The mitochondrial phylogeny of land plants shows support for Setaphyta under composition-heterogeneous substitution models

Filipe Sousa [Corresp., 1], **Peter Civáň** [1, 2, 3], **João Brazão** [1], **Peter Foster** [4], **Cymon Cox** [Corresp. 1]

[1] Centro de Ciências do Mar, Universidade do Algarve, Faro, Portugal

[2] INRA, Université d'Auvergne (Clermont-Ferrand I), Clermont-Ferrand, France

[3] Manchester Institute of Biotechnology, School of Earth and Environmental Sciences, University of Manchester, Machester, United Kingdom

[4] Department of Life Sciences, Natural History Museum, London, United Kingdom

Corresponding Authors: Filipe Sousa, Cymon Cox
Email address: fpsousa@ualg.pt, cymon@ualg.pt

Congruence among analyses of plant genomic data partitions (nuclear, chloroplast and mitochondrial) is a strong indicator of accuracy in plant molecular phylogenetics. Recent analyses of both nuclear and chloroplast genome data of land plants (embryophytes) have, controversially, been shown to support monophyly of both bryophytes (mosses, liverworts, and hornworts) and tracheophytes (lycopods, ferns, and seed plants), with mosses and liverworts forming the clade Setaphyta. However, relationships inferred from mitochondria are incongruent with these results, and typically indicate paraphyly of bryophytes with liverworts alone resolved as the earliest-branching land plant group. Here, we reconstruct the mitochondrial land plant phylogeny from a newly compiled data set. When among-lineage composition heterogeneity is accounted for in analyses of codon-degenerate nucleotide and amino acid data, the clade Setaphyta is recovered with high support, and hornworts are supported as the earliest-branching lineage of land plants. These new mitochondrial analyses demonstrate partial congruence with current hypotheses based on nuclear and chloroplast genome data, and provide further incentive for revision of how plants arose on land.

The mitochondrial phylogeny of land plants shows support for Setaphyta under composition-heterogeneous substitution models

Filipe de Sousa[1], Peter Cíváň[123], João Brazão[1], Peter G. Foster[4], Cymon J. Cox[1*]

[1]Centro de Ciências do Mar, Universidade do Algarve, Gambelas, 8005-319 Faro, Portugal

[2]Manchester Institute of Biotechnology, School of Earth and Environmental Sciences, University of Manchester, Williamson Building, Manchester M13 9PL, United Kingdom

[3]INRA-Université Clermont-Auvergne, UMR 1095 GDEC, Clermont-Ferrand, France

[4]Department of Life Sciences, Natural History Museum, London SW7 5BD, United Kingdom

Corresponding Author: Cymon J. Cox[1]
[1]Centro de Ciências do Mar, Universidade do Algarve, Gambelas, 8005-319 Faro, Portugal
Email address: cymon.cox@googlemail.com

Abstract

Congruence among analyses of plant genomic data partitions (nuclear, chloroplast and mitochondrial) is a strong indicator of accuracy in plant molecular phylogenetics. Recent analyses of both nuclear and chloroplast genome data of land plants (embryophytes) have, controversially, been shown to support monophyly of both bryophytes (mosses, liverworts, and hornworts) and tracheophytes (lycopods, ferns, and seed plants), with mosses and liverworts forming the clade Setaphyta. However, relationships inferred from mitochondria are incongruent with these results, and typically indicate paraphyly of bryophytes with liverworts alone resolved as the earliest-branching land plant group. Here, we reconstruct the mitochondrial land plant phylogeny from a newly compiled data set. When among-lineage composition heterogeneity is accounted for in analyses of codon-degenerate nucleotide and amino acid data, the clade

Setaphyta is recovered with high support, and hornworts are supported as the earliest-branching lineage of land plants. These new mitochondrial analyses demonstrate partial congruence with current hypotheses based on nuclear and chloroplast genome data, and provide further incentive for revision of how plants arose on land.

Introduction

The embryophytes, or land plants, share a green algal ancestor (McCourt, Delwiche & Karol 2004) that colonized terrestrial environments between 515.1- 470.0 Ma (Morris et al. 2018) and comprise gametophyte-dominant lineages, collectively known as bryophytes, and a sporophyte-dominant lineage, the tracheophytes. Establishing the phylogenetic relationships between bryophytes (mosses, liverworts and hornworts) and tracheophytes (a monophyletic lineage that includes lycopods, ferns and seed plants) is therefore fundamental to understanding the evolution of plants on land. However, phylogenetic inferences of relationships among embryophytes drawn from molecular data of the nuclear (Finet et al. 2010; Wodniok et al. 2011; Wickett et al. 2014), chloroplast (Cox et al. 2014; Ruhfel et al. 2014; Zhong et al. 2013; Bell et al. 2020), and mitochondrial (Turmel, Otis & Lemieux 2013; Liu et al. 2014; Bell et al. 2020) genomes have long remained conflicting. These incongruences are likely due to molecular evolutionary processes that are especially apparent at deep timescales, such as multiple substitutions on the same site, that lead to loss of phylogenetic signal, and heterogeneity in substitution process patterns among sites and among lineages (Cox 2018). Phylogenetic patterns commonly observed among embryophytes included a sister-group relationship between hornworts and other embryophytes (Hedderson, Chapman & Rootes 1996; Malek et al. 1996; Nishiyama and Kato 1999; Wickett et al. 2014), between liverworts and other embryophytes (Lewis, Mishler & Vilgalys 1997; Karol et al. 2001; Qiu et al. 2006; Gao, Su & Wang 2010; Karol et al. 2010; Clarke, Warnock & Donoghue 2011), or between embryophytes and a clade uniting mosses and liverworts (Karol et al. 2010).An alternative pattern shows an initial split between the bryophytes and the tracheophytes (Hori, Lim & Osawa 1985; Nishiyama et al. 2004; Goremykin and Hellwig 2005; Cox et al. 2014; Wickett et al. 2014; Puttick et al. 2018; Sousa et al. 2019; Leebens-Mack et al. 2019), implying the monophyly of both lineages. Nevertheless, the results are very much dependent on data and methodology, with authors typically presenting competing hypotheses. For instance, several recent phylogenomic analyses based on large nuclear data sets

and extensive taxon sampling (e.g. Wickett et al. 2014; Leebens-Mack et al. 2019) have been equivocal. These studies presented monophyletic-bryophyte phylogenies based on multi-species coalescent supertrees, but concatenated analyses of the same data resulted in trees in which the bryophytes were paraphyletic. Consequently, the authors were unable to provide arguments for which hypothesis was to be preferred. Indeed, the efficacy and suitability of using multi-species coalescent summary analyses versus concatenated data analyses for phylogenies with deep timescales is currently a topic of considerable debate (e.g. Tonini et al. 2015; Edwards et al. 2016; Springer and Gatesy 2016). However, it should be noted that concatenated analyses of nuclear data do support a monophyletic bryophyte clade when modeling composition heterogeneity across the tree, although restricted analytical conditions, namely reduced taxon and site sampling, are currently necessary to decrease computational complexity when using tree-heterogeneous composition models. For instance, to use these models, Sousa et al (2019) analysed a reduced amino acid data set of 26 taxa and 100 genes, while Puttick et al. (2018) analysed Dayhoff-recoded data, that reduces the amino acid data to only six character states. Nevertheless, these tree-heterogeneous composition models are demonstrably better-fitting and are therefore likely more accurate and reliable than the analyses of larger data sets that used simpler and poorer-fitting models (Cox 2018).

