

Phylogenomic time tree of bryophytes resolves 500 million years of diversification

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Abstract – 200 words

Bryophytes are emerging as the sister-group to extant vascular plants, and their current diversity highlights that their life cycle characterized by a dominating vegetative gametophyte and an unbranched sporophyte composes a successful alternative to that of vascular plants and their dominating sporophyte. The evolutionary history of hornworts, liverworts and mosses remains, however, poorly resolved, due in part to the small character space from which to draw shared ancestry. Our understanding of the diversification of these lineages has been significantly reshaped by inferences from molecular data, highlighting extensive homoplasy in various traits and repeated bursts of diversification. Here, we present the first bryophyte time-tree based on 228 nuclear genes sampled via target capture for 531 species with divergence times estimated based on a comprehensive set of fossil calibrations. Our phylogenetic trees reflect a robust backbone, highlight novel ordinal relationships and circumscriptions, and the recognition of 13 new orders of liverworts and mosses. Our time-tree reveals that most orders originated prior to the Cretaceous, confirms the Cretaceous radiation of the hyperdiverse hypnalean mosses and species-rich liverwort clades. This comprehensive resource provides a robust and expandable phylogenomic framework for evolutionary analyses across lineages in this diverse and important group of land plants.

Keywords: Community resource, Cretaceous diversification, Hornworts, Land plants, Liverworts, Mosses, Phylogenomic, Rapid diversification.

Introduction

The emerging consensus that bryophytes, which comprise hornworts, liverworts, and mosses (Fig. 1), form the sister group to extant vascular plants^{1,2} raises important questions about the morphology and life cycle of the first land plants³. Key elements of land plant development, including meristem function⁴, stomatal development⁵, and hormone responses^{6,7}, are built from a conserved genomic blueprint⁵, and bryophytes hold signatures of divergence and reduction from an ancestor with at least a morphologically more complex sporophyte⁸. Bryophytes are important components of various ecological communities and contribute to local nutrients fluxes and global biogeochemical cycles^{9–11}. Broad evolutionary analyses are key for assessing the significance of vegetative gametophyte trait in these roles. Reconstructing the history of bryophytes and hence of character transformations are therefore critical for understanding the transformations of developmental, physiological and functional morphological traits across land plants^{1,8,12}.

Unraveling the relationships and time of divergence among major lineages of bryophytes has been hampered by the putative low morphological character space, the high homoplasy in the evolution of these traits, the shortage of phylogenetically informative ontogenetic data, the paucity of fossil taxa and the difficulty in reliably assigning these to lineages defined by extant taxa. Furthermore, phylogenetic inferences suffer from insufficient signal, especially across major lineages, due perhaps to their old divergence, and hence substitutional saturation or their rapid diversification. All prior reconstructions rely on either a few loci sampled for perhaps many species, or many markers sampled for a few bryophyte taxa^{8,13–16}.

We present the first comprehensive phylogeny of bryophytes based on 228 markers sampled for 531 species from across all orders, using the GoFlag 408 probe set. We then calibrated this phylogeny based on the most recent assessments of the affinities of liverwort¹⁷ and moss¹⁸ fossils to estimate divergence times. We acquired homologous sequences for virtually all samples and recovered strong support for many relationships between classes and orders. We confirm the persisting phylogenetic ambiguity for others and propose three new liverwort and ten new moss orders. As in vascular plants, significant conflict among genes both within rapidly diversifying groups of bryophytes and among the oldest nodes is likely due to incomplete lineage sorting, ancient hybridization, and lack of signal in explosive radiations. We have unraveled the macroevolutionary history of this key group of land plants using the GoFlag 408 probe set, which emerged as a powerful tool to tackle the complexity of the diversification process at multiple phylogenetic scales across the bryophytes¹⁹.

Results and Discussion

We sampled 533 samples representing 531 species from 499 genera distributed among 72% of families from all 69 orders of bryophytes (Figs 1–3; Extended Data Figs. 1–7; Supplementary Figs. S1–S9; Supplementary Tables S1–S11). The alignment of sequences from 228 targeted nuclear genes varies in length, variable sites, potentially parsimony informative sites and GC content (Supplementary Table S4). The average alignment length was 339.74 bp (min=120, max=1459) with most

($n=146$) over 200 bp long. Parsimony informative sites were correlated with alignment length ($R^2 = 0.9593$) with a mean of 238.42 sites per alignment (min=66, max=1065) and a low percentage of missing data (8.61%, SD=0.43).

Coalescent-based (ASTRAL) and maximum likelihood (ML) concatenated super-matrix analyses (IQ-TREE) of nucleotide and amino-acid data yielded similar results (Extended Data Figs. 1-7). In the following sections, we present the results from the ASTRAL analyses of nucleotide and amino-acid data including quartet values and gene concordance factors (gCF) and site concordance factors (sCF). We refer to a majority of gene trees when more than 50% of these share a given signal, and a plurality of gene trees when the proportion of trees supporting the topology is higher than 33% but less than 50%. The gene concordance factor (gCF) and site concordance factor (sCF) represent the percentage of decisive gene trees and sites containing a specific branch²⁰. ASTRAL quartet values and IQ-TREE concordance factors for nucleotide and amino-acid analyses are presented in the Supplementary information (Supplementary Figs. S1–S8; Supplementary Tables S5–S8).

Phylogenetic relationships within hornworts: The circumscription and relationships of classes and orders within the hornworts, Anthocerotophyta (Fig. 2 and Extended Data Figs. 1 and 4) reflect those inferred from organellar markers and a broad taxon sampling¹³ except for the affinities of the monotypic Leiosporocerotales. The monophyly of subclasses and orders is typically supported by a large majority of loci, whether inferences are based on nucleotides (Extended Data Fig. 1; Supplementary Figs. S1 and S2) or amino-acid data (Extended Data Fig. 4). By contrast, the relationship of *Leiosporoceros* is ambiguous, as it is resolved

as sister to Anthocerotales (i.e., *Anthoceros*) based on nucleotide gene trees (ASTRAL) (Fig. 2 and Extended Data Fig. 1) or to all hornworts based on concatenated nucleotide data (Supplementary Fig. S5) or with ambiguous affinities based amino-acid gene trees (Extended Data Fig. 4).

The position of *Leiosporoceros* as sister to all other hornworts was previously proposed based on few organellar loci sampled for a large set of taxa^{21–23} and from over 400 loci sequenced for selected hornwort placeholders^{19,24}. The novel phylogenetic hypothesis in the ASTRAL analysis would impact ancestral state reconstructions for the hornwort pyrenoid: if *Leiosporoceros* is sister to all hornworts, the pyrenoid-less state would be predicted as the ancestral condition in hornworts¹³, whereas under the alternative topology the presence of the pyrenoid may be ancestral in hornworts with subsequent losses in *Leiosporoceros*. Under either scenario, pyrenoids have been independently lost in all hornwort orders, and gained in the derived Dendrocerotales¹³.

Phylogenetic relationships within liverworts: The earliest split within liverworts segregates the Haplomitriopsida (21 spp.) from the ancestor to the Marchantiopsida (mostly complex thalloid taxa, ca. 543 spp.) and the Jungermanniopsida (simple thalloid and leafy liverworts, over 6870 spp.), a topology supported by a small majority of loci (Fig. 2; Extended Data Figs. 2 and 5). Within the three classes, ordinal circumscriptions and relationships generally reflect prevailing interpretations^{14,25–28}, with most backbone nodes supported by a majority of loci and high posterior probabilities (Fig. 2 and Fig. 3; Extended Data Figs. 2 and 5).

The Haplomitriopsida comprise plants with leaf-like appendages, stems secreting copious mucilage and one primary androgonial initial in early ontogeny²⁹. The earliest split in the Marchantiopsida segregates the Marchantiidae (complex thalloids) from the Blasiidae, a lineage of two simple thalloid species with endophytic cyanobacteria^{30,31}. The Marchantiidae typically develop a complex thallus with air chambers, four primary androgonial initials in early ontogeny, and unlobed sporocytes²⁹. The ordinal relationships within the Marchantiidae vary in support (Fig. 2 and Fig. 3; Extended Data Figs. 2 and 5). The Neohodgsoniales comprise a single species from New Zealand, diagnosed by a uniquely branched carpocephalum, and mark another deep split in the subclass²⁷. The Sphaerocarpales comprise mostly taxa with leafy-like gametophytes and are sister to the Marchantiales, although not supported by most gene trees (Fig. 2 and Fig. 3; Extended Data Figs. 2 and 5). The Marchantiales contain the majority of the species of the class (ca. 497 spp.), including the model species *Marchantia polymorpha*³².

The Jungermanniopsida include species developing simple thalloid or leafy vegetative bodies, all with antheridia developed from two primary androgonial initials in early ontogeny and with lobed sporocytes²⁹. The Jungermanniopsida are typically accommodated in three subclasses, whose monophyly and relationships are not consistently resolved (Figs. 2 and 3) perhaps due to ancient introgression³¹. The Pelliidae comprise two robustly monophyletic orders (i.e., Pallaviciniales and Pelliales) and the Fossombroniales, which are supported by most nucleotide gene trees but only a small plurality of amino-acid loci (Extended Data Figs. 2 and 5). The relationships among the three orders are congruent only among a plurality of gene trees (Extended Data Figs. 2 and 5), with the paraphyly of the subclass caused by the resolution of the Pelliales sister to all other Jungermanniopsida based on

nucleotide data²³ (Figs. 2 and 3; Extended Data Fig. 2) versus sister to other Pelliidae based on amino-acid data with ambiguous signal (Extended Data Fig. 5). Such conflicting topologies were previously recovered based on nucleotide-based nuclear transcriptome and mitochondrial data²⁴, and plastid data^{28,33}, respectively, and may reflect a rapid divergence of the Pelliidae, ancient introgression, incomplete lineage sorting and subsequent saturation in nucleotide data.

The Metzgeriidae compose a robustly monophyletic group comprising the Metzgeriales and Pleuroziales (Fig. 2; Extended Data Figs. 2 and 5), as previously resolved based on a few DNA loci^{25,34,35}, and genomic, transcriptomic and organellar data^{28,33}. The monophyly of the Metzgeriidae is supported by the unique shared lenticular apical cell giving rise to the bilaterally symmetric and thalloid body of the Metzgeriales and the leafy stem of the Pleuroziales²³.

The vast majority of liverworts (ca. 6160 spp.) belong to the well supported clade Jungermanniidae or true leafy liverworts (Figs. 2 and 3; Extended Data Figs. 2 and 5). A shared ancestry of the Ptilidiales and Porellales *sensu lato*, as inferred from nuclear nucleotide data (Fig. 2; Extended Data Fig. 2) was recently argued for based on phylotranscriptomic and plastid evidence³³. By contrast, our amino-acid data resolve the Ptilidiales as sister to the Jungermanniales *s. lato* (Extended Data Fig. 5), a hypothesis also supported by mitochondrial data^{23,33} and prior plastid-based inferences^{25,28}. A majority of genes recover the monophyly of the Porellales *s. lato*, which we propose to accommodate into three resurrected and two new orders (Table 1), each of pre-Cretaceous origin: Frullaniales, Jubulales, Lejeuneales Porellales, and Radulales (Fig. 2; Extended Data Figs. 2 and 5). The Frullaniales (ca. 599 spp.) and Lejeuneales (ca. 1900 spp.) comprise two of the most species-

rich families of liverworts characteristic of epiphytic communities, particularly in tropical rainforests¹⁵.

Within the Jungermanniales *s. lato*, the deepest splits giving rise to the main lineages are estimated to have occurred in or prior to the Triassic, and we therefore propose to recognize these at the ordinal level, resurrecting Perssoniellales³⁶, Lepidoziales and Lophoziales and erecting Myliales (Table 1). The Perssoniellales (ca. 88 spp.) emerged from the earliest split (Fig. 2) in the Carboniferous (Fig. 3) and are sister to the common ancestor shared by the remaining others. The Myliales (5 spp.) mark the next split based on nucleotide data (Figs. 2 and 3; Extended Data Fig. 2) or are sister to the Lophoziales only, based on amino-acid data, although with low support (Extended Data Fig. 5). Either resolution is incongruent with *Myliia* sister to the Jungermanniales *s. str.* based on nuclear transcriptomic and organellar data³³, a hypothesis based, however, on a mislabeled specimen (Y. Liu, pers. com. March 2023).

A majority of nucleotide, and at least a plurality of amino-acid, gene trees resolve the remaining orders of the Jungermanniales *s. lato*, i.e., the Lophoziales (ca. 611 spp.), Lepidoziales (ca. 1943 spp.) and Jungermanniales *s. str.* (ca. 565 spp.) as monophyletic (Extended Data Figs. 2 and 5, respectively). All three suborders are highly heterogenous in terms of leaf morphology (2–4 lobes), branching patterns, form, and position of gametangia³⁷. The sister-group relationship of the latter two suborders most recently supported by nuclear transcriptomic and organellar data³³, emerges here also from a near majority of nucleotide loci but only a weak plurality of amino-acid loci (Extended Data Figs. 2 and 5, respectively). The resolution within suborders seems to vary, especially within species-rich families

(e.g., Lepidoziaceae) and may point to a period of rapid diversification (Extended Data Figs. 2 and 5).

