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ORIGINAL ARTICLE

Exploring the impact of fossil constraints on the divergence time estimates of derived liverworts

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Abstract In this study, we evaluate the impact of fossil assignments and different models of calibration on divergence time estimates carried out as Bayesian analyses. Estimated ages from preceding studies and liverwort inclusions from Baltic amber are used as constraints on a molecular phylogeny of Cephaloziineae (Jungermanniopsida) obtained from sequences of two chloroplast coding regions: *rbc*L and *psb*A. In total, the comparison of 12 different analyses demonstrates that an increased reliability of the chronograms is linked to the number of fossils assigned and to the accuracy of their assignments. Inclusion of fossil constraints leads to older ages of most crown groups, but has no influence on lineage through time plots suggesting a nearly constant accumulation of diversity since the origin of Cephaloziineae in the early to Middle Jurassic. Our results provide a note of caution regarding the interpretation of chronograms derived from DNA sequence variation of extant species based on a single calibration point and/or low accuracy of the assignment of fossils to nodes in the phylogeny.

Keywords Amber fossils · BEAST · Cephaloziineae · Divergence time estimates · Jungermanniopsida

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Introduction

DNA-sequence-based divergence time estimates are now widely employed to study the evolution of various lineages of animals, fungi and plants. The application of these methods resulted in a dramatic improvement of our understanding of evolutionary events and processes, especially in lineages with a poor and patchy fossil record (Hedges and Kumar 2009). However, these results are often controversial, especially when comparisons with the fossil record can be drawn (e.g. Donoghue and Benton 2007; Kenrick et al. 2012; Parham et al. 2012). These controversies are partly caused by confusion about the accuracy of the information obtained by DNA sequence-based or fossil-based reconstruction of lineage histories and ages (Kenrick et al. 2012).

Arguably, one of the most important sources of inaccuracy in molecular-based divergence time estimates is the need to calibrate the molecular clocks using fossil evidence (Donoghue and Benton 2007; Hedges and Kumar 2009; Parham et al. 2012). Other approaches, such as estimates of geological events have been considered as alternatives to fossil-based calibrations, but their usage is restricted to certain cases and could result in misleading estimates (Renner 2005). Early methods of molecular clock dating

used single calibration points despite the fact that the age of a fossil is nearly always documented as a time interval. Improvement has been achieved by the introduction of methods that employ minimum and maximum age constraints beside one or several calibration points (Ho 2007; Benton et al. 2009; Inoue et al. 2010; Magallon 2010; Heled and Drummond 2012; Lukoschek et al. 2012; Sauquet et al. 2012). The most recent innovations allow us to integrate the information given by fossil evidence in form of probability distributions. This kind of calibration is usually part of relaxed molecular clock methods that estimate divergence times based on the DNA sequence data using one or several genomic regions either as a single or several partitions. They allow the integration of several fossil constraints in a single analysis, but require to study the impact of fossil assignments on the sister lineages of the calibrated clades in the tree very careful (Benton et al. 2009; Inoue et al. 2010; Magallon 2010; Parham et al. 2012).

Most authors agree on the importance of the calibration and constraining of molecular clocks with the fossil record (Benton et al. 2009; Hedman 2010; Inoue et al. 2010; Magallon 2010; Pyron 2010; Wilkinson et al. 2011; Lukoschek et al. 2012; Sauquet et al. 2012) and various methods have been made available to integrate this information (Ho 2007; McCormack et al. 2010; Heled and Drummond 2012). So far, relatively few studies explored the impact of these parameters on the estimates obtained from these analyses (e.g., Jacques et al. 2007; Sauquet et al. 2012). However, such studies are required to educate us about the limitations of the currently available methods and to develop guidelines of the employment of parameter models and fossil information (Near and Sanderson 2004; Hedman 2010; Inoue et al. 2010; Lukoschek et al. 2012).

