryaceae, Dicranaceae *s.str.*, Fissidentaceae, and Oncophoraceae *p.p.* The seligerioid and dicranoid types could thus have evolved several times independently, or could represent artificial assemblies of different non-related peristome morphologies. The latter hypothesis is supported by the large variation especially of dicranoid peristomes with respect to the degree of incision and ornamentation of inner and outer surfaces of the peristome teeth, and the presence of peristomes putatively reduced from the dicranoid type, especially in Ditrichaceae (*cf.* Frey & Stech, 2009). Besides, the comparison of mainly seligerioid peristomes by Estébanez *et al.* (2002) showed that peristome movement in relation to histochemical properties can vary greatly between species of the same family, although certain properties (pectin distribution, stages with maximum quantity of phenolics) seemed to characterize the Grimmiaceae. Further comparative morphological, histochemical, and developmental analyses of selected taxa covering the diversity of dicranoid and seligerioid peristome types are clearly necessary to complement earlier studies (e.g. Edwards, 1979; Shaw *et al.*, 1989) and to infer the systematic relevance of characters in these peristome types.

Genus-level phylogenetics

Although phylogenetic analyses in the beginning of the 'molecular era' focussed on higher-level systematic relationships in mosses, most studies published so far tackled systematic and biogeographic relationships between and within genera or single species. Dicranidae comprise about 30% of the total moss species diversity. In the publication record, however, they seem to be underrepresented. Out of a total of 292 molecular systematic studies on mosses, only 65 (22%) deal with haplolepideous taxa (literature compiled in Stech & Quandt, 2010; extended by publications up to the end of 2010). Especially with respect to large genera, Dicranidae seem understudied. The 11 largest haplolepideous genera (100+ species each) are covered by only 18 more detailed molecular phylogenetic publications, six of which deal with *Campylopus* (Stech, 2004; Stech & Dohrmann, 2004; Stech & Wagner, 2005; Frahm & Stech, 2006; Stech *et al.*, 2007, 2010). Relationships within Grimmiaceae are already quite well-studied, with phylogenetic analyses of the largest genera, *Grimmia s.l.* (Streiff, 2006; Hernández-Maqueda *et al.*, 2007, 2008a,b), *Schistidium* (Ignatova *et al.*, 2009; Milyutina *et al.*, 2010), and *Racomitrium s.l.* (Larraín *et al.*, 2011), providing a basis for assessing taxon circumscriptions and relationships. Other large haplolepideous genera, such as *Fissidens*

(*c*. 440 spp.) or *Dicranella*/*Leptotrichella* (*c*. 220 spp.) remain almost unknown at the molecular level (Werner *et al.*, 2009). However, also quite well-studied genera such as *Campylopus* remain a challenge due to incongruence between morphological species circumscriptions and molecular data (Stech *et al.*, 2010 and references therein).