Phylogenetic relationships within mosses: The phylogenetic structure in mosses (Fig. 2; Extended Data Figs. 3 and 6) is congruent with most current supraordinal circumscriptions and ordinal relationships and shaped primarily by innovations in sporangial dehiscence, peristome architecture and development, and by vegetative body plant organization relative to sex organ position^{29,38}. The circumscription and relationships of classes and subclasses sensu^{29,38,39} are typically supported by a majority or a plurality of loci (Fig. 2; Extended Data Fig. 3) or their amino-acid translations (Extended Data Fig. 6), while concordance among nucleotide loci is typically low for clades following the initial splits within the Bryophyta (Fig. 2; Supplementary Tables S5 and S7).

The current classification¹¹ accommodates mosses into four subdivisions, which are here recovered as emerging from the deepest splits, with the Takakiophytina sister to all other extant mosses, followed by the Sphagnophytina and the Andreaeophytina, which comprise the two lineages of lantern mosses (i.e., Andreaeales and Andreaebryales) and are sister to the remaining mosses composing the Bryophytina (Fig. 2; Extended Data Figs. 3 and 6). The latter comprise species potentially developing stomata on their sporangia and a peristome, a set of teeth lining the sporangial mouth and controlling spore dispersal¹². Thus, the lack of stomata in the other subdivisions is considered to result from independent losses⁵, which given the topology occurred independent in all three bryophyte lineages.

Mosses developing peristome teeth made of whole cells (i.e., nematodontous peristome, Fig. 1K) compose a monophyletic group, the Polytrichopsida s. str., as previously suggested³⁸. The monospecific Oedipodiales were resolved either as sister to all peristomate mosses in a small plurality of nucleotide gene trees (Fig. 2, Extended Data Fig. 3) and congruent with signal from organellar loci³⁸, or only to the Polytrichopsida in a small plurality of amino-acid gene trees⁴⁰ (Extended Data Fig. 6). The ambiguous placement of the Oedipodiales from nucleotide data likely emerges from a combination of a relatively rapid divergence (Fig. 3) and saturation in nucleotide substitution, as the more conserved amino-acid data more robustly resolve the Oedipodiales as sister to the Polytrichales and Tetraphidales, similarly to inferences from a distinct set of hundred nuclear loci³⁸.

The remaining mosses (i.e., Bryopsida) typically develop a peristome composed solely of cell plates rather than whole cells (Fig. 1L). Such joined and hence articulate teeth lining the sporangial or capsule mouth upon dehiscence likely constitute a key innovation that optimizes and controls spore release and is developed by the vast majority of mosses. Several arthrodontous peristome types arise from distinct developmental sequences¹² and these largely define monophyletic groups³⁸. However, the resolution of *Catoscopium* as sister to most Dicranidae may call for an amendment of *Dicranum*-peristome type diagnosis⁴¹. Although mature peristomes may be variably reduced, especially along environmental gradients⁴², the fundamental architecture and ontogeny seem highly conserved during the diversification of mosses.

The vast majority of mosses belong to the Bryidae, a lineage diagnosed by its double peristome of alternating inner and outer teeth⁴³. The subclass comprises the

most speciose superorder of mosses, the Hypnanae or pleurocarpous mosses (ca. 4903 spp.), which are characterized by a homogenous anatomy of their midrib, the lateral development of female sex organs, and hence of the sporophyte on the maternal gametophyte^{44,45}. The Hypnanae comprise five orders (i.e., Hypnodendrales, Ptychomniales, Hypopterygiales, Hookeriales and Hypnales), whose relationships match those previously recovered³⁸ but are here, too only supported by a plurality of loci (Fig. 2; Extended Data Figs. 3 and 6). While a lateral shift of the sex organs and the associated shift to monopodial growth has occurred in other moss lineages⁴⁶, such as the Orthotrichales, the persistence of lateral female sex organs in all Hypnanae suggest that this trait is either under strong genetic constraint or selection (see ^{47,48}). Within the Hypnales, the most diverse order of mosses⁴⁹ the backbone relationships are typically shared only by a plurality of gene trees (Fig. 2; Extended Data Figs. 3 and 6), likely reflecting a rapid early radiation of the order⁵⁰. The recurrent phylogenetic pattern of species-rich families being either paraphyletic due to the inclusion of additional, morphologically distinct lineages, or polyphyletic following their phylogenetic fragmentation, and the resolution of various species poorer lineages across the backbone phylogeny, reveals that morphological traits, and primarily the vegetative ones, are evolutionarily highly labile within the Hypnales. Transformations of putative homologous traits appear highly homoplasious, and potentially correlated, yielding morphologically highly heterogenous clades. Such homoplasy may even span across subclasses, as reflected by *Sorapilla*, the sole member of Sorapillaceae, and which was originally placed in Dicranidae, but was then moved to the Hypnales⁵¹. Based on the first DNA sequences obtained for this genus, we resolve the family in the Dicranidae as proposed earlier^{52,53} but in an isolated position within the Dicranalean grade (Fig. 2;

Extended Data Figs. 3 and 6), prompting the proposal for a new order, Sorapillales (Table 1).

The Hypnanae emerged from the acrocarpous Bryidae that typically develop terminal sex organs. The Bryidae are consistently recovered as paraphyletic across all present (Fig. 2; Extended Data Figs. 3 and 6) and prior³⁸ analyses. While all orders are recovered with strong support, the relationships among them are typically supported only by a plurality of loci (Fig. 2; Extended Data Figs. 3 and 6). The relationships among the Rhizogoniales, Orthotrichales, Orthodontiales and Aulacomniales inferred here from nucleotide data (Fig. 2; Extended Data Fig. 3) match those previously proposed³⁸ based on amino-acid sequences. However, the present protein-based topology (Extended Data Fig. 6) differs in that a clade comprising some members of the Rhizogoniales, along with *Hymenodontopsis*, a member of the Aulacomniaceae, are sister to the Hypnanae, whereas the Orthotrichales, Orthodontiales and Aulacomniales, and *Goniobryum* (a member of the Rhizogoniaceae) compose a single lineage sister to the Rhizogoniales and Hypnanae (Extended Data Fig. 6). The relationships of these orders are in all cases supported only by a small plurality of loci. Furthermore, inferences from nucleotide versus amino-acid data resolve incongruent circumscriptions of the Rhizogoniales. *Calomnion*, a genus of a few South Pacific Island species⁵⁴ is reconstructed as sister to the Hypnanae except Hypnodendrales or all Hypnanae based on nucleotide versus amino-acid data, respectively (Fig. 2; Extended Data Figs. 3 and 6). In none of the alternative topologies are the relationships supported by a majority of loci, and hence the circumscription of the Rhizogoniales remains uncertain. The early diversification of the Bryidae gave rise to the Bartramiales, Bryales, Hedwigiales, and Splachnales, which compose robust lineages but of uncertain relationship. The

Helicophyllaceae (Hedwigiales), diagnosed by plagiotropic shoots and dimorphic leaves⁵⁵, are robustly resolved as sister to the Bartramiaceae (Figs. 2 and 3; Extended Data Figs. 3 and 6; see also ⁵⁶).

Within the Dicranidae, the sister group to the Bryidae, the relationships are congruent with previous hypotheses³³ and strengthen the hypothesis of a broadly polyphyletic Dicranales. The earliest splits within the subclass gave rise to the Catoscopiales, followed by the here erected Distichiales and Flexitrichales (Table 1). The latter holds only the Flexitrichaceae, a family here formally recognized (Table 1), and is likely sister to a here not sampled Scouleriales (sensu ⁵⁷; Fig. 2). The next split yields the Grimmeriales, sister to the clade composed of the Archidiales, the dicranalean grade and the Pottiales (Fig. 2; Extended Data Figs. 3 and 6). The circumscription of the Archidiales Limpr. is here broadened to include the Micromitriaceae and Leucobryaceae, as the Archidiaceae are nested between the latter two families (Fig. 2; Extended Data Figs. 3 and 6)⁵⁸.

The Dicranales are consistently resolved as a grade leading to the well supported Pottiales (Fig. 2; Extended Data Figs. 3 and 6), a hypothesis congruent with prior phylogenetic studies^{38,58,59}. To integrate the phylogenetic resolution of the dicranalean grade into the current classification, we propose to restrict the Dicranales (Fig. 2) to only the Dicranaceae, Calymperaceae, Fissidentaceae and Octoblepharaceae, and accommodate the remaining families, which all have an origin no later than the Jurassic (Fig. 3), in the Amphidiales, Bruchiales, Ditrichales, Erpodiales, Eustichiales, Pleurophascales and Rhabdoweisiales (Table 1). Despite these systematic novelties, various families of the Dicranales *s. lato* remain unassigned (e.g., Amphidiaceae, Distichiaceae, etc.) awaiting further study.

Our phylogenomic inferences confirm that the Timmiidae, restricted to *Timmia* characterized by a unique peristomial architecture⁶⁰, compose the sister lineage to the ancestor of the Dicranidae and Bryidae³⁸. The Funariidae, which include the model taxon *Physcomitrium patens*⁶¹ along with the Diphysciidae and Buxbaumiidae, each with a distinct peristome type compose the basal grade within the Bryopsida. Thus, the main transformations of the arthrodontous peristome, yielding the various fundamental types, occurred during the Permian or even late Carboniferous (Fig. 3) rather than in the first half of the Paleozoic as previously proposed (Laenen et al. 2014).

500 million years of bryophyte diversification

Divergence time estimates of the first comprehensive phylogenomic reconstructions of bryophytes confirm that they arose in the Ordovician¹⁶. Speciose lineages arose during the diversification of angiosperms in the Cretaceous (Fig. 3; Extended Data Figs. 7, 10). The common ancestor to extant lineages of mosses and liverworts is traced to 420 [416–424] Ma and 447 [444–450] Ma, respectively, which is consistent with recent estimates^{8,13,15,16,44}. The lineage through time plots (Fig. 3B) suggest a rather steady diversification of both lineages over the last 400 My with a higher net moss diversification near mid Cretaceous. The average crown age of orders as recognized here across all three bryophyte lineages is 150.5 Ma (SD 70 Ma) (Fig. 3C; Supplementary Table S10). The crown age of the most species rich bryophyte order, the Hypnales (491 genera, ca. 4150 species) is 129 [78–173] Ma, and thus younger than previously estimated⁶². The origin of mosses and liverworts (419–447 Ma, crown group Setaphyta) seem to be slightly older than that of extant lycophytes and their sister group, the euphyllophytes¹⁶. The age of most bryophyte

orders coincides with the diversification of angiosperms during the Late Jurassic and Cretaceous⁶³.

Our estimates of family stem ages suggest that most bryophyte families (63% of families, n=166) originated during the Cretaceous (65 families) and Jurassic (40 families). Extant bryophyte families (crown ages, n=80) diversified predominantly during the Late Cretaceous (66–102 Ma, 27 families) and the early Cretaceous (103–145 Ma, 18 families) and the Cenozoic (–65 Ma, 26 families). The average crown age of bryophyte families is 98 Ma (SD 51 Ma) (Extended Data Fig. 10; Supplementary Table S10; Supplementary Fig. S9). In mosses, the crown age of the Pottiaceae (77 genera, ca. 1,209 species) is estimated at 133.3 [115–150] Ma and the Orthotrichaceae (24 genera, ca. 900 species) started to diversify around 139.6 [106–169] Ma. Within liverworts, the diversification of the hyperdiverse Lejeuneaceae (ca. 88 genera, ca. 1900 species) began around 149 [116–188] Ma while that of the Lepidoziaceae (31 genera, ca. 703 species) dates to 121 [114–166] Ma. As previously suggested for bryophyte genera¹⁴, bryophyte diversity may have accrued during the Cretaceous and Cenozoic¹⁴ (Fig. 3C). Our analyses support the Cretaceous terrestrial revolution^{63,64} wherein the majority of bryophyte families diversified during this period (Fig. 3; Extended Data Fig. 10; Supplementary Table S10; Supplementary Fig. S9) contributing to an explosive radiation of life. The Cretaceous expansion of the angiosperm canopy and diversification of leptosporangiate ferns⁶⁵ potentially provided novel habitats for bryophytes to diversify in tropical and subtropical areas. In addition, global and local extinctions, and the climatic fluctuations during the Cretaceous and Cenozoic may explain the burst of diversity in lineages occupying new niches in arid regions (e.g., Pottiales,

Marchantiales) or open areas (e.g., Funariales, Polytrichales) before the explosive diversification of angiosperms and ferns.