Here, we use a main clade of leafy liverworts (Jungermanniidae), the Cephaloziineae, as a model to study the impact of fossil constraints. Generally, liverworts have a rather limited fossil record (Krassilov and Schuster 1984; Taylor et al. 2009), but they are often found as amber inclusions with a very high quality in character preservation (Grolle and Meister 2004a). Unfortunately, amber occurs discontinuously in space and time (Grimaldi 1996). This preservation bias in form of gaps in the record over time has to be taken into consideration when using amber fossils to infer the diversification of organisms (Smith 2001; Pyron 2010; Dornberg et al. 2011; Lloyd et al. 2012). DNA sequence-based divergence time estimates are potentially capable to overcome this shortcoming by integrating the discontinuous amber fossil record into the framework provided by either an already dated phylogeny or preferably into simultaneously inferred divergence time estimates.

The high quality of the preservation of amber fossils allows for a description of many morphological characters that are phylogenetically or taxonomically informative. The exquisite preservation enables the application of roles to the assignment proposed as best practice (Parham et al. 2012) despite some uncertainties concerning the taxonomic interpretation and age of the fossils. Several studies focused on the relationships of amber inclusions of liverworts to extant taxa (Grolle 1983, 1985, 1993; Gradstein 1993; Grolle and Schmidt 2001; Grolle and Meister 2004a, b; Heinrichs and Schmidt 2010; Heinrichs et al. 2011, 2012b). Thus, these fossils can be potentially assigned to particular nodes in the phylogeny of the studied lineage of liverworts and some studies reconstructed the diversification of selected liverwort lineages using either a DNA clock only approach or a DNA plus amber fossil approach (Hartmann et al. 2006, 2007, 2009b; Wilson et al. 2007; Devos and Vanderpoorten 2009). Amber inclusions are, however, not the only source of liverwort fossils (Krassilov and Schuster 1984). Some liverworts fossils have been recorded with different taphonomic background such as *Sinolejeunea* from the Middle Jurassic Yima Formation (Yang and Wu 2010) or anatomical preserved remnants of leafy liverworts from the Eocene of Canada (Steenbock et al. 2011).

Molecular phylogenetic studies indicate that the liverworts (Marchantiophyta) have separated from other lineages in the earliest diversification of plants on land (Qiu et al. 2006). Heinrichs et al. (2007) estimated the age of the leafy liverworts (Jungermanniidae) to the late Carboniferous (308.7 $\pm$ 7.8 Ma), but slightly older ages may need to be considered given the uncertainty of divergence time estimates of the oldest splits of liverworts (Clarke et al. 2011; Kenrick et al. 2012).

In this study, we explore the integration of the amber fossil record in studies focusing on the diversification of a derived lineage of liverworts, the Jungermannialean lineage Cephaloziineae (Crandall-Stotler et al. 2009), which comprises the families Cephaloziaceae, Cephaloziellaceae, Hygrobiellaceae, Adelanthaceae s. l. and Scapaniaceae s. l. We are particularly interested in the impact of alternative assignments that reflect uncertainties in the interpretation of the fossils' relationships (see also Sauquet et al. 2012). This study will also provide us with empirical evidence about the performance of recently established methods to estimate divergence times in the context of phylogenetic uncertainty concerning the relationships of the taxa preserved in the fossil record (Drummond et al. 2006). All analyses were carried out using BEAST, a Bayesian MCMC method capable to obtain time-measured phylogenies with confidence intervals using relaxed molecular clocks and various parameters to integrate information about the evolutionary processes and the fossil record

(Drummond et al. 2006). The main focus of this study is on the performance of relaxed clock methods using state-of-the-art calibration tools (Ho 2007; Battistuzzi et al. 2010; Sauquet et al. 2012). In particular, we investigate the impact of different calibrations on the estimates of diversification rate changes through time.