Unravelling relationships within Pottiaceae *s.str.* are particularly difficult because it is the largest moss family, with about 1425 species in 83 genera (Frey & Stech, 2009), and because it includes several large and taxonomically difficult genera (e.g. *Barbula*, *Didymodon*, *Hyophila*, *Syntrichia*, *Tortella*, *Tortula*, *Trichostomum*, and *Weissia*). For some of these genera such as *Barbula* and *Hyophila*, molecular data are almost unavailable. The few published sequences included in, e.g. Werner *et al.* (2004) and Köckinger & Kučera (2011), seem to indicate that *Barbula* is polyphyletic, but a combined molecular–morphological analysis is clearly needed. Other genera like *Didymodon* seem to be monophyletic, but their subgeneric taxonomy based on morphological characters is not supported by nrITS data (Werner *et al.*, 2005a). Especially complex is the circumscription of *Tortula*. While the available data support the view that a part of the species traditionally included in the genus *Pottia* are indeed morphologically reduced members of *Tortula*, also *Crossidium*, *Phascum*, *Pterygoneurum*, and *Stegonia* are part of a *Tortula s.l.* clade (Werner *et al.*, 2002), with several well-supported clades being formed by species of both *Crossidium* and *Tortula*. Aside from general considerations of how to treat such molecular topologies, further molecular phylogenetic analyses of *Tortula* and related genera based on additional markers should be performed. In contrast, *Syntrichia* is molecularly clearly separated from *Tortula*, although some species like *Tortula subulata* Hedw. show some similarity with *Syntrichia* on a morphological basis (Werner *et al.*, 2002, 2003). The subfamily Trichostomoideae is particularly complex at all taxonomic levels. On the one hand, molecular data in many cases contradict traditional generic delimitations, for example between *Weissia*, *Trichostomum*, *Pottiopsis*, *Tortella*, *Pleurochaete*, *Oxystegus*, *Chionoloma*, and *Pseudosymblepharis*. On the other hand, the genus *Weissia* seems to evolve extremely fast morphologically as compared with the degree of molecular variation. Even rapidly evolving molecular markers like nrITS show almost identical sequences in samples that some authors separated into different genera, e.g. *Weissia* [*Astomum*] *levieri* (Limpr.) Kindb. and *W. controversa* Hedw. (Werner *et al.*, 2005b). In summary, despite molecular efforts to resolve relationships of Pottiaceae at (supra-)generic level (e.g. Werner *et al.*, 2002, 2004, 2005a,b; Grundmann *et al.*, 2006; Zander, 2006), a more complete analysis of Pottiaceae, and especially of the larger genera, based on a comprehensive taxon and marker sampling, is still missing. Besides, several remarkable new species and genera were recently described based on molecular and/or morphological data (Hedderson & Zander, 2007, 2008a,b; Jiménez & Cano, 2007, 2008a,b; Gallego & Cano, 2007, 2009; Erdağ & Kürschner, 2009; Cano *et al.*, 2010; Jiménez *et al.*, 2010; Köckinger *et al.*, 2010; Akiyama & Goffinet, 2011; Zander & Hedderson, 2011), indicating that the total diversity within Pottiaceae is still insufficiently known.

Genetics and genomics

Apart from the most prominent 'genetic model moss' *Physcomitrella patens* (Hedw.) Bruch & Schimp. (Funariidae) (reviewed in Beike *et al.*, 2010), research on genetic mechanisms and genomic structure in mosses has so far mostly focussed on haplolepideous taxa, namely *Ceratodon purpureus* (Hedw.) Brid. and *Syntrichia* species. *Syntrichia ruralis* (Hedw.) F. Weber & D.Mohr is the second moss species, after *P. patens* (Sugiura *et al.*, 2003), from which the complete chloroplast genome was sequenced (Oliver *et al.*, 2010), and *Syntrichia* species are well-known as a model for research on sex ratio variation (Bowker *et al.*, 2000), sexual dimorphism (Stark *et al.*, 2001), and desiccation tolerance (e.g. Oliver *et al.*, 2005; Stark *et al.*, 2006). *Ceratodon purpureus* is amenable to mutagenesis and growth under laboratory conditions, and is widely used as a model for the study of developmental responses to light and gravity (Cove *et al.*, 1996; Sack *et al.*, 2001; Thornton *et al.*, 2005; Cove & Quatrano, 2006). Besides, *C. purpureus* is the only eukaryote, other than yeast and *P. patens*, that is known to undergo efficient gene targeting via homologous recombination (Brucker *et al.*, 2005; Trouiller *et al.*, 2007; Mittmann *et al.*, 2009). In work with natural populations, Jules & Shaw (1994) demonstrated that *C. purpureus* can adapt to growth on heavy metal containing soils, and Shaw & Beer (1999) and McDaniel (2005) conducted the most in-depth description of within and among-population quantitative genetic variation in a moss species in *C. purpureus* as well. The study of hybridization has a long history in the haplolepideous mosses, with several studies documenting hybrid sporophyte morphology and spore germination patterns in the families Ditrichaceae, Pottiaceae, Dicranaceae, and Grimmiaceae (reviewed in Natcheva & Cronberg, 2004). More recently, McDaniel *et al.* (2007, 2008) used a genetic map to dissect the genetic architecture of spore inviability and abnormal development in the progeny from a cross between a temperate and a tropical population of *C. purpureus*. Increasing the resolution of the phylogeny of

Dicranidae will enable evolutionary biologists to develop sophisticated tests for hypotheses of character correlations derived from developmental or population studies, as well as provide a critical framework for studying gene family and genome evolution across this group.