Conclusions

Bryophytes are the second-most diverse group of land plants, compose the sister group to extant vascular plants^{8,24} and contribute significant ecosystem functions in communities where they are abundant. Bryophytes are thus critical to understand the polarity of character transformations early in the evolution of land plants, and contemporary ecological processes, in particular, local and global nutrient flows. In parallel to the loss of complexity of the vascular plant gametophyte, this puts renewed importance on comparative studies of gametophyte morphology and function. The ordinal-level phylogeny of the bryophytes, based on the GoFlag408 probe set, provides an expandable resource upon which to build such studies.

Our results are largely consistent with previous efforts based on fewer loci or representative taxon sampling, with some interesting exceptions (Fig. 2) suggesting that the bryophyte phylogenetic studies are converging on a relatively stable set of relationships. The incongruence found in several nodes along the backbone implies that different genes coalesce to variable and conflicting topologies, especially in recent radiations (e.g., Hypnales), or are remnants of potential ancient introgressions³³ (Fig. 3; Extended Data Figs. 8 and 9) or incomplete lineage sorting. The current challenge facing the community is to fill in unsampled genera, species, or populations of focal taxa. At shallower phylogenetic depth, the data could be expanded to the flanking intron and spacer regions that are generally more variable

than the exonic regions, and hence suitable for resolving population genomic problems within or between populations of single species, or groups of closely related species. The growing GoFlag 408 database of sequences used here, and in other studies, provides a sturdy scaffold in which to place more focused studies.

Methods

Taxon sampling

We sampled in total 531 bryophyte species belonging to 499 genera (Supplementary Table S3), comprising 362 moss species representing 338 genera (34 %); 159 liverwort species that can be assigned to 151 genera (37 %), and 12 hornwort species from 10 genera (100 %). Sampling percentage values were calculated using recent checklists and classification literature^{66–68}. Hornworts were assumed to be the sister group of the other two bryophyte clades based on²⁴.

DNA Extraction

We used the modified cetyltrimethylammonium bromide (CTAB) extraction protocol⁶⁹ described in¹⁹ for most of the samples. This includes lysing the cells by centrifuging them and performing two rounds of chloroform/isoamyl 24:1 wash followed by cold isopropanol precipitation and an ethanol 70% wash. Finally, we added 2 µl of 10 mg/ml RNase A (QIAGEN, Valencia, California, United States) to remove RNA contamination between chloroform washes. For a few specimens (Lejeuneaceae)

the Invisorb Spin Plant Mini Kit (Strattec Molecular GmbH, Berlin, Germany) was used following the kit protocol.

Target Enrichment and Sequencing Assembly

We generated a multi-locus nuclear sequence dataset with a target enrichment approach using the GoFlag probe sets. We assembled published data from 36 samples generated using the GoFlag 451 probe set and generated new data from 497 samples using the GoFlag 408 probe set, an optimized subset of the GoFlag 451 probe set consisting of 53,306 probes covering 408 exons from single or low copy nuclear loci. The library preparation, target enrichment, and sequencing were done by RAPiD Genomics (Gainesville, FL USA) using protocols described in ¹⁹, with the enriched, pooled libraries sequenced on an Illumina HiSeq 3,000 platform (Illumina; 2 × 100 bp). The paired end raw reads are available in the NCBI SRA database. We assembled phylogenetic sequence alignments for each locus using the iterative baited assembly pipeline¹⁹. In some loci, the pipeline retains more than one copy per sample, where the Bridger⁷⁰ assembler interprets greater than simple allelic variation. To control this, we removed all copies of a sample in a locus alignment (script "rmall.pl"). Second, we removed any columns in the alignments that had nucleotide data from fewer than ten samples in all output PHYLIP files. We also pruned out sequences associated with excessively long branches in the locus trees. First, we inferred maximum likelihood (ML) trees from the locus alignments using RAxML using the GTR CAT model, and we rooted the resulting gene trees with Newick Utilities⁷¹ using the hornworts as the outgroup. We eliminated one locus for which hornworts were not monophyletic. For the others, we calculated the root to tip

distance for each taxon and pruned out any sequences that had a root to distance >3 standard deviations more than the average for that locus. This removed 750 out of 178,650 sequences. We then for each locus examined the trees with branch lengths >1 and removed 35 additional long branch sequences. All loci correspond to exons, and some which are part of the same gene¹⁹. Thus, to create gene alignments, we concatenated any loci found in the same gene, resulting in 228 gene alignments that were used in our analyses. We also created amino-acid alignments for each gene. To do this, we took the original locus alignments and removed any columns sequence alignments with only one nucleotide, most likely representing sequencing error. We used AliView⁷² to make edits by hand to put the alignments in frame; usually this required minimal editing to getting the alignments in line. We then saved the corresponding amino-acid alignments and concatenated the loci from the same gene.

Phylogenomic analyses

We performed Maximum Likelihood phylogenetic inferences of the concatenated nucleotide dataset implemented in IQ-TREE v.2.1⁷³. Loci located within the same gene were concatenated and the dataset was partitioned by genes (228 genes) and were used for nucleotide and amino-acid analyses. The best-scoring ML tree and a rapid bootstrap analysis with 100 replicates was run. In all cases a GTRGAMMA model of sequence evolution was chosen. Moreover, we used IQ-TREE v.2.1⁷³ for phylogenetic inference on the translated matrix dataset (into amino-acids), with “-S option” that load all alignment files within the directory. We performed auto model

selection (ModelFinder) and tree inference separately for each gene. Branch support was assessed using ultrafast bootstrapping (1000 replicates)⁷⁴.

We conducted species tree analyses using ASTRAL-III v.5.7.7⁷⁵. Gene trees were estimated in RAxMLv.8.2.9^{76,77} under the following settings for the nucleotide dataset: the default RAxML tree search algorithm (-f d) was implemented and a GTRGAMMA model selected. The loci belonging to the same gene were combined prior to the gene tree estimation, resulting in 228 unrooted gene trees. For amino-acids dataset gene trees were estimated in IQ-TREE v.2.1, with “-S option” (see above). ASTRAL-III was employed for species tree inference on both gene tree datasets using the default settings and computing local posterior probabilities⁷⁸ as well as quartet scores for all three resolutions per branch (-t 8). The resulting ASTRAL topologies were annotated with quartet support values⁷⁸ for the main topology (q1), the first alternative topology (q2), and the second alternative topology (q3). We also used the built-in functionality of ASTRAL to compute the percentage of gene trees that agreed with each branch in the species tree, by finding the average number of gene-tree quartets defined around the branch (choosing one taxon from each side) that were congruent with the species tree. Similar phylogenetic analyses were conducted on the translated matrix (into amino-acids). We used DiscoVista⁷⁹ to visualize the results.

In addition to bootstrap (IQ-TREE) and local posterior probabilities (ASTRAL), we implemented the gene (gCF) and site (sCF) concordance factors to investigate topological conflict around each branch of the species tree for the nucleotide and amino-acid datasets in IQ-TREE, using the “-gcf and -scf ” options²⁰, particularly for

the backbone nodes. Likewise, we compared the species and gene trees generated with ASTRAL (gCGAst, sCFast, sDFPAst) and IQ-TREE (maximum likelihood, gCGmL, sCFmL, gDFPmL) to evaluate possible divergences. These analyses measured every branch of the species tree; the gCF and sCF represents the percentage of decisive gene trees and sites. The program also estimates gDFP (gene discordant factor due to paraphyly) or the gene discordance factor due to lack of information in the genes or paraphyly of the quartet. For measures of support gene concordance factor/site concordance factor (gCF/sCF) was categorized as follows” weak < 33, moderate 33–50, strong > 50 (following ^{20,80}). Using the ASTRAL output, we analyzed whether the discordance among gene trees or sites regarding the neutral ILS model implemented in IQ-TREE. We carried out χ^2 tests comparing the number of trees or sites supporting discordant topologies with roughly equal quartet values. Under the assumption of ILS, the discordant topologies should be supported by an equal number of gene trees or sites (Supplementary Table 12). Equal quartets, gcf or scf (~33%) for the tree quartets point out to incomplete lineage sorting or ancient introgression²⁰. We performed correlation analyses between branch lengths, divergence times (see below) and concordance factor values using linear regressions.

Divergence time estimation

Divergence time estimation was performed in treePL⁸¹ according to the analysis guidelines of ⁸². The best-ML tree as input for treePL was selected from the maximum likelihood analysis (see above). In order to be able to infer node bars on the dated tree, a set of bootstrap replicates were run through the

“Bootstrap+Consensus” workflow of RAxML-HPC2 on XSEDE on the CIPRES Science Gateway⁸³, whereby the best ML tree served as constraint, the option to print branch lengths on the BS replicates was selected, a partition scheme by genes (i.e., loci were grouped together according to their relatedness to genes) was implemented as well as a GTR+G substitution model and 1000 bootstrap iterations. All trees were rooted with hornworts set to be the sister group of other bryophytes, using the program pxrr in phyx⁸⁴ that was also used in the dating analysis. We calibrated 16 liverwort and 13 moss nodes stemming from fossil evidence (Supplementary Table S2) and implemented a maximum age constraint of the root of 515 Ma¹⁶. Priming and cross-validation analyses were performed using the best-ML tree and all 29 calibrations. Best optimization parameters were indicated as the follows: opt = 2, optad = 2, optcvad = 5. Cross-validation used these parameters and was done four times, indicating stable values of a smoothing parameter = 10. To obtain confidence intervals on the dated tree, we ran the treePL analysis with the bootstrap replicates using the same calibration, optimization and cross-validation values as outlined above. The set of trees was summarized in TreeAnnotator v.2.6.6⁸⁵; (part of the BEAST package) with mean node heights and 0% of burnin. Trees were visualized in FigTree v.1.4.3⁸⁶. Lineage-through-time plots were conducted on the treePL chronogram for all bryophyte taxa together, as well as liverworts and mosses separated using the R-package “ape”^{87,88}. We visualized the temporal distribution of order and family ages using the average stem and crown age. Monotypic families or orders were excluded from the calculations (Supplementary Table S10). Bryophyte species number were obtained from ^{66–68}.

Absolute rates were estimated with the same calibrations and targeted ML trees using r8s⁸⁹. Among-lineage rate variation, as expected across such a dataset,

is high. The lowest absolute substitution rate in mosses characterizes the Orthodontiales (1.34×10^{-04} subst/site/Ma) and the highest the Polytrichales (5.44×10^{-04} subst/site/Ma). In liverworts, the variation spans from 3.53×10^{-04} subst/site/Ma in the simple thalloid Pallaviciniales to 7.85×10^{-04} subst/site/Ma in the complex thalloids (Marchantiopsida,) (Fig. 3; Supplementary Table S9). The rate variation observed in our dataset set the stage to test specific hypotheses correlating natural history traits (e.g., perennial vs annual taxa) and molecular rates across genes and all bryophyte groups.

Data Availability Section

All bryophyte gene sequences, alignments for each gene and concatenated alignments will be available from FigShare.

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G.B., S.M., D.B., G.P.-B., J.B., B.G., J.C.V.A. conceptualized the study.

B.G, J.C.V.A. wrote the first draft.

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A.B, A.M.S.P., A.S.-V., C.Z., C.D., D.C.C., I.D., J.J.L., J.L., L.L., M.v.K., S.C., S.H., D.L., L.E., L.L.F., P.L., R.G., G.E.L., K.F., J.B., G.P.-B., S.M., D.B., J.B., J.C.V.A. participated in the specimen collections.

L.L., L.E., G.E.L., J.B., J.C.V.A. participated in data collection and laboratory work.

G.B., J.B., G.P.-B., A.M.S.P., B.G., J.C.V.A. performed the analyses.

G.B., C.D., E.S., S.M., J.C.V.A. procured funding.

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Table 1: Description of novel families and orders of liverworts and mosses.

MARCHANTIOPHYTA (LIVERWORTS)

Lejeuneales Bechteler, A.M. Sierra, D. Bell & D.G. Long ord. nov.

Leaves mostly 2-lobed, the ventral lobule forming a *Lejeunea*-type water sac, gynoecia with 1 archegonium and a single series of bracts and bracteoles, seta 4 cells in diameter, commonly articulate; Type: Lejeuneaceae Rostovzev, New Phytol. 9: 291. 1910; Lejeuneaceae.

Frullaniales D. Bell & D.G. Long ord. nov.

Leaves 3-lobed, the median lobule forming a *Frullania*-type water sac, underleaves bifid, gynoecia with multiple archegonia, bracts and bracteoles in 3 or 4 series, seta up to 12 cells in diameter, non-articulate; Type: Frullaniaceae Lorch in G.Lindau, Krypt.-Fl. Anf. 6: 174. 1914; Frullaniaceae.

Myliales D.G. Long & D. Bell ord. nov.

Leaves unlobed, branching mostly terminal, rhizoids numerous, leaves succubous, simple, gemmiferous, with reduced underleaves, dioicous, sporophyte enclosed in shoot calyptra and perianth; Type: Myliaceae Schljakov, Novosti Sist. Nizsh. Rast. 12: 308. 1975; Myliaceae.