Most analyses of land plant relationships have been based on chloroplast data and have typically shown the bryophytes to be paraphyletic (see Table 1 in Cox 2018). More recent phylogenetic analyses using models that account for saturation and composition tree-heterogeneity have, however, indicated that the bryophytes form a monophyletic group, and the authors provided arguments as to why these analyses using better-fitting models are to be preferred (Cox et al. 2014). In contrast, few land plant analyses of mitochondrial data have been presented, but all have indicated that the bryophytes form a paraphyletic group (i.e. Duff and Nickrent 1999; Groth-Malonek et al. 2004; Liu et al. 2014). The most recent and extensive phylogenetic analyses of plant mitochondrial genomes using tree-homogeneous composition models have shown that protein-coding nucleotide data place mosses as the sister-group to the remaining embryophytes, whereas amino acid data show a split between liverworts and the remaining embryophytes (Liu et al. 2014). However, this placement of liverworts as the sister-group to the remaining embryophytes contradicts recent nuclear and chloroplast phylogenies

which show high support for the clade Setaphyta, that groups liverworts with mosses (Cox et al. 2014; Puttick et al. 2018; Sousa et al. 2019).

Our confidence in any evolutionary hypotheses regarding land plant relationships would increase greatly if phylogenetic inferences made from all three plant genomic compartments were not in conflict. In this study we investigate the mitochondrial phylogeny of land plants by applying better-fitting evolutionary models that account for composition tree-heterogeneity to a newly compiled data set of mitochondrial loci that includes sequences from three newly assembled genomes from the Coleochaetales and Zygnematales. We assemble a mitochondrial land plant data set of 26 taxa, which includes all major lineages of land plants and one of the putative most closely-related lineages to land plants, the Zygnematales. Assuming that both bryophytes and tracheophytes are likely monophyletic on the species tree (Sousa et al. 2019), the taxon sampling is deliberately restricted to include a proportional selection of bryophytes (11 taxa) and tracheophytes (10) as symmetrical trees improve estimation (Huang and Knowles 2009) and minimise long branch attraction (Philippe and Laurent 1998). Where possible, taxa were chosen to span what is currently considered the ancestral node of each bryophyte lineage (or the oldest ancestral node possible given the currently available data), thereby attempting to minimise the length of the sub-tending branch of each bryophyte clade and reduce long-branches. Most importantly, a smaller data set enables us to use better-fitting models that account for among-lineage and among-site composition heterogeneity that are computationally intractable for large data sets. This reduced sampling is in contrast to other studies which typically include many more taxa from highly derived lineages (especially angiosperms) whose inclusion, we maintain, has little impact on the resolution among major lineages (the question under consideration), but severely restricts the complexity of models that can be used and therefore the reliability of the inferences. Notably, a recent large-scale analysis of plant transcriptomes, despite the inclusion of 1155 taxa and 410 genes, was unable to resolve many of the long-standing contentious phylogenetic relationships, such as the relationships among the major lineages of land plants (Fig. 3 in Leebens-Mack et al. 2019). Indeed, rather than just including all available data injudiciously, and thereby restrict the complexity of models that can be applied for phylogenetic inference, it is important to consider which data should be included in a particular analysis: the choice of data should reflect carefully the question being addressed and its suitability for analysis by better-fitting models of molecular evolution.

Materials & Methods

Sampling of mitochondrial genomes

We sampled 21 taxa representing the major lineages of land plants, plus 5 green algae species as outgroup taxa. The taxa sampled for this study were: Coleochaetales (*Chaetosphaeridium globosum, Coleochaete scutata*), Zygnematales (*Closterium baillyanum, Gonatozygon brebissonii, Roya anglica)*, liverworts (*Aneura pinguis*. *Marchantia polymorpha*, *Pleurozia purpurea, Treubia lacunosa*), mosses (*Atrichum angustatum*, *Bartramia pomiformis*, *Physcomitrella patens*, *Sphagnum palustre*, *Tetraphis pellucida*), hornworts (*Megaceros aenigmaticus*, *Phaeoceros laevis*), lycophytes (*Huperzia squarrosa, Isoetes engelmannii*), pteridophytes (*Ophioglossum californicum*, *Psilotum nudum*), and spermatophytes (*Brassica napus*, *Cycas taitungensis*, *Ginkgo biloba*, *Liriodendron tulipifera, Oryza sativa*, *Welwitschia mirabilis*).

Algal cultures for *Coleochaete scutata* (SAG 3.91) and *Gonatozygon brebissonii* (SAG 292) were obtained from the Culture Collection of Algae (SAG) (Georg-August-Universität Göttingen, Germany), and the algal culture of *Roya anglica* (ACOI 799) was obtained from the Coimbra Collection of Algae (ACOI) (Universidade do Coimbra, Coimbra, Portugal). The mitochondrial genomes of the three taxa were sequenced and assembled *de-novo* using standard methods as described in (Civáň et al. 2014), and annotated with the aid of Mitofy (Alverson et al. 2010) and NCBI BLAST analyses (Altschul et al. 1990). The remainder of the genomes were retrieved from NCBI GenBank (Bethesda, USA). The list of species samples, their classification, and the source and accession numbers of the sequences used are shown in Table 1.

Alignment and model testing

The sequences of each of 43 mitochondrial protein-coding genes were aligned using the program MAFFT (vers. 6.8; Katoh and Toh 2008). Nucleotide alignments were manually edited in Geneious (vers. 9; http://www.geneious.com) to remove out-of-frame indels, misaligned portions, premature stop codons, positions with less than 50% taxon occupancy, and ambiguities. Genes that were missing with the algal outgroup taxa (Coleochaetales and Zygnematales) or

were under 200 bp in length were discarded. The final data set consisted of 36 genes for 26 taxa, with a missing gene occupancy of 9.5%. Amino acid alignments were generated by translation from each of the 36 nucleotide matrices using SeaView (vers. 4.5.4; Gouy, Guindon & Gascuel 2009). The best-fitting substitution models for the 36 amino acid alignments were inferred in PartitionFinder (Lanfear et al. 2012) using the BIC selection criterion. The stmtREV (Liu et al. 2014) with a gamma-distribution of among-site rates (+G) model was the best-fitting model for all genes but one, for which the chosen model was JTT+G (Jones, Taylor & Thornton 1992).