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Appendix: Voucher information and GenBank accession numbers of the specimens analysed in the present study

Species	Voucher	Acc. no. *rps*4-*trn*F	Acc. no. *atp*B-*rbc*L
Amphidiaceae			
Amphidium lapponicum (Hedw.) Schimp.	Ignatov 14.6.1989 (L)/ Kürschner 1-4647 (*herb.* Frey)	JQ690740	JQ690698
Amphidium mougeotii (Bruch & Schimp.) Schimp.	Frahm s.n. (BONN)	AF127187	AY159894
Aongstroemiaceae			
Aongstroemia longipes (Sommerf.) Bruch & Schimp.	Stech B970828.2 (L)	AF135091	JQ690700
Diobelonella palustris (Dicks.) Ochyra	Frahm s.n. (BONN)	AF135090	JQ690699
Archidiaceae			
Archidium alternifolium (Hedw.) Mitt.	Frahm s.n. (BONN)	AF135114	EU186597
Bryoxiphiaceae			
Bryoxiphium norvegicum (Brid.) Mitt.	Stech 04-242 (L)/Koponen 36664 (B)	JQ690736/AF135101	EU186590
Catoscopiaceae			
Catoscopium nigritum (Hedw.) Brid.	Stech B970828.13 (L)/Genbank	EU186545/AF497128	EU186592
Calymperaceae			
Calymperes erosum Müll.Hal.	Capesius s.n. (sterile culture)	JQ690739/DQ238541	JQ690702
Calymperes motleyi Mitt.	L0090735 (L)/Genbank	JQ690738/DQ238533	JQ690701
Syrrhopodon gardneri (Hook.) Schwägr.	Bryotrop project 7904 (BSB)	AF135087	JQ690703
Dicranaceae			
Chorisodontium wallisii Müll.Hal.	Frahm & Gradstein 300 (BONN)	AF135071	JQ690704
Dicranoloma plurisetum Müll.Hal. ex Dixon	Frey & Pfeiffer 98-T99 (CHR)	DQ462606	–
Dicranum polysetum Sw.	Stech B970518.1 (L)	AF129587	AY159895
Leucoloma procerum Renauld	Magill & Pócs 11222 (BONN)	AF135072	JQ690705
Paraleucobryum longifolium (Hedw.) Loeske	Stech B891114.1 (L)	AF135076	JQ690706