BRYOPHYTA (MOSESSES)

Amphidiales D. Bell & Goffinet ord. nov.

Plants acrocarpous, forming dense cushions, leaves crisped when dry, capsules gymnostomous, emergent to shortly exserted; Type: Amphidiaceae M. Stech, Nova Hedwigia 86: 14, 2008; Amphidiaceae.

Bruchiales Goffinet ord. nov.

Plants erect, acrocarpous, capsule with well-developed neck, peristome haplolepideous; Type: Bruchiaceae Schimp., Coroll. Bryol. Eur.: 6, 1856; Bruchiaceae.

Distichiales D. Bell & Goffinet ord. nov.

Plants acrocarpous, leaves distichous, spreading from broad sheathing base, costa excurrent, monoecious, peristome haplolepideous; Type: Distichiaceae Schimp., Syn. Musc. Eur. 135. 1860; Distichiaceae.

Ditrichales D. Bell & Goffinet ord. nov.

Plants erect, acrocarpous, with costate and mostly lanceolate leaves, sporangium exserted with haplolepideous peristome, or immersed and cleistocarpous. Type: Ditrichaceae Limpr., Laubm. Deutschl. 1: 482, 1887; Ditrichaceae.

Erpodiales Goffinet ord. nov.

Plants small, plagiotropic, leaves ecostate, cladocarpous, peristome haplolepideous; Type: Erpodiaceae Broth., Nat. Pflanzenfam. I(3): 706. 1905; Erpodiaceae.

Eustichiales Goffinet ord. nov.

Plants erect, leaves single costate, distichous with sheathing laminae, with lateral sporophytes and erect capsules; Type: Eustichiaceae Broth., Nat. Pflanzenfam. (ed. 2) 10: 420, 1924; Eustichiaceae.

Flexitrichaceae Ignatov & Fedosov ex Goffinet fam. nov. for Flexitrichaceae Ignatov & Fedosov invalid

Plants acrocarpous; leaves straight to flexuose, from sheathing bases, dioecious, peristome haplolepideous; Type: *Flexitrichum* Ignatov & Fedosov, Bot. J. Linn.

Soc. 181(2): 152. 2016; *Flexitrichum*.

Flexitrichales D. Bell & Goffinet ord. nov.

Plants acrocarpous; leaves straight to flexuose, from sheathing bases, dioecious;

Type: Flexitrichaceae Ignatov & Fedosov ex D. Bell & Goffinet; Flexitrichaceae.

Pleurophascales Goffinet ord. nov.

Plants robust, stems plagiotropic, branches erect, leaves acostate, sporophyte exerted, capsule globose, cleistocarpous; Type: Pleurophascaceae Broth. Nat.

Pflanzenfam. I(3): 774, 1906; Pleurophascaceae.

Rhabdoweisiales D. Bell & Goffinet ord. nov.

Plants small, erect, acrocarpous, leaves unicostate, unistratose, peristome

haplolepideous; Type: Rhabdoweisiaceae Limpr. Laubm. Deutschl. 1: 271, 1886;

Rhabdoweisiaceae⁵² and Rhachithecaceae.

Sorapillales Goffinet ord. nov.

Plants monopodial, vaginant leaves distichous, complanate, sporophyte lateral,

immersed, peristome haplolepideous with vestigial exostome; Type: Sorapillaceae

M. Fleisch, Musci Buitenzorg 3: 847, 1908; Sorapillaceae.

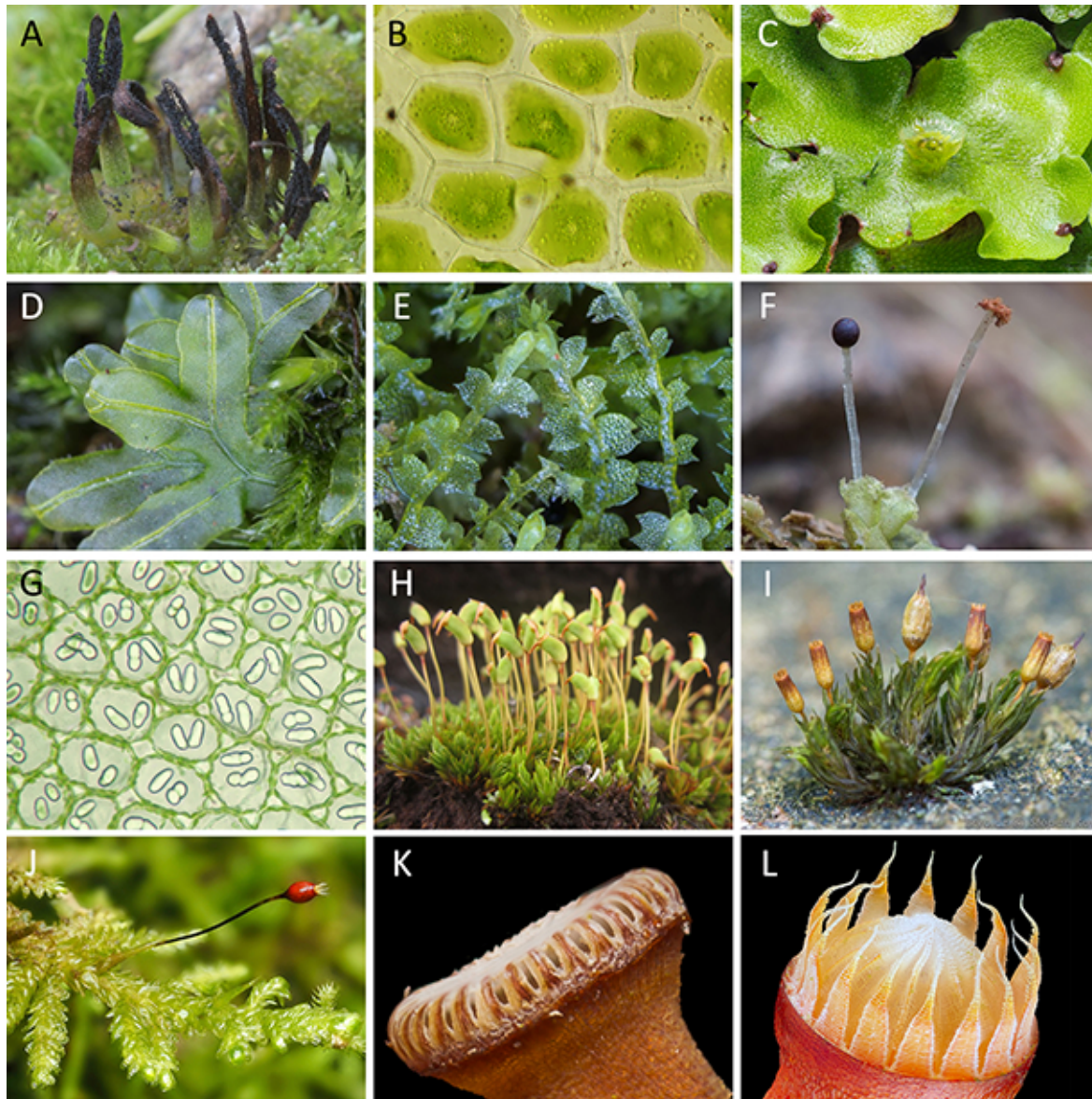


Figure 1. Representatives of major lineages of bryophytes and examples of traits unique to hornworts, liverworts and mosses. A. Hornwort with longitudinally dehiscent sporophyte: *Anthoceros neesii*. B. Pyrenoid (arrowheads) of the hornwort *Phaeoceros perpusillus*. C. Complex thalloid liverwort: *Marchantia paleacea*. D. Simple thalloid liverwort: *Metzgeria conjugata*. E. Leafy liverwort: *Leiocolea badensis*. F. Liverwort sporophyte before (left) and after (right) dehiscence: *Fossombronia pusilla*. G. Oil bodies of *Gymnocolea inflata*. H. Polytrichopsida moss: *Atrichopsis trichodon*. I. Acrocarpous moss: *Orthotrichum anomalum*. J. Pleurocarpous moss: *Exsertotheca intermedia*. K. Nematodontous peristome of *Polytrichum ohioense*. L. Arthrodontous peristome of *Bryum capillare*. Pictures kindly shared by Štěpán Koval (A, D, E, F & I), Sahut Chantanaorrapint (B), Des Callaghan (C, J & L), Bernard Goffinet (H), Jerry Jenkins (K) and David Wagner (G).

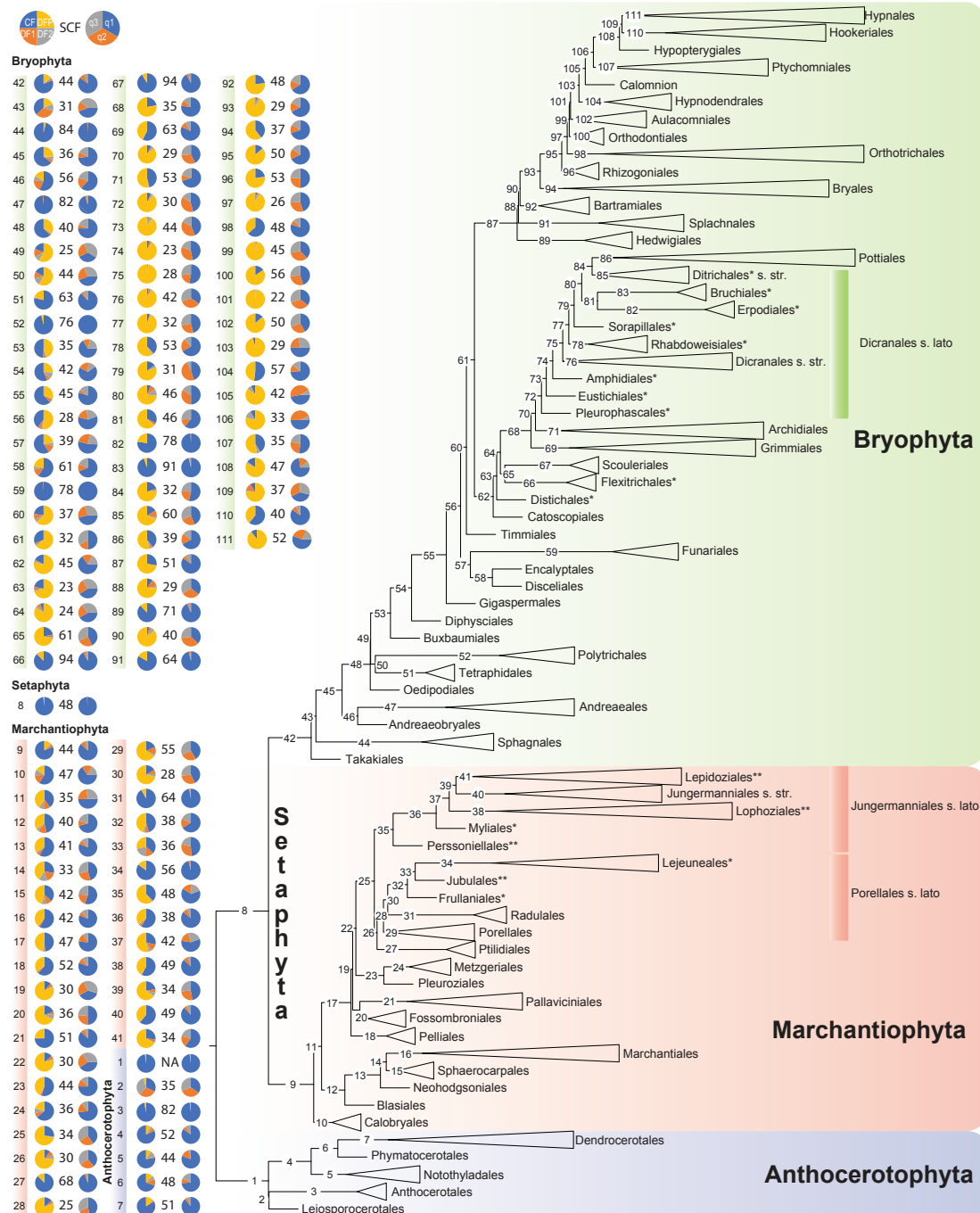


Figure 2. Phylogenetic inferences for 531 species from 499 genera belonging to orders from all three bryophyte groups (mosses, liverworts and hornworts) based on ASTRAL analysis of 228 single-copy nuclear genes (Supplementary Tables S1 and S3). The phylogram shows internal branch lengths relative to coalescent unites between branching events, as estimated by ASTRAL-III v.5.7.7. The nodes of interests have been numbered to illustrate quartet values from ASTRAL analyses presented as pie-charts (right) and quartet values from concordance factor values (left), while the number in the center is the site concordance factor (sCF). ASTRAL pies are divided into q1 (blue), q2 (orange) and q3 (grey) with the percentage for q1 included in the pie diagram; see Supplementary Fig. S1 and Fig. S2 for quartet values for all other nodes and local posterior probabilities. Pies for quartet values from concordance factor values: CF (topology shown) and alternative options (DF1, DF2, DFP); see Supplementary Tables S4-S6 for the concordance factors for all nodes. * marks newly proposed order and ** resurrected order.