Gene tree estimation and monophyly test

Gene trees were estimated from individual nucleotide matrices, using the general time-reversible model (Rodriguez et al. 1990) with a gamma rate distribution and estimated base frequencies (GTR+G+Fest), and from amino acid matrices, using the best-fitting models inferred inPartitionFinder (stmtREV+G+F_{est}, JTT+G+F_{est}). Bayesian MCMC analyses were performed with P4 (Foster 2004) using tree-homogeneous (henceforth referred to as CV1, i.e. one composition vector) and tree-heterogeneous (NDCH) composition models. Each analysis had two independent runs which were assessed for convergence by calculation of the marginal likelihood (chains were considered to have converged if they differed by <10 units) and the average standard-deviation of split support between trees sampled from the posterior distributions (chains were considered to have converged if <0.01). Posterior predictive simulations of the X^2 test statistic of composition homogeneity was used to assess composition fit (Foster 2004). For each gene, 50% majority-rule consensus trees were generated from the best-fitting analyses of the nucleotide and amino acid data.

Gene tree topologies inferred from each of the 36 amino acid alignments were tested using "gene genealogy interrogation" (GGI; Arcila et al. 2017) to ascertain whether the non-monophyly of the five major lineages under scrutiny (hornworts, liverworts, mosses, tracheophytes, and the outgroups) was statistically supported by the data. We were to consider any gene that supported the non-monophyly of one of these clades as aberrant and not suitable for inclusion in the combined analyses, as the monophyly of hornworts, liverworts, mosses, tracheophytes and embryophytes has been consistently recovered in molecular phylogenies (e.g. Qiu *et al.* 2006; Liu *et al.* 2014). Optimal trees for each gene were compared to each of fifteen

constraint trees representing all possible topologically resolved combinations of the five monophyletic groups, using a nonparametric bootstrapping test. The results were assessed for statistical significance with the Approximately Unbiased (AU) test (Shimodaira 2002) in CONSEL (vers. 0.1k; Shimodaira and Hasegawa 2001). Optimal trees were estimated in RAxML (MPI-compiled vers. 8.2.8; Stamatakis 2014) using the model stmtREV+G+F_{est} and starting from 20 random trees. Constraint trees were written in Newick format with internal branches within each of the five major clades collapsed to a polytomy. Each constraint tree was optimized in RAxML under the stmtREV+G+F_{est} model. Constraint topologies were considered statistically supported by the data if the p-value of the AU test was equal or greater than 0.05, meaning that the monophyly of each of the five lineages could not be rejected. In every gene, at least one of the constraint topologies was supported by the data, meaning that the monophyly of each clade could not be rejected, thus all 36 genes were included in downstream analyses.

Analyses of concatenated nucleotide data

A nucleotide alignment with 24,864 characters was obtained from the concatenation of the 36 individual genes. A second concatenated nucleotide matrix was constructed by codon-degenerate recoding of the data where ambiguity codes are used to negate synonymous substitutions (Criscuolo and Gribaldo 2010; Cox et al. 2014). Bayesian MCMC analyses were performed on the concatenated and codon-degenerate nucleotide matrices using a tree-homogeneous composition model (CV1; 2 replicates) and the tree-heterogeneous composition (NDCH2; 4 replicates) model, as implemented on P4. In contrast to the original NDCH model (Foster 2004) that allows an *a priori* defined number of compositions to evolve on the tree, the NDCH2 model estimates a separate composition for each node of the tree, constrained by a sampled Dirichlet prior on how much the composition vectors may differ from the empirical composition. Model fit to composition heterogeneity was inferred during the Bayesian MCMC with posterior predictive simulations of the X^2 statistic of composition homogeneity, where p-values equal or greater than 0.05 indicate acceptance of the model. The GTR+G+F_{est} model of substitution was used for for all MCMC of nucleotide and degenerate nucleotide data. Marginal likelihoods were estimated in P4 according to the Eqn 16 method of Newton and Raftery (1994).

Analyses of concatenated amino acid data

An amino acid alignment with 8,288 characters was obtained by concatenation of the amino acid translations of the 36 genes. Bayesian MCMC analysis was performed on the concatenated amino acid data using both tree-homogeneous composition (stmtREV+G+F_{CV1}; 2 replicates) and tree-heterogeneous composition (stmtREV+G+F_{NDCH2}; 4 replicates) models, with the fit of the composition evaluated by posterior predictive simulations as described for the nucleotide data. In addition, a Bayesian MCMC analysis was also performed using PhyloBayes (MPI-compiled vers. 1.6; Lartillot, Lepage & Blanquart 2009) under the model stmtREV+G+F_{CAT}.

Alignments of individual genes, the concatenated data, and the resulting tree files of each analysis are available on Zenodo (doi: 10.5281/zenodo.3554149). All ML analyses of the concatenated nucleotide and amino acid data sets were consistent with the homogeneous Bayesian MCMC analyses and are not reported here, but the resulting tree files are also available on Zenodo.

Results

Nucleotide and codon-degenerate data

All individual genes were best-fit by a tree-heterogeneous composition model with two composition vectors on the tree (CV2; Table S1). Majority-rule consensus trees resulting from the best-fitting Bayesian MCMC analyses of individual genes had low resolution in general, but liverworts were supported (> 95% posterior probability (PP)) as the earliest-branching lineage in two genes (*nad*3 and *nad*5), whereas the mosses were supported as the earliest-branching lineage in one gene (*ccm*C). All other resolutions of the bryophyte lineages relative to the tracheophyte clade were not statistically supported, and the Setaphyta clade was not resolved in any gene tree.

Bayesian MCMC analysis of the concatenated nucleotide data set, assuming a homogeneous composition (CV1), resulted in a tree with mosses as the sister-group to the remaining land plants (PP= 1.0; Figure 1a; Figure S1), and hornworts as sister-group to the tracheophytes (PP= 1.0). In contrast, the analysis of the degenerate data set under a homogeneous base composition (Figure 1c; Figure S2) returned a tree with liverworts the earliest-branching lineage of embryophytes (PP= 1.0) and hornworts the sister-group to tracheophytes (PP= 0.93). However, the homogeneous model was rejected for both data sets by the posterior predictive simulation of the X^2 statistics of homogeneity, with a tail-area probability of 0.0, thereby indicating that the data were not composition homogeneous. The tree-

heterogeneous composition analysis (NDCH2) of the concatenated nucleotide data also resulted in mosses supported as the earliest-diverging land plant lineage (PP= 0.98; Figure 1b; Figure S3), but placed liverworts as the sister-group to tracheophytes (PP= 0.94). The NDCH2 model was a good fit to the data according to the posterior predictive simulation (X^2 p= 0.99). When analysing the codon-degenerate data, the NDCH2 model recovered hornworts as the sister-group to the remaining land plants with full branch support (PP= 1.0; Figure 1d; Figure S4), and mosses fully supported (PP= 1.0) as the sister-group to liverworts (i.e. the clade Setaphyta). The NDCH2 model was not a good statistical fit to the data for the best scoring MCMC run, according to the X^2 posterior predictive test (X^2 p= 0.038; Figure S4).