Materials and methods

Taxon sampling and assembling of DNA sequence data

For this study, we obtained chloroplast DNA *rbc*L- and *psb*A-sequences of most genera that are assigned to the Cephaloziineae based on the morphological studies or on the molecular phylogenetic results of several preceding works (Forrest et al. 2006; He-Nygrén et al. 2006; de Roo et al. 2007; Heinrichs et al. 2007; Feldberg et al. 2009, 2010; Vilnet et al. 2010). In addition to a DNA dataset of Adelanthaceae s. l. (Feldberg et al. 2010), we obtained *rbc*L and *psb*A sequences of related lineages via DNA sequencing or GenBank.

For DNA sequencing plant tissue from the distal portions of a few shoots was isolated from herbarium collections. Total genomic DNA was purified using Invisorb Spin Plant Mini Kit (Invitek, Berlin, Germany) prior to amplification. Protocols for PCR were carried out as described in the previous publications: *rbc*L from Hentschel et al. (2006), and *psb*A from Forrest and Crandall-Stotler (2004).

Bidirectional sequences were generated using an MegaBACE 1000 automated sequencing machine using DYEnamic ET Primer DNA Sequencing Reagent (Amersham Biosciences, Little Chalfont, UK). Newly generated sequences were assembled and edited using SeqAssem (Hepperle 2004).

Initial phylogenetic analyses

All sequences (Table 1) were aligned manually in BioEdit version 5.0.9 (Hall 1999). Ambiguous positions were excluded from the alignment; missing nucleotides in the aligned sequences were coded as missing. jModelTest 0.1 (Posada 2008) was employed to choose nucleotide substitution models each for the *rbc*L as well as the *psb*A dataset and the combined matrices. The Akaike information criterion supported the GTR + Γ + I model as the best fit for both partitions and also for the unpartitioned dataset. Topological congruence of the two datasets was explored by comparing visually phylogenetic trees obtained from maximum likelihood-based bootstrap analyses with Garli 0.96 (Zwickl 2006).

All divergence time analyses were carried out using BEAST package version 1.5.4 (Drummond and Rambaut 2007). With BEAUti 1.5.4 (BEAST package) two partitions were created, one for each marker, in addition to a combined dataset. Because previous analyses rejected a strict clock for the leafy liverworts (Heinrichs et al. 2007), we used the uncorrelated relaxed clock model (Drummond et al. 2006) with a lognormal distribution of rates estimated during the analyses. For each analysis, one run with 100 million generations, and sampling of every 10,000th generation was undertaken. All analyses were carried out with the models selected by jModelTest 0.1 (Posada 2008), with the age estimates and a birth–death model implemented. Tracer 1.5 (Rambaut and Drummond 2003–2009) was used to check the effective sampling size (ESS) for all parameters. The results were considered reliable when the ESS exceeded 500 for all parameters. Age estimates were summarized in a mean consensus chronogram built with TreeAnnotator 1.5.4 (BEAST package) after exclusion of trees recovered during the burn-in-phase. FigTree 1.3.1 (Rambaut 2006–2009) was used to display the consensus tree created via TreeAnnotator. All analyses were carried out with calibrations described below. To achieve comparability, only calibrations were modified.

Assignment of fossil evidence

We tested several approaches to calibrate the tree. First, a series of analyses with only an age constraint for the root node was employed. BEAST performs more reliable under the assumption of a basal constraint (Drummond et al. 2006; McCormack et al. 2010), but since no fossil is known that could serve as maximum age constraint the age for this node was taken from a previous divergence time estimate for liverworts (Heinrichs et al. 2007). We chose the minimum age of 158.0–500.0 Ma for the analyses with uniform distributed priors. Based on the results of this analysis, we chose the estimated age of 171.1 ± 8.3 Ma and a normal distributed prior for following analyses.