Species	Voucher	Acc. no. *rps*4-*trn*F	Acc. no. *atp*B-*rbc*L
Dicranellaceae			
Campylopodium medium (Duby) Giese & J.-P.Frahm	Eggers CEL2/3 (BONN)	AF135088	JQ690707
Dicranella cerviculata (Hedw.) Schimp.	Stech B970824.1 (L)	AF129597	EU186591
Dicranella heteromalla (Hedw.) Schimp.	Stech 08-380 (L)/Stech B960905.1 (L)	JQ690737/AF129596	–
Leptotrichella flaccidula (Mitt.) Ochyra	Schultze-Motel 3209 (B)	AF136637	JQ690709
Microcampylopus khasianus (Griffiths) Giese & J.-P.Frahm	Schäfer-Verwimp & Verwimp 20891 (BONN)	AY545564	JQ690708
Ditrichaceae			
Ceratodon purpureus (Hedw.) Brid.	N.N. (sterile culture)/Genbank	AF135096	EU053087
Cheilothela chloropus (Brid.) Broth.	Churchill et al. 13415 (B)	AF135097	JQ690710
Ditrichum flexicaule (Schwägr.) Hampe	Stech B890430.2 (L)/Genbank	AF135095	DQ397160
Pleuridium acuminatum Lindb.	Frey 1-4991 (*herb.* Frey)	EU186546	EU186596
Trichodon cylindricus (Hedw.) Schimp.	Düll 337/2[e] (B)	AF135099	JQ690711
Drummondiaceae			
Drummondia prorepens (Hedw.) E.Britton	Allen 6192 (L)	JQ690728	–
Erpodiaceae			
Aulacopilum cf. *abbreviatum* Mitt.	L0094498 (L)	JQ690730	JQ690712
Erpodium biseriatum (Austin) Austin	L0093906 (L)	JQ690729	–
Eustichiaceae			
Eustichia longirostris (Brid.) Brid.	L0472902 (L)	JQ690731	JQ690713
Fissidentaceae			
Fissidens bryoides Hedw.	Darmer 13107 (BSB)	AF135105	EU186586
Fissidens fontanus (Bach Pyl.) Steud.	Haapasaari 22.8.1997 (L)	AF135107	EU186585
Grimmiaceae			
Racomitrium canescens (Hedw.) Brid.	Kortselius 2008.11.0002 (L)	JQ690732	JQ690714
Schistidium apocarpum (Hedw.) Bruch. & Schimp.	Stech B970226.2 (L)	AF127185	EU186588
Hypodontiaceae			
Hypodontium dregei (Hornsch.) Müll.Hal.	L0472355 (L)	JQ690733	JQ690715
Hypodontium pomiforme (Hook.) Müll.Hal.	Viviers 105 (L)	JQ690734	JQ690716
Leucobryaceae			
Atractylocarpus alticaulis (Broth.) Williams	Frahm 8070 (BONN)	AF129592	JQ690717
Brothera leana (Sull.) Müll.Hal.	Koponen 37142 (B)	AF135077	JQ690719
Campylopodiella flagellacea (Müll.Hal.) J.-P.Frahm & Isoviita	Allen 9172 (BONN)	AF135078	JQ690718
Campylopus flexuosus (Hedw.) Brid.	Stech B960905.2 (L)	AF129593	AY159919
Dicranodontium denudatum (Brid.) Britt.	Frahm s.n. (L)	AF129591	JQ690720
Leucobryum juniperoideum (Brid.) Müll.Hal.	Frahm s.n. (L)	AF135084	JQ690722
Ochrobryum gardneri (Müll.Hal.) Mitt.	Allen 13706 (L)	JQ690735	JQ690721
Pilopogon africanus Broth.	Frahm 8079 (BONN)	AF129595	JQ690723
Oncophoraceae			
Cynodontium polycarpum (Hedw.) Schimp.	Stech B930721.2 (L)	AF129599	EU186595
Hymenoloma crispulum (Hedw.) Ochyra	Stech B970828.2 (L)	AF135074	JQ690724
Oncophorus virens (Hedw.) Brid.	Stech B960801.1 (L)	AF129598	EU186593
Oncophorus wahlenbergii Brid.	Stech B970828.3 (L)	AF135094	JQ690725
Oreoweisia bogotensis (Hampe) Mitt.	Philippi P-275 (B)	AF129600	JQ690726
Rhabdoweisia crenulata (Mitt.) Jameson	Frahm s.n. (BONN)	AF127181	EU186594
Pottiaceae			
Cinclidotus riparius (Host ex Brid.) Arn.	Stech B920517.4 (L)	EU186544	EU186587
Syntrichia ruralis (Hedw.) F.Weber & D.Mohr	Genbank	FJ546412	FJ546412
Tortula muralis Hedw.	Stech B970226.3 (L)	AF135108	AY159892
Ptychomitriaceae			
Ptychomitrium polyphyllum (Sw.) Bruch & Schimp.	Stech 04-040 (L)	EU186542	EU186583
Seligeriaceae			
Blindia acuta (Hedw.) Bruch & Schimp.	Frahm s.n. (L)	AF135109	JQ690727
Timmiaceae (outgroup)			
Timmia austriaca Hedw.	Stech B970831.4 (L)/Genbank	EU186543/AF229892	EU186584
Encalyptaceae (outgroup)			
Encalypta streptocarpa Hedw.	Stech B060412.2 (L)/Genbank	EU186541/HM148898	EU186582