Amino acid data

Individual mitochondrial protein alignments were best-fit by both homogeneous and heterogeneous composition models, with some being best-fit by a model with up to four compositions (CV4), indicating that they are highly heterogeneous in composition among lineages (Table S1). Majority-rule consensus trees of best-fitting Bayesian MCMC analyses of individual proteins were poorly supported regarding relationships among bryophyte lineages. Indeed, only one tree (*ccm*C) showed any statistically supported resolution and placed the mosses as the earliest-branching lineage of embyrophytes. Although not statistically supported, one amino-acid tree showed bryophytes as a monophyletic group (*rps*7), and the clade Setaphyta was present in three others (*atp*4, *rpl*2, and *sdh*4).

When the concatenated amino acid data were analysed with a tree-homogeneous composition model (CV1), the resulting tree recovered liverworts as the sister-group to the remaining embryophytes without statistically significant support (PP= 0.82), and hornworts as the sister-group to tracheophytes, also without support (PP= 0.82; Figure 1e; Figure S5). The homogeneous composition model did not fit the data (X^2 p= 0.0). The Phylobayes stmtREV+G+F_{CAT} analysis (stationary, data-heterogeneous composition model) of the concatenated amino acid data resulted in an unsupported placement of liverworts as the sister-group to the remaining embryophytes (PP= 0.89; Figure S6). A posterior predictive composition homogeneity test using Phylobayes (*readpb_mpi* parameter *-comp*) showed that the data rejected the model and that the data were therefore composition tree-heterogeneous (p= 0.0). When the tree-heterogeneous composition NDCH2 model was used to model the concatenated protein data,

the separate analyses did not converge on the same tree topology, although the NDCH2 model was a good fit (X^2 p= 0.1022). The tree obtained from the analyses with the best marginal likelihood (-lnLh 142829.1129) supported hornworts as the earliest-branching lineage (PP= 1.0), with the liverworts as the sister-group to mosses (PP=1.0; Figs.1f, 2, S7).

Discussion

Gene tree discordance in mitochondrial data is likely due to mis-modeling and insufficient phylogenetic signal

Alternative hypothesis testing using nonparametric and parametric bootstrapping has been applied before to the mitochondrial land plant phylogeny to test the fit of the data (Liu et al. 2014). Here we chose a different approach, and used the optimized likelihood of constraint trees to identify genes that did not support the monophyly of the major embryophyte lineages (hornworts, liverworts, mosses, and tracheophytes) and of the outgroup. The optimal trees of the 36 mitochondrial genes, inferred under maximum-likelihood, show varied topologies, among which none is predominant. Because the four major land plant lineages are known to be monophyletic (as shown by many studies, e.g. Wickett et al. 2014) our concern was to identify gene trees that showed non-monophyly of one of these groups. The strategy we adopted allowed us to discern whether such topologies truly reflect underlying data or whether they are one among different topologies supported by the data. The AU test indicated, in all genes, that the monophyly of each land plant lineage was not statistically contradicted. This result suggests that the observed phylogenetic conflict among mitochondrial gene trees is unlikely to be explained by biological processes, such as horizontal gene transfer or duplication-loss, affecting specific lineages within each of the four major groups, and that any observed paraphyly of major groups on gene trees is probably the result of inadequate data modeling or paucity of phylogenetic signal.

Synonymous substitutions are responsible for the placement of mosses as the earliest-branching lineage of embryophytes

The tree inferred from the concatenated nucleotide data set of 36 mitochondrial genes shows mosses as the sister-group to the remaining land plants, as previous analyses of mitochondrial

nucleotide data have shown (Liu et al. 2014). However, the mosses are replaced by the liverworts in the same position when analysing codon-degenerate recoded data. Codon degenerate recoding is used to eliminate synonymous substitutions, which are unconstrained by selection at the protein level and therefore can be subject to high rates of substitution, and ultimately saturation and loss of phylogenetic signal. Indeed, as the exclusion of synonymous substitutions is sufficient to eliminate phylogenetic signal that supports mosses as the sister-group to the remaining land plants, these results illustrate that despite being the slowest evolving genomic compartment in plants, phylogenetic inferences from highly divergent mitochondrial genomes are also affected by substitutional saturation due to the effect of high substitution rates. Moreover, this observation implies that caution should be taken when invoking biological explanations (e.g. hybridisation, incomplete lineage sorting) to explain incongruence among phylogenies when inadequate modeling of the substitution process may represent a simpler explanation.

Codon degenerate nucleotide data and protein data support Setaphyta under tree-heterogeneous models of substitution

With the nucleotide data, both the tree-homogeneous and NDCH2 tree-heterogeneous models support mosses as the earliest-diverging group in the embryophytes. The likely incorrect placement of mosses as the earliest-diverging group that is recovered with the best-fitting tree-heterogeneous NDCH2 model suggests that homoplasy driven by high nucleotide substitution rates (saturation) may overwhelm the ability of the model to correct for composition bias, hence the need to use codon-degenerate recoded data in combination with tree-heterogeneous models. Indeed, when codon-degenerate recoded data are used, contrasting supported relationships are obtained under tree-homogeneous and tree-heterogeneous composition models. Whereas using a homogeneous model for the analysis of the codon-degenerate data shows liverworts well supported as the sister-group to other embryophytes, the tree-heterogeneous analysis (NDCH2) model places liverworts as the sister-group to the mosses (clade Setaphyta), with maximum support, and hornworts as the sister-group to all other embryophytes, also with maximum branch support. These results demonstrate that the phylogenetic signal contained in non-synonymous sites is also subject to composition biases and that tree-heterogeneous composition models are required to model the data effectively. Contrasting results were also obtained when the amino

acid data were analysed with tree-homogeneous and tree-heterogeneous models. The tree inferred under the poorly-fitting (P=0.0) homogeneous model (CV1) resolves liverworts as the sister-group to the remaining land plants (PP=0.82). By contrast, the Bayesian MCMC run with the highest marginal likelihood under the NDCH2 (fitting) model shows strong support for the Setaphyta clade (PP= 1.0 – mosses plus liverworts) with the hornworts as the earliest-branching lineage of embryophytes (PP= 1.0). Liu et al. (2014) observed topological congruence between mitochondrial nucleotide and protein data that placed liverworts as the sister-group to all other embryophytes, but this placement of liverworts received low branch support in different analyses, and thus no firm conclusions regarding these cladogenic events were put foward. Importantly, in that study no tree-heterogeneous analyses of the codon-degenerate data were performed, nor was the protein data analysed with more than two composition vectors on the tree. In contrast, our analyses of the codon-degenerate nucleotide data and amino acid data using a better-fitting tree-heterogeneous model resulted in well supported, congruent topologies, strengthening the argument in favour of the analyses presented here, that show support for the clade Setaphyta.