Beside analyses with single root node calibrations, we carried out several analyses with additional fossil-based calibrations. Three inclusions from Eocene Baltic amber (35–48 Ma; Standke 1998) are applicable to the tree: *Scapania hoffeinsiana* Grolle, *Lophozia kutscheri* Grolle, and *Cylindrocolea dimorpha* (Casp.) Grolle (Grolle and Schmidt 2001; Grolle and Meister 2004a, b; Frahm 2006). All three fossils are sufficiently well preserved and their taxonomic relationships have been thoroughly discussed by Grolle and Meister (2004a).

Both gametophyte and sporophyte of *S. hoffeinsiana* have been well preserved and indicated it as a member of the crown-group of *Scapania*. It resembles *Scapania* (subg. *Scapania*) *umbrosa* (Schrad.) Dumort. in several aspects (Grolle and Schmidt 2001; Grolle and Meister 2004a). The perianth is dorsally eplicate, a common character of

Table 1 Geographic origins, vouchers, and GenBank accession numbers of the taxa investigated

Species	Voucher and herbarium	Origin	GenBank acc. nos.	
			*rbc*L	*psb*A
Adelanthus falcatus (Hook.) Mitt.	Engel and von Konrat 23859 (GOET)	New Zealand	GQ900278	GQ900069
A. lindenbergianus (Lehm.) Mitt.	Jácome JJ1045 (GOET)	Bolivia	GQ900284	GQ900076
Alobiella husnotii (Gottsche) Schiffn.	Schäfer-Verwimp and Verwimp 17800 (GOET)	Dominica	**KC184698**	**KC184767**
Anastrophyllum auritum (Lehm.) Steph.	Churchill and Linneo 24571 A (GOET)	Bolivia	**KC184699**	**KC184768**
A. bidens (Reinw., Blume and Nees) Steph.	Gradstein 12067 (GOET)	Indonesia	**KC184700**	**KC184769**
A. donnianum (Hook.) Steph.	Norris 96581 (GOET)	Alaska	**KC184701**	**KC184770**
A. leucocephalum (Taylor ex Lehm.) Steph.	Schäfer-Verwimp et al. 24471 (GOET)	Ecuador	**KC184702**	**KC184771**
A. nigrescens (Mitt.) Steph.	Schäfer-Verwimp et al. 24444 (GOET)	Ecuador	**KC184703**	**KC184772**
A. piligerum (Nees) Steph.	Schäfer-Verwimp et al. 24271 (GOET)	Ecuador	**KC184704**	**KC184773**
A. tubulosum (Nees) Grolle	Schäfer-Verwimp et al. 24464 (GOET)	Ecuador	**KC184705**	**KC184774**
Andrewsianthus australis J.J. Engel	Schäfer-Verwimp and Verwimp 23734 (GOET)	Thailand	**KC184706**	**KC184775**
A. perigonialis (Hook.f. and Taylor) R.M.Schust.	Engel and von Konrat 27283 (GOET)	New Zealand	**KC184707**	**KC184776**