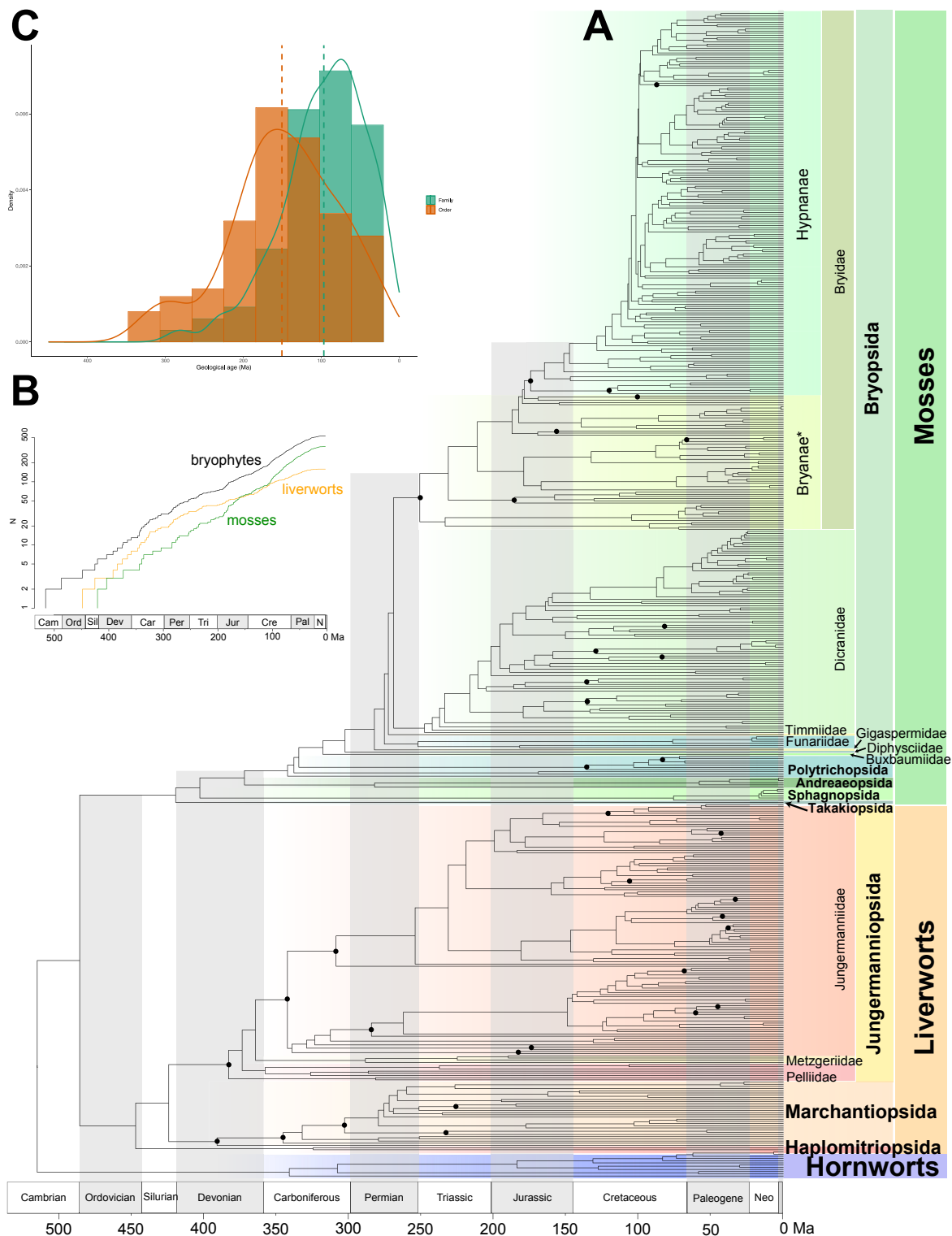


Figure 3. A. Divergence time estimates for bryophytes inferred by penalized likelihood using 29 fossil calibrations. The three bryophyte groups (hornworts, liverworts, mosses) are included with their major suprafamilial taxonomic ranks including classes, subclasses, orders and suborders. Black dots scattered in the tree represent calibration points (Supplementary Table S2). Detailed chronograms with mean node ages including confidence intervals are reported in three parts: liverworts and hornworts in Extended Data Figure 7-1, mosses Extended Data Figure 7-2 and Extended Data Figure 7-3. **B.** Lineage through time plots of all bryophytes (black line), liverworts (orange line) and mosses (green line), reflecting the slowdown in diversification in liverworts during the Late Cretaceous when

compared to mosses. **C.** Histogram of the frequency of orders and families estimated from crown age divergences of all bryophytes (hornworts, liverworts, and mosses). Kernel density plots depict the probability distribution (95% confidence interval) of crown age divergences. The green dotted line represents the average of family crown ages and orange dotted line represents the average of order crown ages. See also Extended Data Fig. 10, Supplementary Table S10 and Supplementary Fig. S9.

Extended data

Phylogenomic time tree of bryophytes resolves 500 million years of diversification

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Extended Data Figure 2. Annotated ASTRAL phylogeny of liverworts inferred from nucleotide data, highlighting suprafamilial relationships.

Extended Data Figure 3. Annotated ASTRAL phylogeny of mosses inferred from nucleotide data, highlighting suprafamilial relationships.

Extended Data Figure 4. Annotated ASTRAL phylogeny of hornworts inferred from amino acid data, highlighting (supra)ordinal relationships.

Extended Data Figure 5. Annotated ASTRAL phylogeny of liverworts inferred from amino acid data, highlighting suprafamilial relationships.

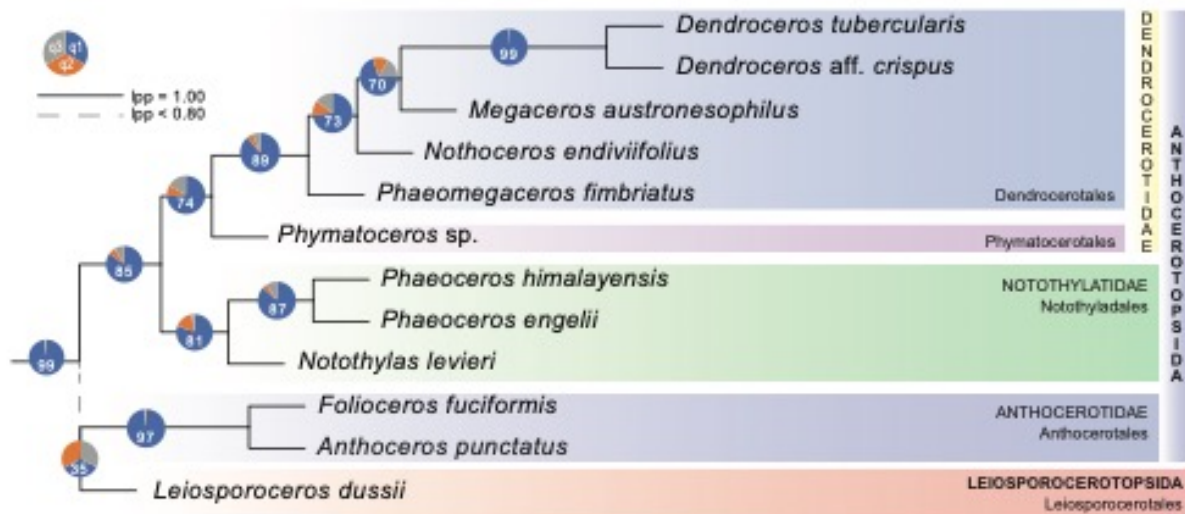
Extended Data Figure 6 Annotated ASTRAL phylogeny of mosses inferred from amino acid data, highlighting suprafamilial relationships.

Extended Data Figure 7. Divergence times estimates for bryophytes inferred by penalized likelihood using 29 fossil calibrations.

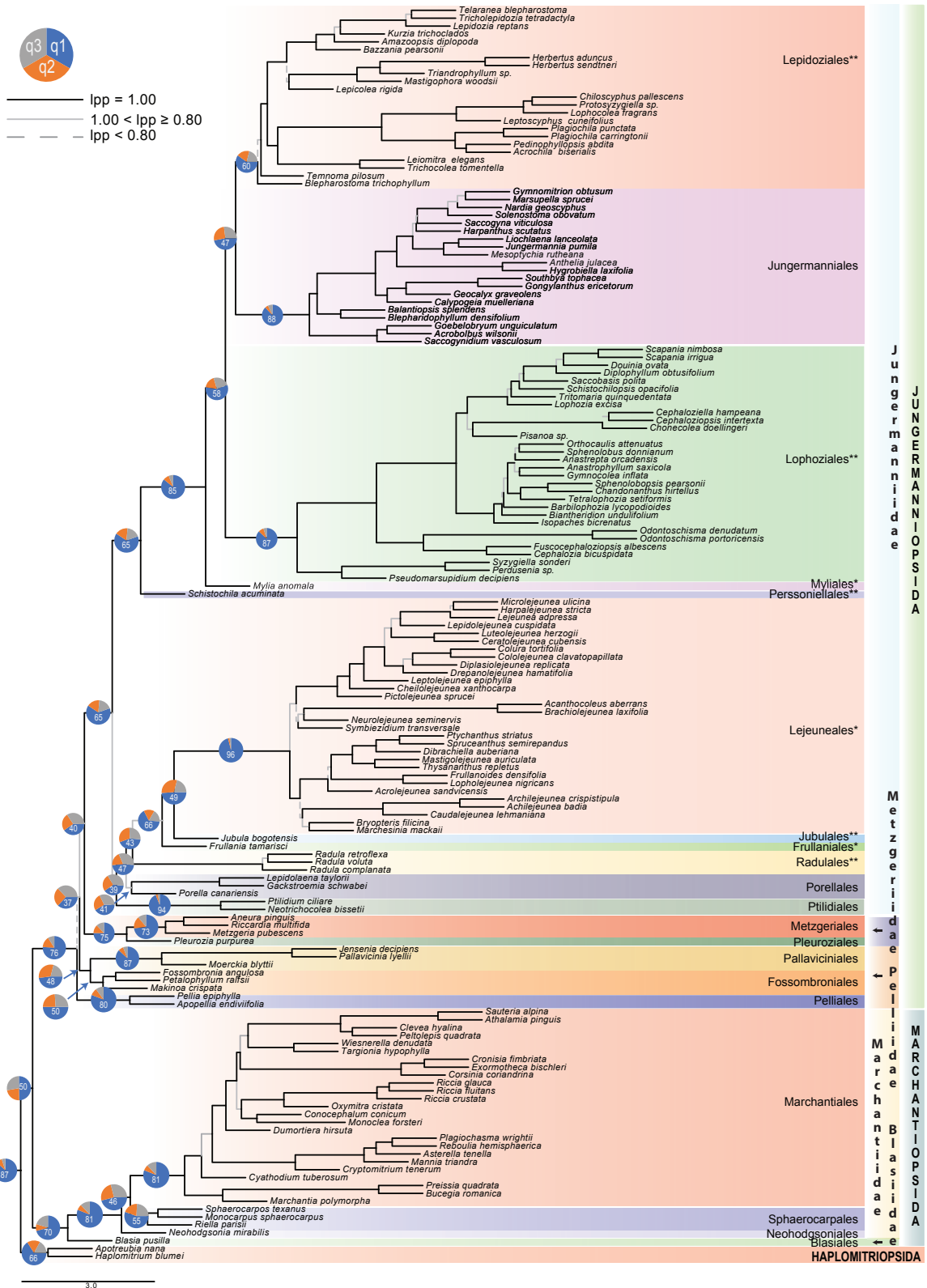
Extended Data Figure 8. Correlation between gene concordance factor inferred by IQ-TREE and number of genes in the nucleotide dataset supporting a node.

Extended Data Figure 9. Correlation between gene concordance factor inferred by IQ-TREE and mean divergence times for backbone nodes (between 130-450 Ma) inferred from the nucleotide data.

Extended Data Figure 10. Histogram of the frequency of orders and families (A) stem and (B) crown estimated age divergences of three bryophyte phyla (Anthocerotophyta, Bryophyta, and Marchantiophyta).