The land plant mitochondrial phylogeny is partially congruent with nuclear and chloroplast phylogenies

In contrast to previous analyses of the land plant mitochondrial phylogeny, we show that both nucleotide and amino acid data carry signal that joins mosses and liverworts as sister lineages (clade Setaphyta). This phylogenetic signal is typically obscured due to substitution saturation in the nucleotide data and among-lineage composition bias in both the nucleotide and amino acid data. In the nucleotide data, phylogenetic signal supporting mosses as the sister-group to the remaining land plants is eliminated when codon-degenerate recoded data is analysed, and instead the liverworts are found as the sister-group to all the remaining embryophytes under tree-homogeneous composition models. However, it is only when a combination of codon-degenerate recoding and a better-fitting tree-heterogeneous composition model is used that the mosses and liverworts appear resolved as sister taxa, therefore suggesting that both substitutional saturation and among-lineage composition heterogeneity are important evolutionary processes to be modeled in the nucleotide data. Similarly, the unsupported placement of liverworts as the earliest-branching lineage is obtained using tree-homogeneous composition models with the

amino acid data, but better-fitting tree-heterogeneous composition models again support the mosses plus liverwort clade.

Support for the moss-liverwort sister-group relationship has been found in trees previously inferred from nuclear and chloroplast protein-coding data (e.g. Nishiyama et al. 2004; Cox et al. 2014; Puttick et al. 2018; Sousa et al. 2019). The clade can be resolved by mitochondrial data with our analyses, and therefore avoids the necessity of calling upon biological explanations, such as hybridisation, to account for incongruence among the phylogenies of the three plant genomes regarding the placement of mosses and liverworts. However, if the placement of the hornworts as the earliest-branching lineage of embryophytes does indeed reflect the true mitochondrial topology, then it is in conflict with the nuclear and chloroplast data which suggest the bryophytes are likely monophyletic. A biological process involving a rapid divergence of the hornworts from other bryophytes, after the tracheophyte-bryophyte split, and the retention of a copy of the mitochondrion that was lost in all other embryophyte lineages, could be invoked to explain the observed phylogenetic conflict. However, the incongruence of the mitochondrial data could, of course, still be a result of mis-modeling or lack of phylogenetic signal. It is likely that further sampling of mitochondrial genomes from hornworts and other bryophyte lineages may aid resolution of the phylogeny, but such analyses would only be informative if they were in combination with the heterogeneous composition models that have been shown here to be necessary to correctly model the underlying processes of mitochondrial evolution.

Conclusions

The main contribution of this study is the demonstration that liverworts are not the sister-group to embryophytes in the land plant mitochondrial phylogeny, unlike earlier analyses of mitochondrial genomes suggested (Liu et al. 2014). Instead, strong support is found for the clade Setaphyta, corroborating support for this clade found in nuclear and plastid genomes, and showing that the mitochondrial phylogeny of land plants is not strongly incongruent with the nuclear and plastid phylogenies. Although the best-scoring tree found by analyses of amino acid data places hornworts as sister-group to embryophytes, the monophyly of bryophytes, which is

supported by evidence from nuclear and plastid genomes, cannot be strongly rejected. Importantly, this study also shows that modeling of composition tree-heterogeneity in amino acid data must not be disregarded, even in slower-evolving genomic regions such as plant mitochondria.

References

Alverson AJ, Wei XX, Rice DW, Stern DB, Barry K, Palmer JD. 2010. Insights into the evolution of mitochondrial genome size from complete sequences of *Citrullus lanatus* and *Cucurbita pepo* (Cucurbitaceae). *Molecular Biology and Evolution* 27: 1436–1448.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. *Journal of Molecular Biology* 215: 403–410.

Arcila D, Ortí G, Vari R, Armbruster JW, Stiassny ML, Ko KD, Sabaj MH, Lundberg J, Revell LJ, Betancur-R R. 2017. Genome-wide interrogation advances resolution of recalcitrant groups in the tree of life. *Nature Ecology & Evolution* 1: 0020.

Bell, D., Lin, Q., Gerelle, W. K., Joya, S., Chang, Y., Taylor, Z. N.,Rothfels, C.J., Larsson, A., Villarreal, J.C., Li, F.-W., Pokorny, L., Szövényi, P., Crandall-Stotler, B., DeGironimo, L., Floyd, S.K., Beerling, D.J., Deyholos, M.K., von Konrat, M., Ellis, S., Shaw, A.J., Chen, T., Wong, G.K.-S., Stevenson, D.W., Palmer, J.D., Graham, S.W. (2020). Organellomic data sets confirm a cryptic consensus on (unrooted) land-plant relationships and provide new insights into bryophyte molecular evolution. *American Journal of Botany* 107: 91-115.

Civáň P, Foster PG, Embley MT, Seneca A, Cox CJ. 2014. Analyses of charophyte chloroplast genomes help characterize the ancestral chloroplast genome of land plants. *Genome Biology and Evolution* 6: 897–911.

Clarke JT, Warnock RCM, Donoghue PCJ. 2011. Establishing a time-scale for plant evolution. *New Phytologist* 192: 266–301.

436 Cox CJ, Li B, Foster PG, Embley TM, Civáň P. 2014. Conflicting phylogenies for early land
437 plants are caused by composition biases among synonymous substitutions. *Systematic*
438 *Biology* 63: 272–279.
439 Cox CJ. 2018. Land plant molecular phylogenetics: a review with comments on evaluating
440 incongruence among phylogenies. *Critical Reviews in Plant Sciences* 37: 113–127.
441 Criscuolo A, Gribaldo S. 2010. BMGE (Block Mapping and Gathering with Entropy): a new
442 software for selection of phylogenetic informative regions from multiple sequence
443 alignments. *BMC Evolutionary Biology* 10: 210.
444 Duff RJ, Nickrent DL. 1999. Phylogenetic relationships of land plants using mitochondrial small
445 subunit rDNA sequences. *American Journal of Botany* 86: 372-386.
446 Edwards SV, Xi Z, Janke A, Faircloth BC, McCormack JE, Glenn TC, Zhong B, Wu S, Lemmon
447 EM, Lemmon AR, Leaché AD. 2016. Implementing and testing the multispecies
448 coalescent model: a valuable paradigm for phylogenomics. *Molecular Phylogenetics and*
449 *Evolution* 94: 447–462.
450 Finet C, Timme RE, Delwiche CF, Marlétaz F. 2010. Multigene phylogeny of the green lineage
451 reveals the origin and diversification of land plants. *Current Biology* 20: 2217–2222.
452 Foster PG. 2004. Modeling compositional heterogeneity. *Systematic Biology* 53: 485–495.
453 Gao L, Su YJ, Wang T. 2010. Plastid genome sequencing, comparative genomics, and
454 phylogenomics: Current status and prospects. *Journal of Systematics and Evolution* 48:
455 77–93.
456 Goremykin VV, Hellwig FH. 2005. Evidence for the most basal split in land plants dividing
457 bryophyte and tracheophyte lineages. *Plant Systematics and Evolution* 254: 93-103.
458 Gouy M, Guindon S, Gascuel O. 2009. SeaView version 4: a multiplatform graphical user
459 interface for sequence alignment and phylogenetic tree building. *Molecular Biology and*
460 *Evolution* 27: 221–224.
461 Groth-Malonek M, Pruchner D, Grewe F, Knoop V. 2004. Ancestors of trans-splicing
462 mitochondrial introns support serial sister group relationships of hornworts and mosses
463 with vascular plants. *Molecular Biology and Evolution* 22: 117–125.
464 Hedderson TA, Chapman RL, Rootes, WL. 1996. Phylogenetic relationships of bryophytes
465 inferred from nuclear-encoded rRNA gene sequences. *Plant Systematics and Evolution*
466 *200*: 213-224.