Barbilophozia barbata (Schmidel ex Schreb.) Loeske	Hentschel Bryo 0753 (GOET)	Bulgaria	DQ312477	GQ900081
B. hatcheri (A. Evans) Loeske	Bakalin 53 (GOET)	Russia	**KC184708**	**KC184777**
B. lycopodioides (Wallr.) Loeske	Schäfer-Verwimp and Verwimp 27242 (GOET)	France	**KC184709**	**KC184778**
Cephalozia badia (Gottsche) Steph.	Schäfer-Verwimp and Verwimp 10867/A (GOET)	Argentina	**KC184710**	**KC184779**
C. bicuspidata (L.) Dumort.	Hentschel Bryo 0362 (GOET)	Germany	AM392307	AM396186
C. crassifolia (Lindenb. and Gottsche) Fulford	Churchill et al. 21621 (GOET)	Bolivia	**KC184711**	**KC184780**
C. infuscata R.M.Schust.	Gradstein 8936 (GOET)	Colombia	**KC184712**	–
C. otaruensis Steph.	Tsubota 220 (GOET)	Japan	**KC184713**	**KC184781**
C. pachycaulis R.M.Schust.	Bakalin 30 (GOET)	Russia	**KC184714**	**KC184782**
Cephaloziella divaricata (Sm.) Schiffn.	Hentschel Bryo 01159 (GOET)	Germany	DQ312481	AM396180
C. granatensis (J.B.Jack ex Steph.) Fulford	Dauphin et al. 1548 (GOET)	Panama	**KC184715**	**KC184783**
C. microphylla (Steph.) Douin	Schäfer-Verwimp and Verwimp 16291 (GOET)	Thailand	**KC184716**	**KC184784**
C. spinicaulis Douin	Deguchi 119 (GOET)	Japan	**KC184717**	**KC184785**
C. stellulifera (Taylor ex Gottsche, Lindenb. and Nees) Schiffn.	Doyle 11250 (GOET)	USA	**KC184718**	**KC184786**
C. turneri (Hook.) Müll.Frib.	Shevock 27856 (GOET)	USA	**KC184719**	**KC184787**
Cephaloziopsis intertexta (Gottsche) R.M. Schust.	Linneo et al. 424 (GOET)	Bolivia	**KC184720**	**KC184788**
Cladopodiella fluitans (Nees) H.Buch	Heinrichs et al. 2058 (GOET)	Germany	**KC184721**	**KC184789**
Cuspidatula flaccida (Steph.) Feldberg, Váňa, Hentschel and Heinrichs	Gradstein and Ariyanti 11025 (GOET)	Indonesia	GQ900288	GQ900080
C. monodon (Hook.f. and Taylor) Steph.	Pócs and Streimann 99189/B (EGR)	Australia	GQ900299	GQ900091
Cylindrocolea recurvifolia (Steph.) Inoue	Yamaguchi 28949 (GOET)	Japan	**KC184722**	**KC184790**
Diplophyllum albicans (L.) Dumort.	Hentschel Bryo 0240 (GOET)	Germany	AM392309	AM396190
D. obtusifolium (Hook.) Dumort.	Hentschel Bryo 02592 (GOET)	Germany	**KC184723**	**KC184791**
Douinia ovata (Dicks.) H.Buch	Schofield 1809 (GOET)	Canada	**KC184724**	**KC184792**
Gottschelia schizopleura (Spruce) Grolle	Ah-Peng R96 (GOET)	Madagascar	FJ984940	KC184793