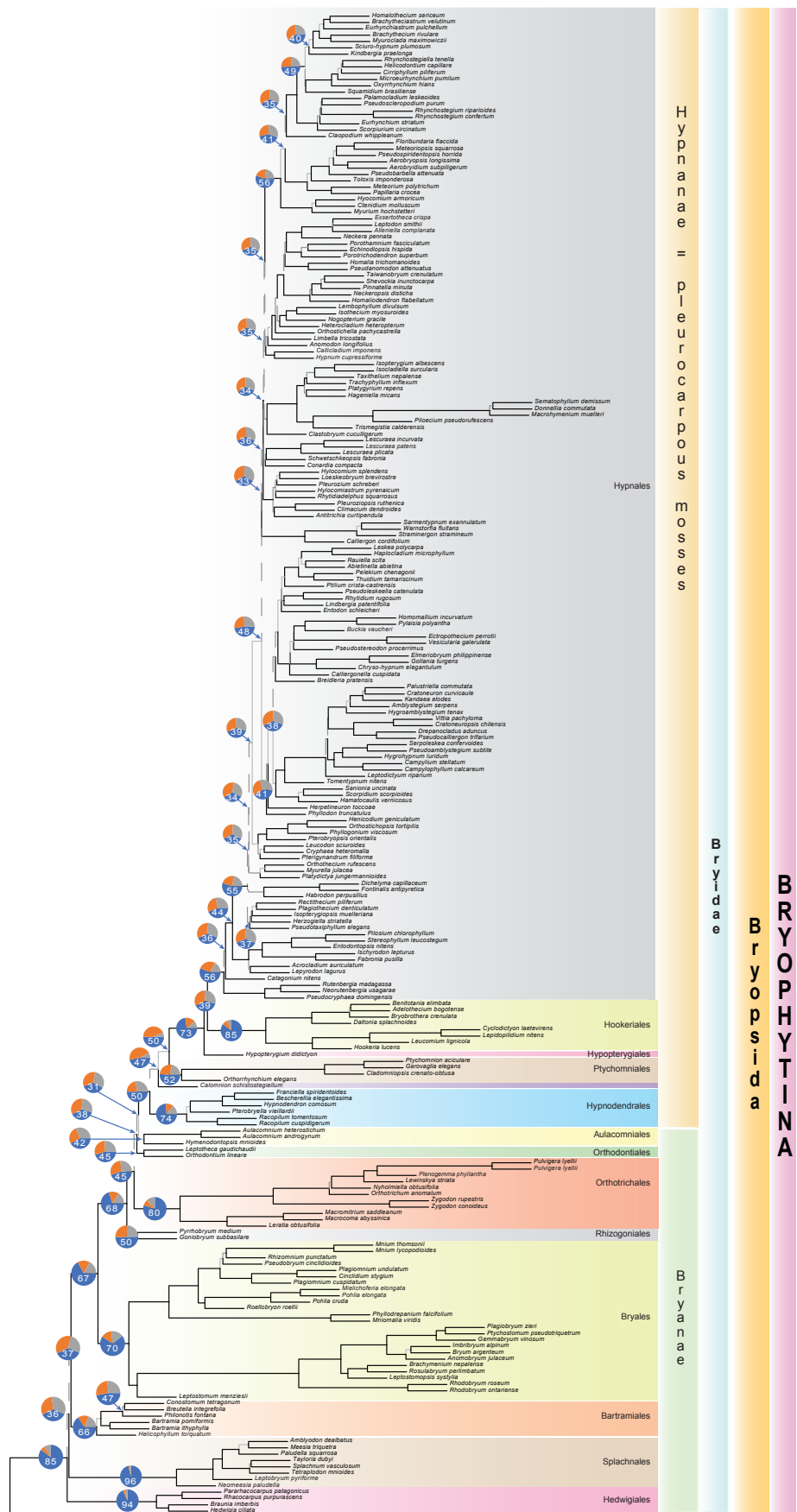
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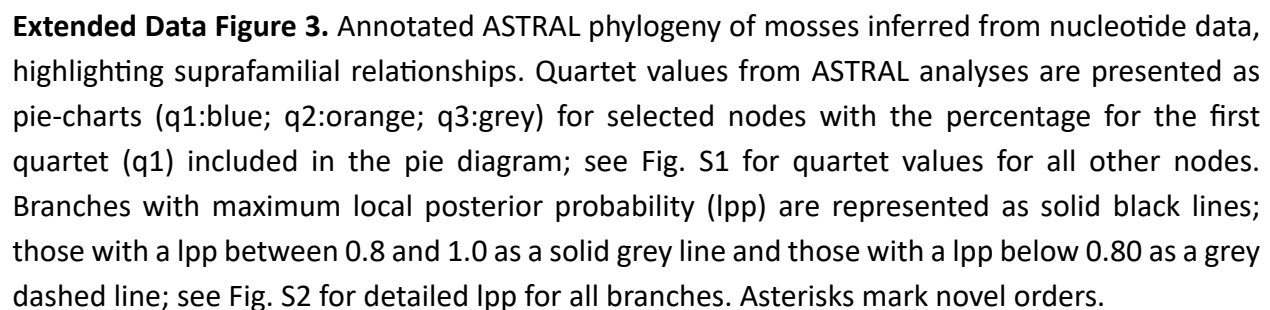


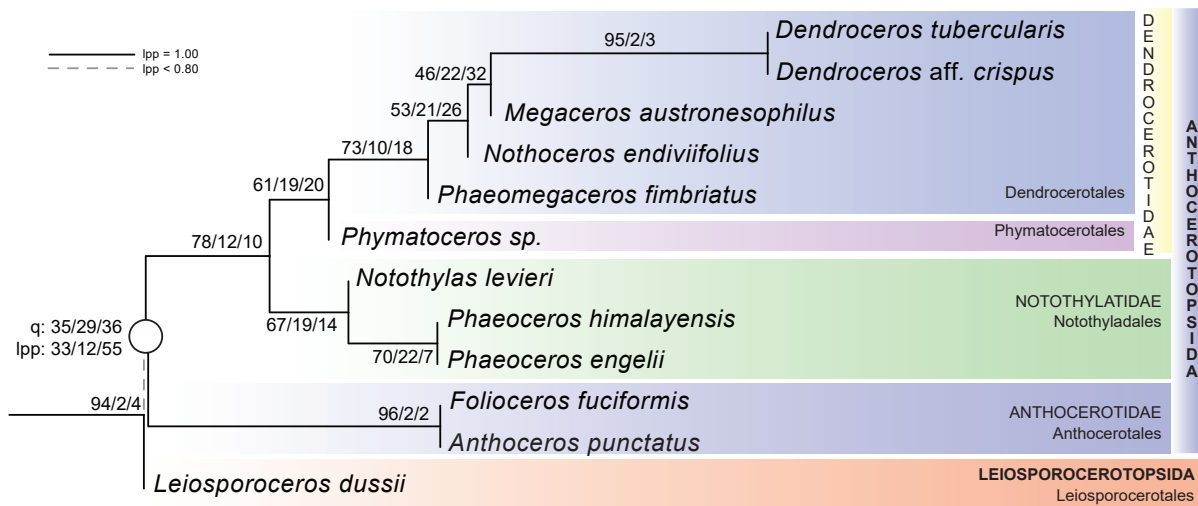
Extended Data Figure 2. Annotated ASTRAL phylogeny of liverworts inferred from nucleotide data, highlighting suprafamilial relationships. Quartet values from ASTRAL analyses are presented as pie-charts (q1:blue; q2:orange; q3:grey) for selected nodes with the percentage for the first quartet (q1) included in the pie diagram; see Fig. S1 for quartet values for all other nodes. Branches with maximum local posterior probability (lpp) are represented as solid black lines; those with a lpp between 0.8 and 1.0 as a solid grey line and those with a lpp below 0.80 as a grey dashed line; see Fig. S2 for detailed lpp for all branches. Asterisk marks novel order, and double Asterisk resurrected order.



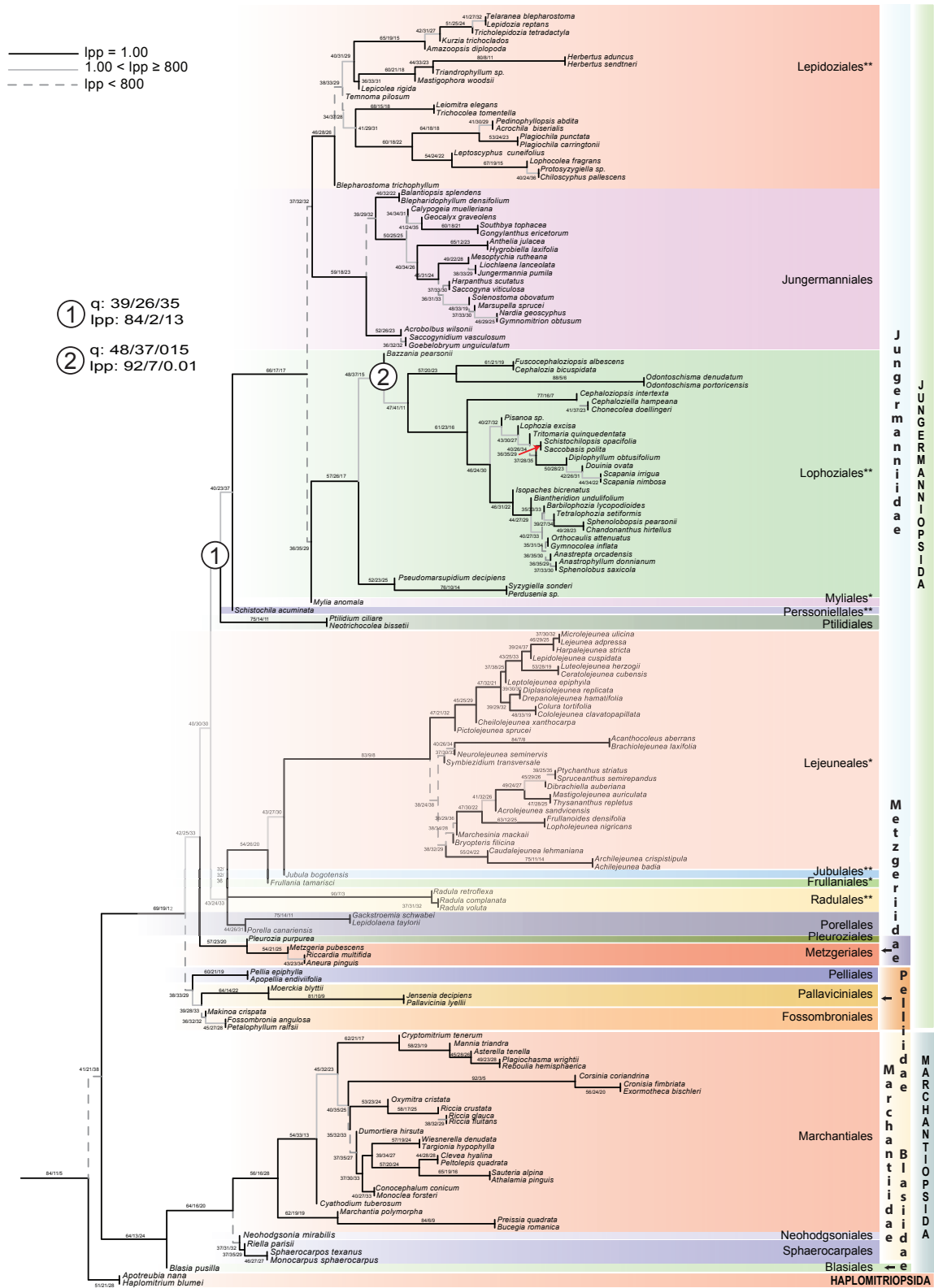
— lpp = 1.00
— 1.00 < lpp ≥ 0.80
— lpp < 0.80