Hori H, Lim B-L, Osawa S. 1985. Evolution of green plants as deduced from 5S rRNA sequences. *Proceedings of the National Academy of Sciences U. S. A.* 82: 820–823.

Huang H, Knowles LL. 2009. What is the danger of the anomaly zone for empirical phylogenetics? *Systematic Biology* 58: 527–536.

Jones DT, Taylor WR, Thornton JM. 1992. The rapid generation of mutation data matrices from protein sequences. *Bioinformatics* 8: 275-282.

Karol KG, McCourt RM, Cimino MT, Delwiche CF. 2001. The closest living relatives of land plants. *Science* 294: 2351–2353.

Karol KG, Karol KG, Arumuganathan K, Boore JL, Duffy AM, Everett KD, Hall JD, Hansen SK, Kuehl JV, Mandoli DF, Mishler BD, Olmstead RG. 2010. Complete plastome sequences of *Equisetum arvense* and *Isoetes flaccida*: implications for phylogeny and plastid genome evolution of early land plant lineages. *BMC Evolutionary Biology* 10: 321.

Katoh K, Toh H. 2008. Recent developments in the MAFFT multiple sequence alignment program. *Brief. Bioinformatics* 9: 286–298.

Lanfear R, Calcott B, Ho SY, Guindon S. 2012. PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Molecular Biology and Evolution* 29: 1695–1701.

Lartillot N, Lepage T, Blanquart S. 2009. PhyloBayes 3: a Bayesian software package for phylogenetic reconstruction and molecular dating. *Bioinformatics* 25: 2286–2288.

Leebens-Mack J, Barker M, Carpenter E, Deyholos M, Gitzendanner M, Graham S, Grosse I, Li Z, Melkonian M, Mirarab S, Porsch M, Quint M, Rensing S, Soltis D, Soltis P, Stevenson D, Ullrich K, Wickett N, DeGironimo L, Edger P, Jordon-Thaden I, Joya S, Liu T, Melkonian B, Miles N, Pokorny L, Quigley C, Thomas P, Villarreal J, Augustin M, Barrett M, Baucom R, Beerling D, Benstein R, Biffin E, Brockington S, Burge D, Burris J, Burris K, Burtet-Sarramegna V, Caicedo A, Cannon S, Cebi Z, Chang Y, Chater C, Cheeseman J,Chen T, Clarke N, Clayton H, Covshoff S, Crandall-Stotler B, Cross H, DePamphilis C, Der J, Determann R, Dickson R, Di Stilio V, Ellis S, Fast E, Feja N, Field K, Filatov D, Finnegan P, Floyd S, Fogliani B, Garcia N, Gateble G, Godden G, Goh Q.-Y, Greiner S, Harkess A,Heaney J, Helliwell K, Heyduk K, Hibberd J, Hodel R, Hollingsworth P, Johnson M, Jost R,Joyce B, Kapralov M, Kazamia E, Kellogg E, Koch M, Von Konrat M, Konyves K, Kutchan T, Lam V, Larsson A, Leitch A, Lentz R, Li F.-W, Lowe A, Ludwig

M, Manos P, Mavrodiev E, McCormick M, McKain M, McLellan T, McNeal J, Miller R, Nelson M, Peng Y, Ralph P,Real D, Riggins C, Ruhsam M, Sage R, Sakai A, Scascitella M, Schilling E, Schlosser E.-M, Sederoff H, Servick S, Sessa E, Shaw A, Shaw S, Sigel E, Skema C, Smith A, Smithson A, Stewart C, Stinchcombe J, Szovenyi P, Tate J, Tiebel H, Trapnell D, Villegente M, Wang C.-N, Weller S, Wenzel M, Weststrand S, Westwood J, Whigham D, Wu S, Wulff A, Yang Y, Zhu D, Zhuang C, Zuidof J, Chase M, Pires J, Rothfels C, Yu J, Chen C, Chen L, Cheng S, Li J, Li R, Li X, Lu H, Ou Y, Sun X, Tan X, Tang J, Tian Z, Wang F, Wang J, Wei X, Xu X, Yan Z, Yang F, Zhong X, Zhou F, Zhu Y, Zhang Y, Ayyampalayam S, Barkman T, Nguyen N.-P, Matasci N, Nelson D, Sayyari E, Wafula E, Walls R, Warnow T, An H, Arrigo N, Baniaga A, Galuska S, Jorgensen S, Kidder T, Kong H, Lu-Irving P, Marx H, Qi X, Reardon C, Sutherland B, Tiley G, Welles S, Yu R, Zhan S, Gramzow L, Theissen G, Wong G. K.-S. 2019. One thousand plant transcriptomes and the phylogenomics of green plants. *Nature* 574: 679–685.

Lewis LA, Mishler BD, Vilgalys R. 1997. Phylogenetic relationships of the liverworts (Hepaticae), a basal embryophyte lineage, inferred from nucleotide sequence data of the chloroplast gene rbcL. *Molecular Phylogenetics and Evolution* 7: 377–393

Liu Y, Cox CJ, Wang W, Goffinet B. 2014. Mitochondrial phylogenomics of early land plants: Mitigating the effects of saturation, compositional heterogeneity, and codon-usage bias. *Systematic Biology* 63: 862–878.

Malek O, Lättig K, Hiesel R, Brennicke A, Knoop V. 1996. RNA editing in bryophytes and a molecular phylogeny of land plants. *The EMBO Journal* 15: 1403–1411.

McCourt RM, Delwiche CF, Karol KG. 2004. Charophyte algae and land plant origins. *Trends in Ecology & Evolution* 19: 661–666.

Morris JL, Morris JL, Puttick MN, Clark JW, Edwards D, Kenrick P, Pressel S, Wellman CH, Yang Z, Schneider H, Donoghue PC. 2018. The timescale of early land plant evolution. *Proceedings of the National Academy of Sciences U. S. A.* 115: E2274–E2283.

Newton MA, Raftery AE. 1994. Approximate Bayesian inference with the weighted likelihood bootstrap. *Journal of the Royal Statistical Society B* 56: 3–48.