Table 1 continued

Species	Voucher and herbarium	Origin	GenBank acc. nos.	
			*rbc*L	*psb*A
Hygrobiella laxifolia (Hook.) Spruce	Bakalin 61 (GOET)	Russia	**KC184725**	**KC184794**
Kymatocalyx dominicensis (Spruce) Váňa	Schäfer-Verwimp and Verwimp 22451 (GOET)	Guadeloupe	**KC184726**	**KC184795**
Lophozia ascendens (Warnst.) R.M. Schust.	Klama et al. 171 (GOET)	Poland	**KC184727**	**KC184796**
L. lantratoviae Bakalin	Bakalin P-72-19-05 (GOET)	Russia	**KC184728**	**KC184797**
Lophoziopsis excisa (Dicks.) Konstant. and Vilnet	Bakalin P-74-17-05 (GOET)	Russia	**KC184729**	**KC184798**
L. longidens (Lindb.) Konstant. and Vilnet	Bakalin 65 (GOET)	Russia	**KC184730**	**KC184799**
Metahygrobiella albula (Steph.) Grolle	Deguchi 219 (GOET)	Japan	**KC184731**	**KC184800**
Metahygrobiella macgregorii (Steph.) R.M.Schust.	Koponen 35296 (GOET)	Papua New Guinea	**KC184732**	**KC184801**
Neoorthocaulis attenuatus (Mart.) L.Söderstr., De Roo and Hedd.	Strebel 226 (GOET)	Poland	**KC184733**	**KC184802**
Neoorthocaulis floerkei (F.Weber and D.Mohr) L. Söderstr., De Roo and Hedd.	Drehwald s.n. (GOET)	Germany	**KC184734**	**KC184803**
Nowellia dominicensis Steph.	Schäfer-Verwimp and Verwimp 17954/A (GOET)	Dominica	**KC184735**	**KC184804**
N. curvifolia (Dicks.) Mitt.	Schäfer-Verwimp and Verwimp 26658 (GOET)	Dominican Republic	**KC184736**	**KC184805**
Odontoschisma denudatum (Nees) Dumort.	Churchill et al. 24480 (GOET)	Ecuador	**KC184737**	**KC184806**
Odontoschisma elongatum (Lindb.) A. Evans	Bakalin 40 (GOET)	Russia	**KC184738**	**KC184807**
Odontoschisma falcifolium Steph.	Gradstein 8535 (GOET)	Colombia	**KC184739**	**KC184808**
Odontoschisma longiflorum (Taylor) Trevis.	Churchill et al. 24307 (GOET)	Ecuador	**KC184740**	**KC184809**
Odontoschisma portoricensis (Hampe and Gottsche) Steph.	Gradstein 5000 (GOET)	Guyana	**KC184741**	–
Pleurocladula albescens (Hook.) Grolle	Schäfer-Verwimp and Verwimp 18194 (GOET)	Austria	**KC184742**	**KC184810**
Plicanthus hirtellus (F.Weber) R.M. Schust.	Gradstein 10388 (GOET)	Malaysia	**KC184743**	**KC184811**
Pseudomarsupidium decipiens (Hook.) Grolle	Wigginton 05/613 (GOET)	St. Helena	FJ984934	GQ900113
Scapania ampliata Steph.	Yokoyama 11576 (GOET)	Japan	**KC184744**	**KC184812**
S. aspera M. Bernet and Bernet	Hentschel Bryo 0762 (GOET)	Bulgaria	AM392310	AM396191
S. bolanderi Austin	Whittemore 6738 (GOET)	USA	**KC184745**	**KC184813**
S. calcicola (Arnell and J.Perss.) Ingham	Hentschel Bryo 01300 (GOET)	Germany	**KC184746**	**KC184814**
S. curta (Mart.) Dumort.	Hentschel Bryo 03174 (GOET)	Germany	**KC184747**	**KC184815**
S. cuspiduligera (Nees) Müll.Frib.	Long 13996 (GOET)	USA	**KC184748**	**KC184816**
S. hyperborea Jørg.	Hentschel Bryo 03230 (GOET)	Norway	**KC184749**	**KC184817**
S. javanica Gottsche	Schäfer-Verwimp and Verwimp 16900 (GOET)	Indonesia	**KC184750**	**KC184818**
S. nemorea (L.) Grolle	Schäfer-Verwimp and Verwimp 28792 (GOET)	Germany	**KC184751**	**KC184819**
S. ornithopodioides (With.) Waddel	Rycroft 1511 (GOET)	UK	**KC184752**	**KC184820**
S. paludosa (Müll.Frib.) Müll.Frib.	Schäfer-Verwimp and Verwimp19613 (GOET)	Germany	**KC184753**	**KC184821**
S. portoricensis Hampe and Gottsche	Churchill 24297 (GOET)	Ecuador	**KC184754**	**KC184822**
S. sphaerifera H.Buch and Tuom.	Konstantinova 110802 (GOET)	Russia	**KC184755**	**KC184823**
S. uliginosa (Sw. ex Lindenb.) Dumort.	Schäfer-Verwimp and Verwimp 18181 (GOET)	Austria	**KC184756**	**KC184824**
S. umbrosa (Schrad.) Dumort.	Eckstein 6509 (GOET)	Germany	**KC184757**	**KC184825**
S. undulata (L.) Dumort.	Schäfer-Verwimp and Verwimp 27551 (GOET)	Italy	**KC184758**	**KC184826**
Schiffneria hyalina Steph.	Schäfer-Verwimp and Verwimp 24869 (GOET)	Indonesia	**KC184759**	–