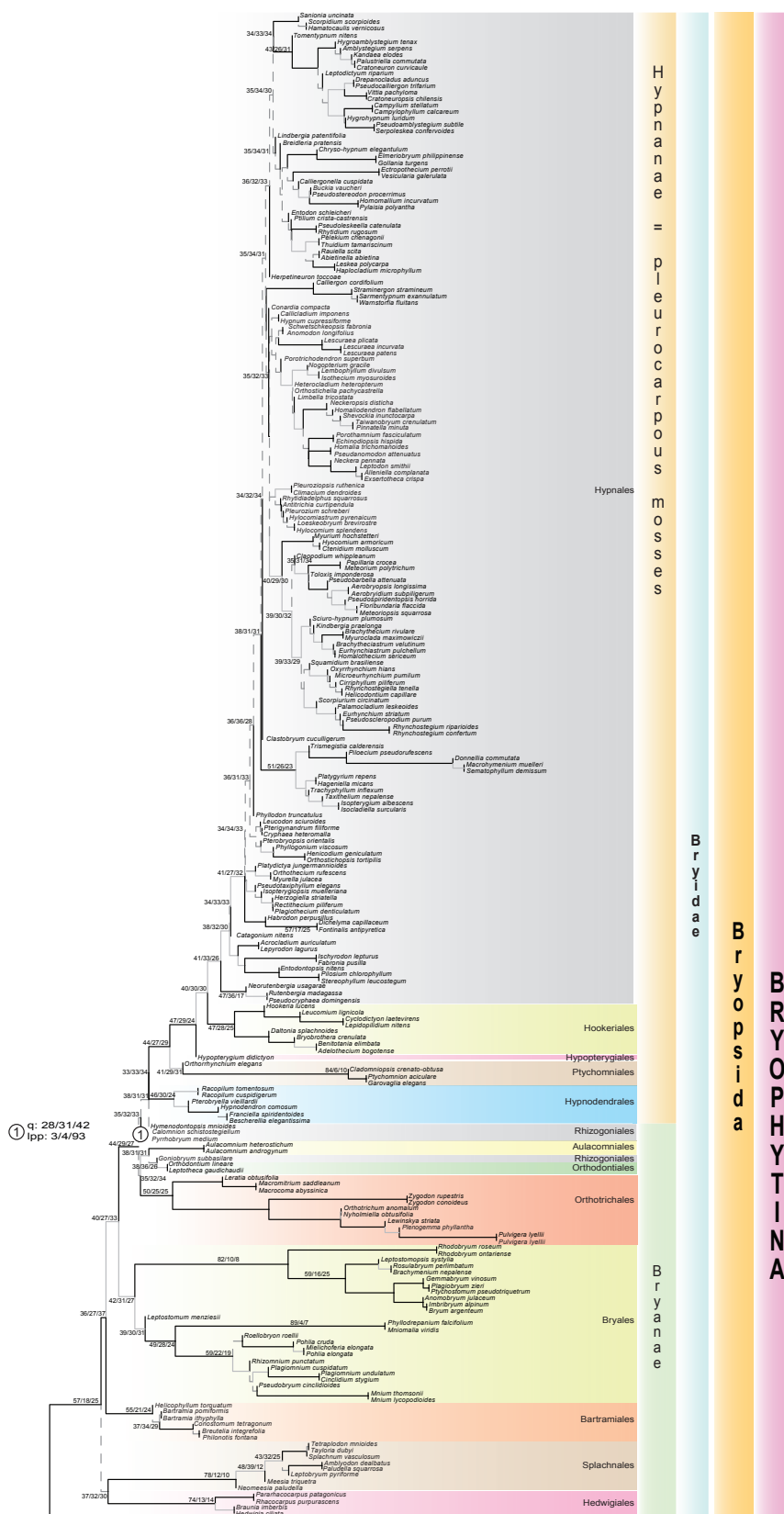


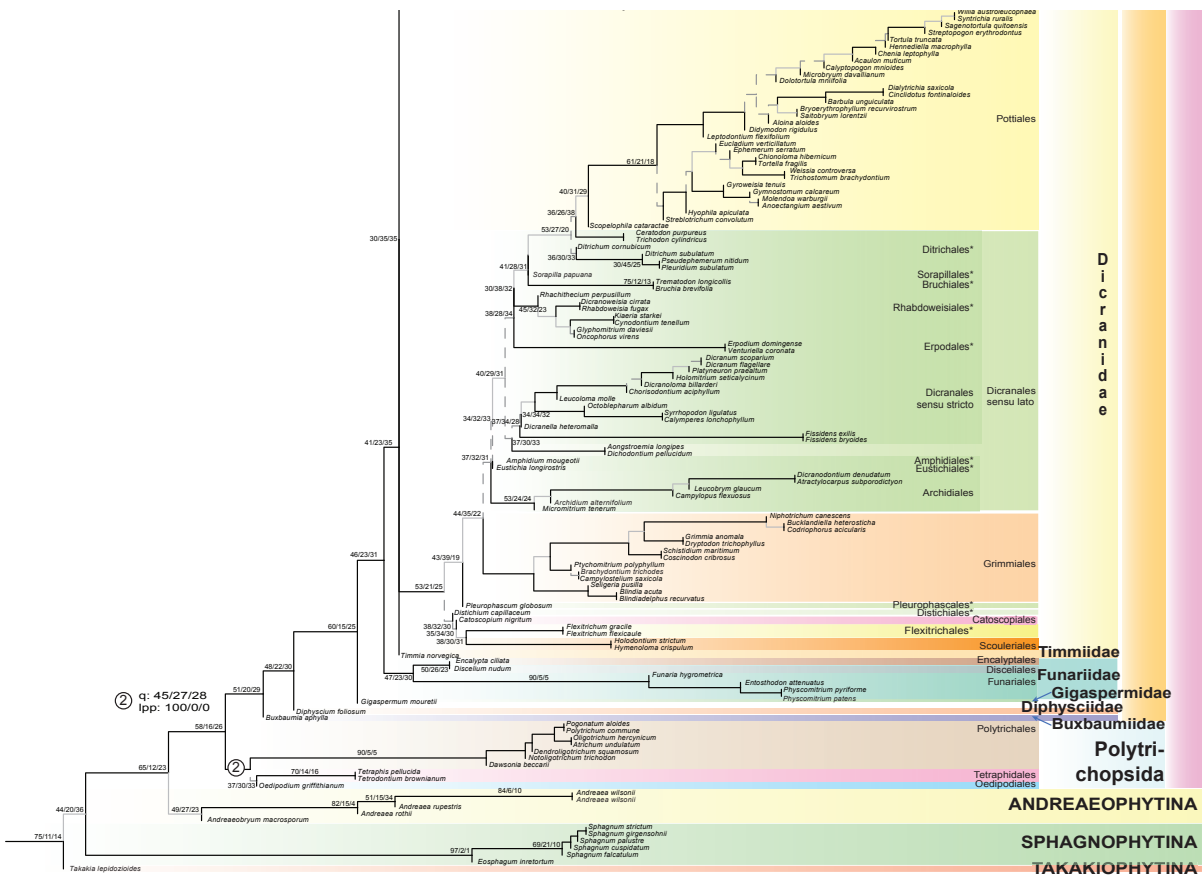


Extended Data Figure 4. Annotated ASTRAL phylogeny of hornworts inferred from amino acid data, highlighting (supra)ordinal relationships. Quartet values for the three possible topologies are presented as percentages near the node. Branches with maximum local posterior probability (lpp) are represented as solid black lines; those with a lpp below 0.80 as a grey dashed line; see Fig. S7 for detailed lpp for all branches.



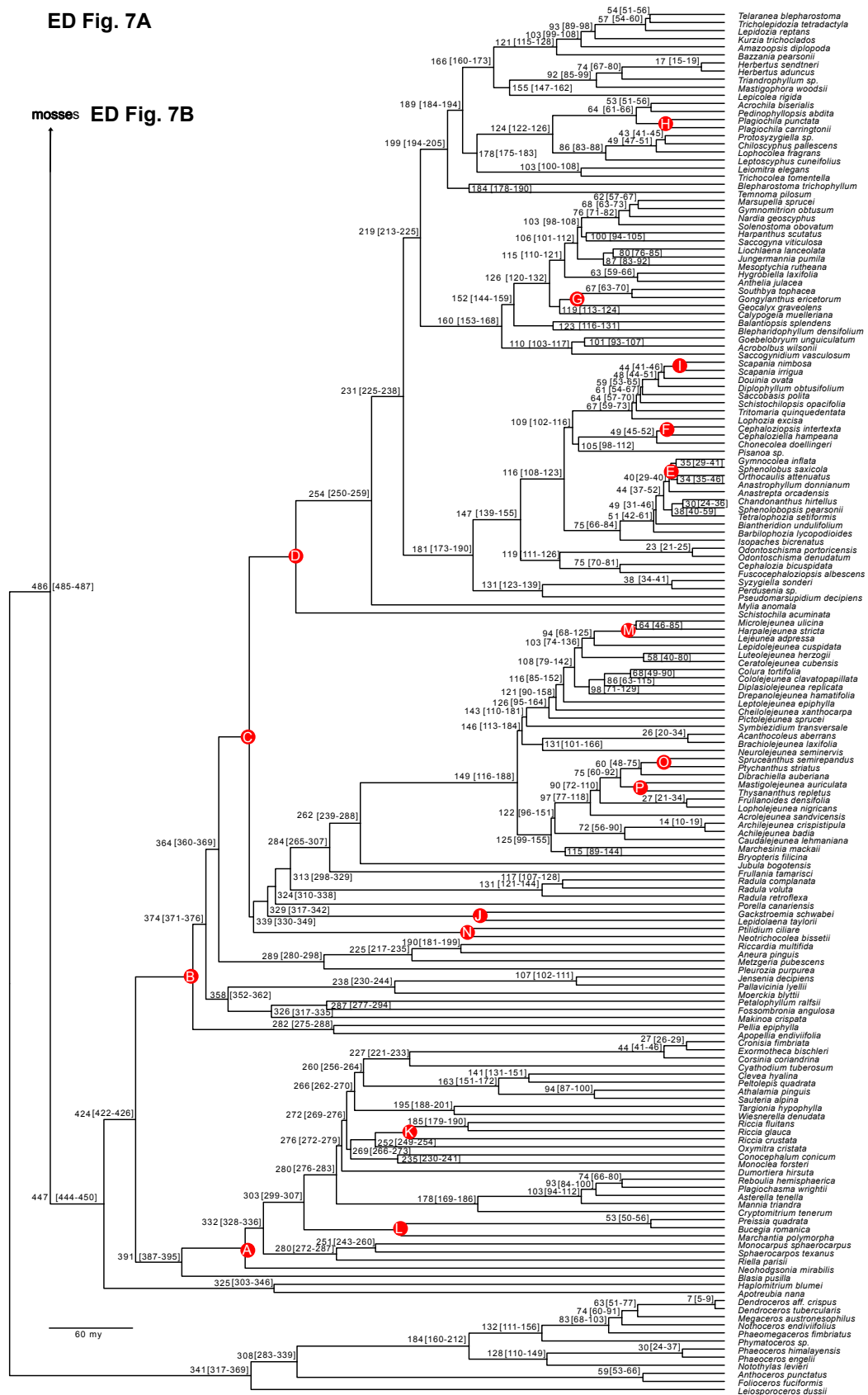
Extended Data Figure 5. Annotated ASTRAL phylogeny of liverworts inferred from amino acid data, highlighting suprafamilial relationships. Quartet values for the three possible topologies are presented as percentages near the node. Branches with maximum local posterior probability (lpp) are represented as solid black lines; those with a lpp between 0.8 and 1.0 as a solid grey line and those with a lpp below 0.80 as a grey dashed line; see Fig. S7 for detailed lpp for all branches. Asterisk marks novel order, and double Asterisk resurrected order.



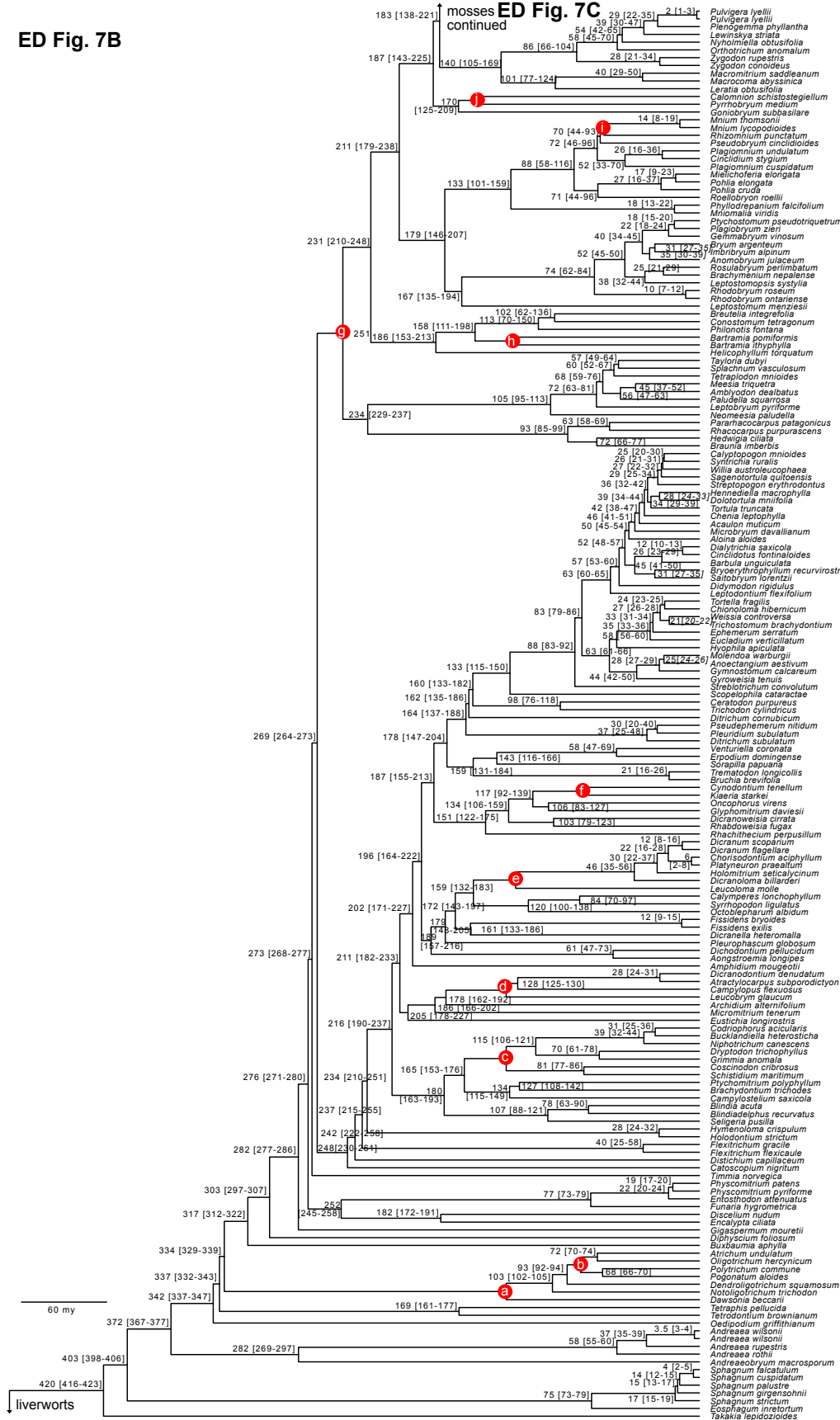


Extended Data Figure 6 Annotated ASTRAL phylogeny of mosses inferred from amino acid data, highlighting suprafamilial relationships. Quartet values for the three possible topologies are presented as percentages near selected nodes; see Fig. S6 for quartet values for all other branches. Branches with maximum local posterior probability (lpp) are represented as solid black lines; those with a lpp between 0.8 and 1.0 as a solid grey line and those with a lpp below 0.80 as a grey dashed line; see Fig. S7 for detailed lpp for all branches. Asterisks mark novel orders.