Nishiyama T, Nishiyama T, Wolf PG, Kugita M, Sinclair RB, Sugita M, Sugiura C, Wakasugi T, Yamada K, Yoshinaga K, Yamaguchi K, Ueda K. 2004. Chloroplast phylogeny indicates that bryophytes are monophyletic. *Molecular Biology and Evolution* 21: 1813–1819.

529 Nishiyama T, Kato M. 1999. Molecular phylogenetic analysis among bryophytes and
530 tracheophytes based on combined data of plasmid coded genes and the 18S rRNA gene.
531 *Molecular Biology and Evolution* 16: 1027–1036.
532 Philippe H, Laurent J. 1998. How good are deep phylogenetic trees? *Current Opinion in*
533 *Genetics and Development* 8: 616–623.
534 Puttick MN, Morris JL, Williams TA, Cox CJ, Edwards D, Kenrick P, Pressel S, Wellman CH,
535 Schneider H, Pisani D, Donoghue PC. 2018. The interrelationships of land plants and the
536 nature of the ancestral embryophyte. *Current Biology* 28: 733–745.
537 Qiu Y-L*,* Li L, Wang B, Chen Z, Knoop V, Groth-Malonek M, Dombrovska O, Lee J, Kent L,
538 Rest J, Estabrook GF. 2006. The deepest divergences in land plants inferred from
539 phylogenomic evidence. *Proceedings of the National Academy of Sciences U. S. A.* 103:
540 15511–15516.
541 Rodriguez F, Oliver JL, Marin A, Medina JR. 1990. The general stochastic model of nucleotide
542 substitution. *Journal of Theoretical Biology* 142: 485-501.
543 Ruhfel BR, Gitzendanner MA, Soltis PS, Soltis DE, Burleigh JG. 2014. From algae to
544 angiosperms--inferring the phylogeny of green plants (Viridiplantae) from 360 plastid
545 genomes. *BMC Evolutionary Biology* 14: 23.
546 Shimodaira H. 2002. An approximately unbiased test of phylogenetic tree selection. *Systematic*
547 *Biology* 51: 492–508.
548 Shimodaira H, Hasegawa M. 2001. CONSEL: for assessing the confidence of phylogenetic tree
549 selection. *Bioinformatics* 17:1246–1247.
550 Sousa F, Foster PG, Donoghue PC, Schneider H, Cox CJ. 2019. Nuclear protein phylogenies
551 support the monophyly of the three bryophyte groups (Bryophyta Schimp.). *New*
552 *Phytologist* 222: 565–575.
553 Springer MS, Gatesy J. 2016. The gene tree delusion. *Molecular Phylogenetics and Evolution*
554 94, 1-33.
555 Stamatakis A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of
556 large phylogenies. *Bioinformatics* 30: 1312-1313.
557 Tonini J, Moore A, Stern D, Shcheglovitova M, Ortí G. 2015. Concatenation and species tree
558 methods exhibit statistically indistinguishable accuracy under a range of simulated
559 conditions. *PLoS currents,* 7.

Turmel M, Otis C, Lemieux C. 2013. Tracing the evolution of streptophyte algae and their mitochondrial genome. *Genome Biology and Evolution* 5: 1817–1835.

Wickett NJ, Mirarab S, Nguyen N, Warnow T, Carpenter C, Matasci N, Ayyampalayam S, Barker MS, Burleigh JG, Gitzendanner MA, Ruhfel BR, Wafula E, Der JP, Graham SW, Mathews S, Melkonian M, Soltis DE, Soltis PS, Miles NW, Rothfels C-J, Pokorny L, Shaw AJ, DeGironimo L, Stevenson DW, Surek B, Villarreal JC, Roure B, Philippe H, dePamphilis CW, Chen T, Deyholos MK, Baucom RS, Kutchan TM, Augustin MM, Wang J, Zhang Y, Tian Z, Yan Z, Wu X, Sun X, Wong GK-S, Leebens-Mack J. 2014. Phylotranscriptomic analysis of the origin and early diversification of land plants. *Proceedings of the National Academy of Sciences U. S. A.* 111: E4859–E4868.

Wodniok S, Brinkmann H, Glöckner G, Heidel AJ, Philippe H, Melkonian M. Becker B. 2011. Origin of land plants: do conjugating green algae hold the key? *BMC Evolutionary Biology* 11: 104.

Zhong B, Xi Z, Goremykin VV, Fong R, Mclenachan PA, Novis PM, Davis CC, Penny D. 2013. Streptophyte algae and the origin of land plants revisited using heterogeneous models with three new algal chloroplast genomes. *Molecular Biology and Evolution* 31: 177-183.

Table 1(on next page)

Accession table of the 26 samples used in this study.

For each species, the corresponding taxonomic group and NCBI GenBank accession numbers are shown. Accessions marked with an asterisk (*) correspond to newly assembled genomes.

1

species	taxonomic group	Accession
Closterium baillyanum	Zygnematales	NC_022860
Gonatozygon brebissonii	Zygnematales	MK720950 *
Roya anglica	Zygnematales	MK720948 *
Chaetosphaeridium globosum	Coleochaetales	NC_004118
Coleochaete scutata	Coleochaetales	MK720949 *
Megaceros aenigmaticus	hornworts	NC_012651
Phaeoceros laevis	hornworts	NC_013765
Aneura pinguis	liverworts	NC_026901
Marchantia polymorpha	liverworts	NC_001660
Pleurozia purpurea	liverworts	NC_013444
Treubia lacunosa	liverworts	NC_016122
Atrichum angustatum	mosses	NC_024520
Bartramia pomiformis	mosses	NC_024519
Physcomitrella patens	mosses	NC_007945
Sphagnum palustre	mosses	NC_024521
Tetraphis pellucida	mosses	NC_024290
Welwitschia_mirabilis	seed plants	NC_029130
Brassica napus	seed plants	NC_008285
Liriodendron tulipifera	seed plants	NC_021152
Oryza_sativa	seed plants	NC_011033
Cycas taitungensis	seed plants	NC_010303
Ginkgo biloba	seed plants	NC_027976

Ophioglossum californicum	ferns	NC_030900
Psilotum nudum	ferns	NC_030952, KX171639
Huperzia squarrosa	lycophytes	NC_017755
Isoetes engelmannii	lycophytes	FJ010859, FJ176330, FJ390841, FJ536259, FJ628360

2

3

Figure 1

A schematic representation of the topologies obtained from tree-homogeneous and tree-heterogeneous analyses of nucleotide, codon-degenerate nucleotide, and amino acid translation data.

Analyses of nucleotide data place mosses as the earliest-branching lineage of the embryophytes (1a, 1b). Analyses of codon-degenerate and amino acid data under tree-homogeneous models place liverworts as the sister-group to the remaining embryophytes (1c, 1e), whereas analyses under tree-heterogeneous models show support for the clade Setaphyta (1d, 1f).