Table 1 continued

Species	Voucher and herbarium	Origin	GenBank acc. nos.	
			*rbc*L	*psb*A
Schistochilopsis incisa (Schrad.) Konstant.	Shevock 27788 (GOET)	USA	**KC184760**	**KC184827**
Sphenolobus minutus (Schreb.) Berggr.	Hentschel Bryo 0421 (GOET)	Spitsbergen	DQ312475	GQ900117
S. saxicola (Schrad.) Steph.	Heinrichs et al. JH3734 (GOET)	Germany	**KC184761**	**KC184828**
Syzygiella anomala (Lindenb. and Gottsche) Steph.	Heinrichs et al. K4 (GOET)	Ecuador	GQ900328	GQ900124
S. autumnalis (DC.) Feldberg, Váňa, Hentschel and Heinrichs	Schröder 8327/2 (JE)	Germany	GQ900301	GQ900093
S. jacquinotii (Mont.) Hentschel, Feldberg, Váňa and Heinrichs	Drehwald and Mues 970061 (GOET)	Argentina	GQ900321	GQ900115
S. liberata Inoue	Churchill 22577 (GOET)	Bolivia	FJ984936	GQ900143
S. perfoliata (Sw.) Spruce	Costa and Gradstein 3888 (GOET)	Brazil	GQ900353	GQ900152
S. pseudocclusa (E.A.Hodgs.) Feldberg, Váňa, Hentschel and Heinrichs	Engel and von Konrat 27179 (GOET)	New Zealand	GQ900292	GQ900085
S. rubricaulis (Nees) Steph.	Sauer MS-E 251 (GOET)	Ecuador	GQ120508	GQ900156
S. securifolia (Nees ex Lindenb.) Inoue	Ilkiu-Borges et al. 2970 (GOET)	Malaysia	GQ900361	GQ900160
S. tasmanica (Hook.f. and Taylor) Feldberg, Váňa, Hentschel and Heinrichs	Engel 20486 (GOET)	New Zealand	GQ900316	GQ900107
Tetralophozia cavallii (Gola) Váňa	Pócs and Ochyra 88152/C (GOET)	Tanzania	**KC184762**	**KC184829**
T. pilifera (Steph.) R.M.Schust.	Gradstein 11015 (GOET)	Indonesia	**KC184763**	**KC184830**
T. setiformis (Ehrh.) Schljakov	Faubert 268.3 (GOET)	Canada	**KC184764**	**KC184831**
Tritomaria exsecta (Schmidel ex Schrad.) Loeske	Schäfer-Verwimp and Verwimp 21816(GOET)	Russia	**KC184765**	**KC184832**
T. exsectiformis (Breidl.) Loeske	Schäfer-Verwimp and Verwimp 21849/A (GOET)	Russia	**KC184766**	**KC184833**
T. quinquedentata (Huds.) H. Buch	Heinrichs 2978 (GOET)	Germany	AY700003	AM396189
Wettsteinia inversa (Sande Lac.) Schiffn.	Gradstein 11014 (GOET)	Indonesia	FJ984935	GQ900168
Wettsteinia schusteriana Grolle	Engel 23131 (GOET)	New Zealand	GQ900369	GQ900169

Accession numbers of new sequences are in bold

members of the derived subgenus *Scapania*. *Scapania* developed from an ancestor with a plicate perianth, however, not only most members of the derived subg. *Scapania* have eplicate perianths, but also some early diverging species (Heinrichs et al. 2012a). We assume that the different perianth-forms of *Scapania* derived from the plicate perianth type and that the eplicate perianth is an apomorphy of the crown group. In combination with the leaf characters of *S. hoffeinsiana* it indicates a position within *S.* subg. *Scapania* and can be distinguished from independently evolved similar structures in some earlier diverging lineages of *Scapania*. Therefore, *S. hoffeinsiana* may not only be assigned as the ancestor of the whole genus *Scapania*, but also as the ancestor of the subgenus *Scapania*. We performed several analyses with only the basal constraint and different assignments of *S. hoffeinsiana* to test if this significantly influenced the ages of the sister lineages.