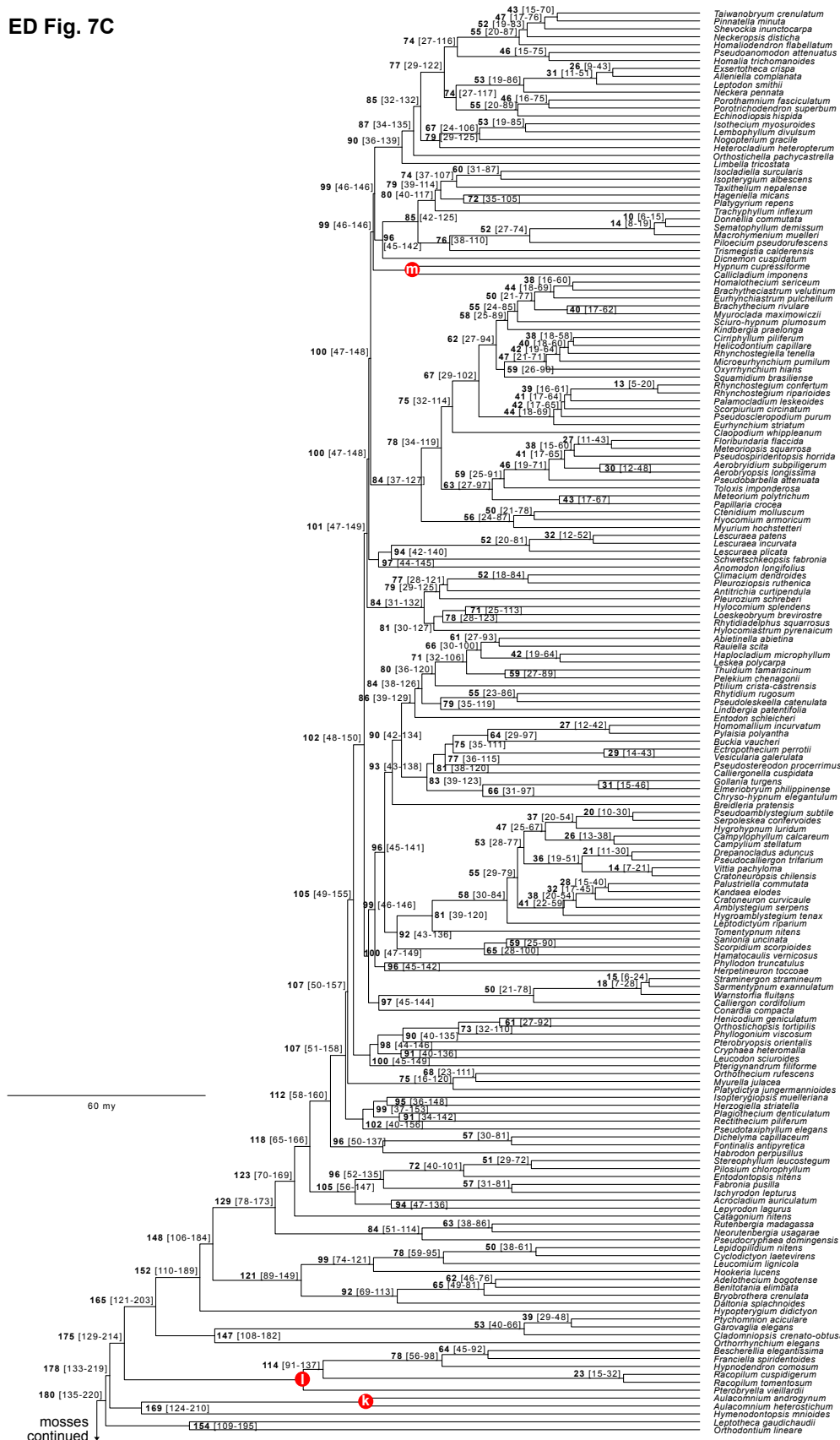
ED Fig. 7A



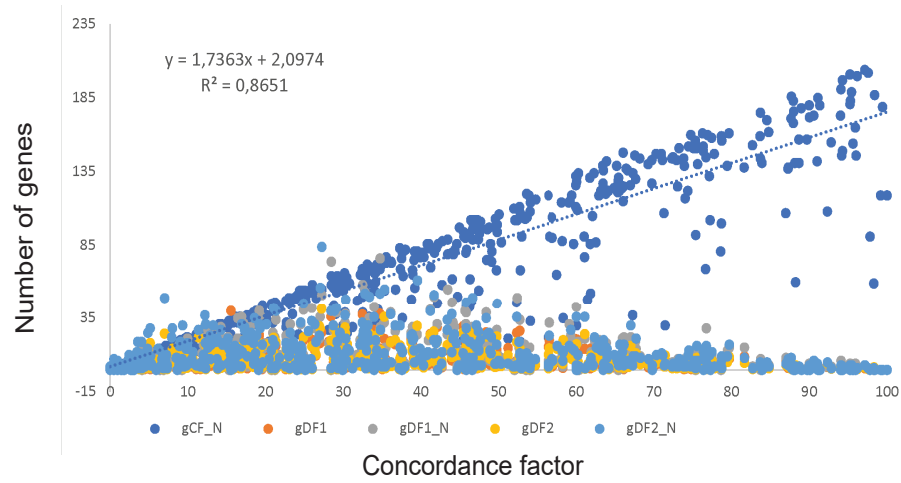
ED Fig. 7B



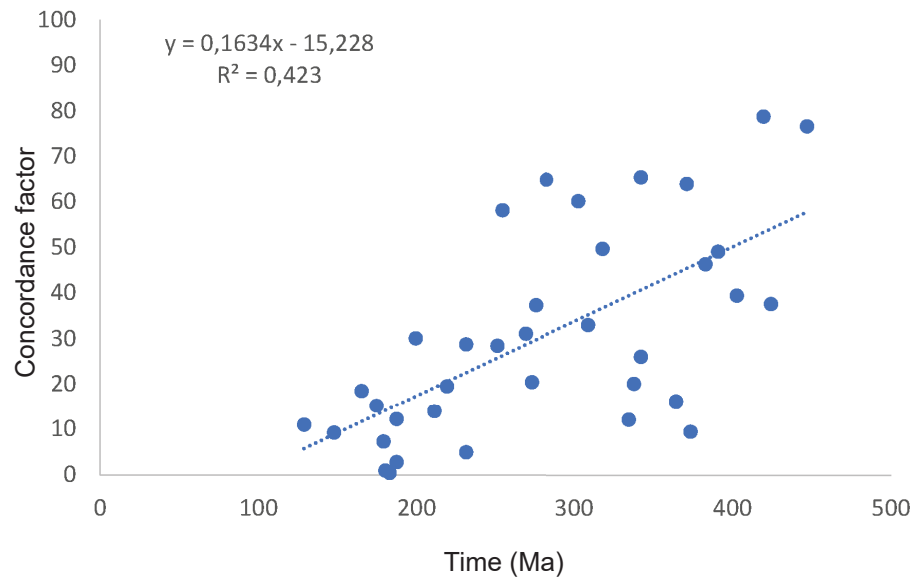
ED Fig. 7C



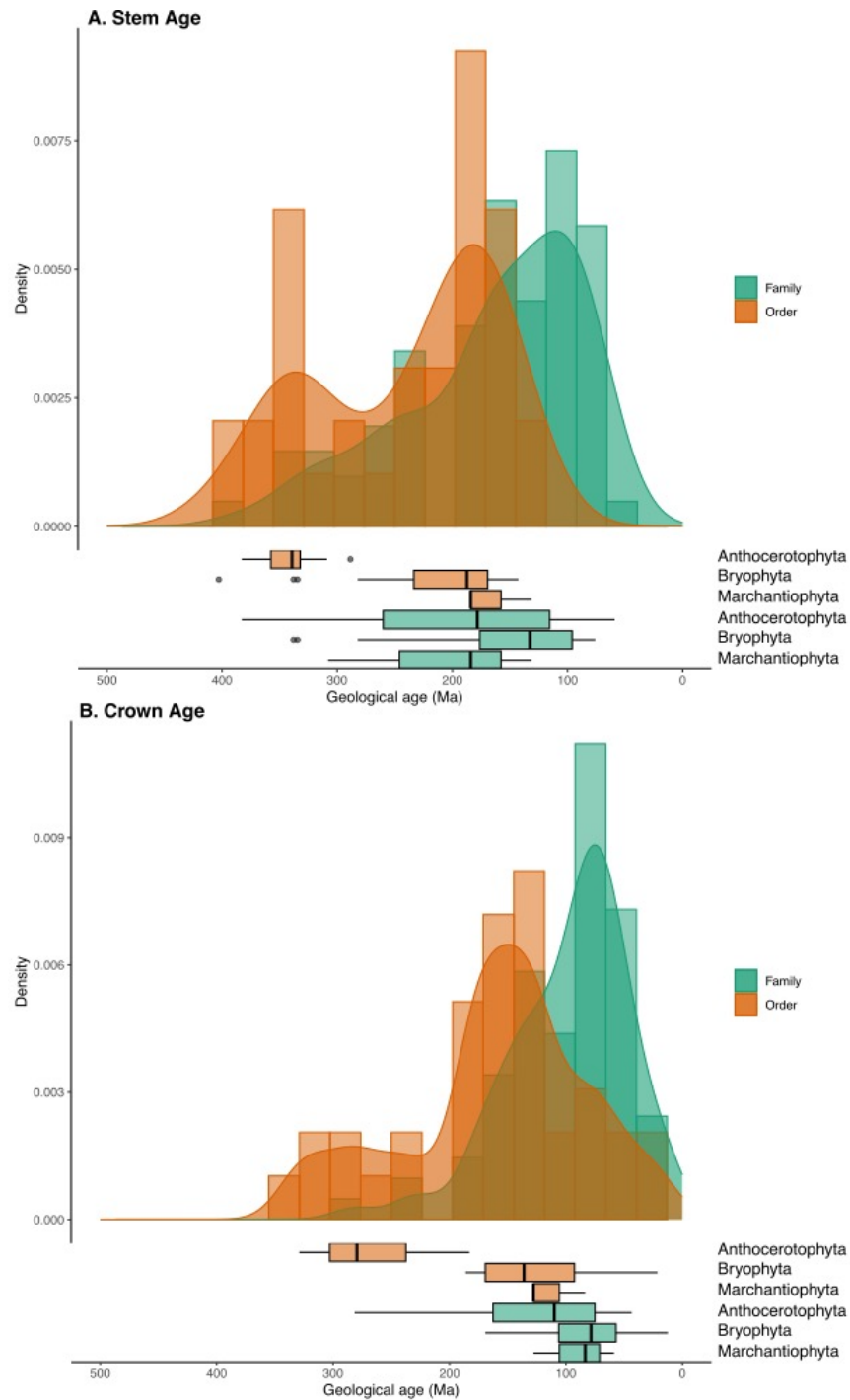
Extended Data Figure 7. Divergence times estimates for bryophytes inferred by penalized likelihood using 29 fossil calibrations. Mean node ages (in bold) including confidence intervals (in brackets) reported in millions of years are stemming from a treePL analysis. Letters in red circles indicate fossil calibrations as outlined in Table S2. The figure is divided in three parts: liverworts and hornworts: Extended Data Figure 7A, mosses Extended Data Figures 7B & C.



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