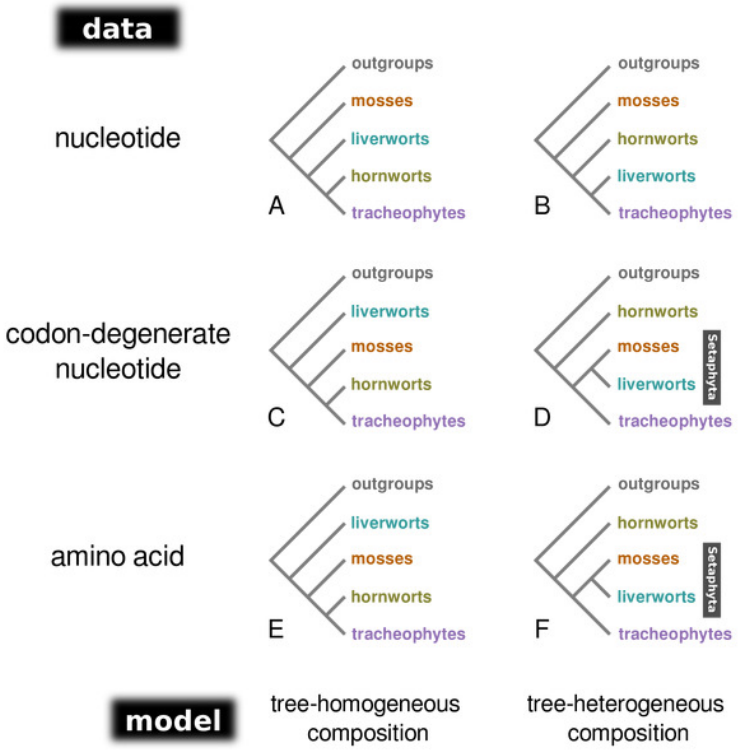
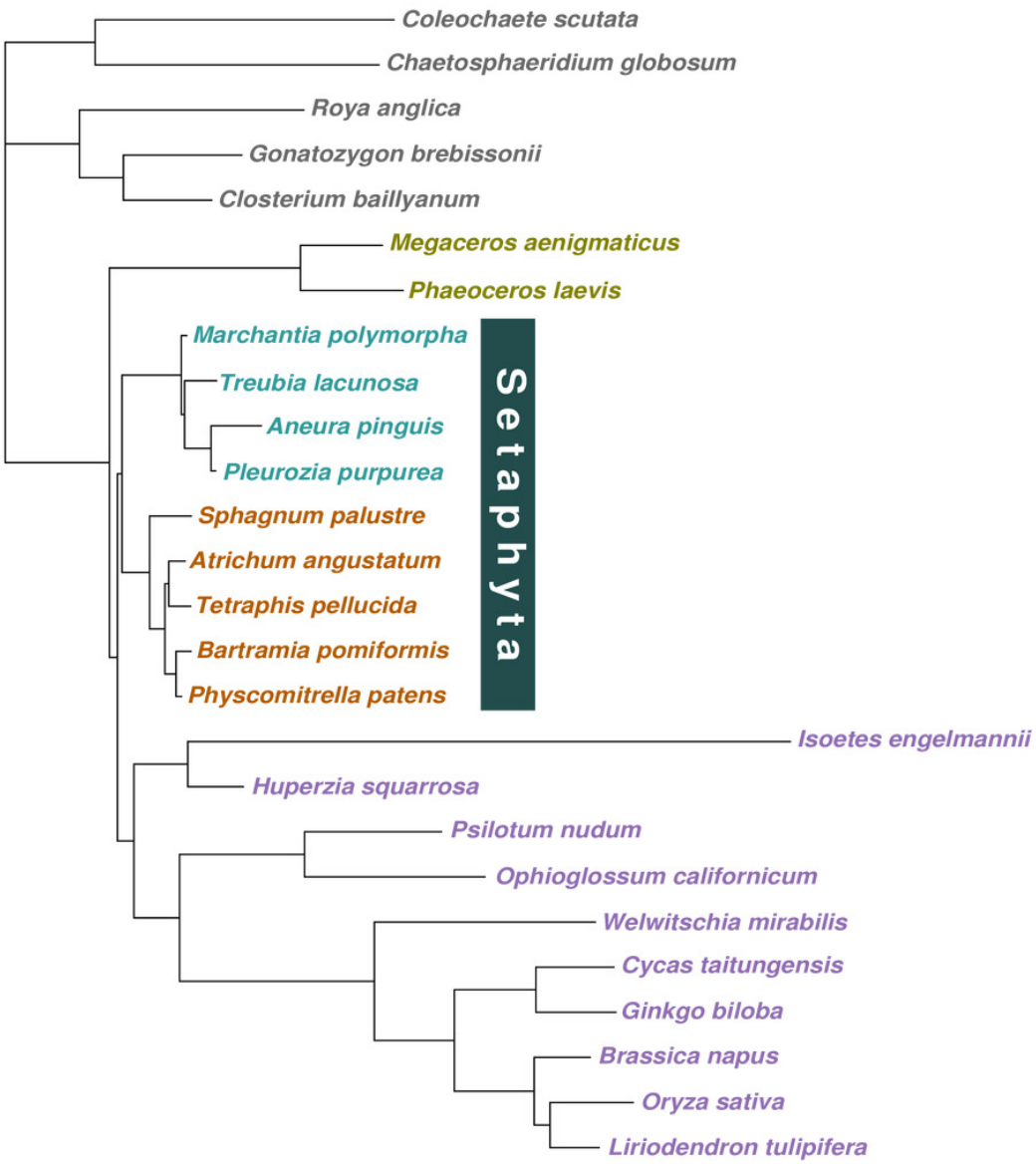


Figure 2

Majority-rule consensus tree inferred from the 36 gene, 26 taxon concatenated amino acid data.

Bayesian MCMC with a tree-heterogeneous composition model NDCH2, marginal likelihood - L_h= 142829.112. Additional analysis statistics can be found in the legend of Fig. S7. All branches fully supported (PP=1.0). Taxa indicated as follows: outgroups, grey; hornworts, olive green; liverworts, cyan blue; mosses, orange; vascular plants, violet.


Coleochaete scutata
Chaetosphaeridium globosum
Roya anglica
Gonatozygon brebissonii
Closterium baillyanum
Megaceros aenigmaticus
Phaeoceros laevis
Marchantia polymorpha
Treubia lacunosa
Aneura pinguis
Pleurozia purpurea
Sphagnum palustre
Atrichum angustatum
Tetraphis pellucida
Bartramia pomiformis
Physcomitrella patens
Setaphyta
Isoetes engelmannii
Huperzia squarrosa
Psilotum nudum
Ophioglossum californicum
Welwitschia mirabilis
Cycas taitungensis
Ginkgo biloba
Brassica napus
Oryza sativa
Liriodendron tulipifera