For the other fossils, it was not possible to test varying assignments to higher or lower ranking clades, so we placed them near to the most similar extant relative to perform analyses with all three fossils. Based on the morphological treatment in Grolle and Meister (2004a) *C. dimorpha* was assigned to crown group node B containing *C. recurvifolia* (Steph.) Inoue, and *L. kutscheri* was assigned to the base of the *Barbilophozia* clade, since it is most similar to *Barbilophozia hatcheri* (A. Evans) Loeske.

Divergence times estimates and lineages through time plots

Three of the 12 analyses were performed with a root node-constraint only: 1.A: a partitioned dataset with uniform prior distribution of 158.0–500.0 Ma, 2.A: a partitioned dataset with normal distributed prior distribution of 171.1 ± 8.3 Ma and 3.A: an un-partitioned dataset with normal prior distribution of 171.1 ± 8.3 Ma.

The Eocene amber fossils were added as uniform minimum age constraints of 35.0–100.0 Ma. Lineages through time plots for the ingroup (A) of the analyses 2.A and 2.D were performed with Tracer 1.5.

Table 2 Results of the analyses using age constraints (bold face) with uniform distributed minimum age priors for the Cephaloziineae (A) and the fossil constraints (B*, C*, D1*, D2*)

Partitioned analyses: uniform constraints (minimum age)				
	1.A tmrca (A) = 158.0–500.0	1.B tmrca (A) = 158.0–500.0 tmrca (D2) = 35.0–100.0	1.C tmrca (A) = 158.0–500.0 tmrca (D1) = 35.0–100.0	1.D tmrca (A) = 158.0–500.0 tmrca (B) = 35.0–100.0 tmrca (C) = 35.0–100.0 tmrca (D2) = 35.0–100.0
Cephaloziineae (calibration node A)	**208.68*** (158.01–371.77)	**453.82*** (228.19–480.58)	**186.39*** (160.97–191.46)	**404.87*** (283.58–499.9)
C.0 (Cephaloziaceae)	193.06 (120.49–326.48)	396.08 (197.6–425.47)	151.58 (134.37–175.65)	349.05 (228.5–439.34)
C.I	73.4 (42.75–155.6)	168.99 (74.72–208.54)	58.09 (49.78–99.56)	127.74 (92.03–224.69)
C.II	113.59 (68.70–205.01)	232.46 (118.71–268.89)	102.82 (80.07–119.14)	208.34 (138.64–281.67)
AD.0 (Adelanthaceae)	94.03 (63.23–209.21)	235.2 (109.55–281.35)	106.69 (75.39–135.96)	209.89 (127.2–296.39)
AD.I (Adelanthoideae)	58.16 (32.70–118.73)	101.48 (57.05–155.98)	66.53 (37.77–79.28)	139.49 (70.32–171.72)
AD.II (Jamesonielloideae)	49.89 (38.36–125.35)	131.68 (68.45–166.11)	77.64 (45.09–80.89)	139.39 (78.75–170.57)
Syzygiella	47.89 (34.85–111.71)	124.99 (61.94–149.69)	67.21 (42.12–72.44)	122.58 (71.62–153.01)
CE.0 (Cephaloziellaceae)	51.15 (28.07–95.03)	95.23 (48.71–126.65)	45.11 (33.63–63.49)	90.31 (63.68–137.02)
Calibration node B*	25.17 (10.96–43.14)	36.19 (18.83–58.36)	21.57 (12.12–29.98)	**47.88*** (35–63.45)
S.0 (Scapaniaceae s. l.)	90.51 (57.89–177.34)	156.22 (103.36–234.97)	94.5 (71.47–109.89)	174.53 (118.67–244.92)
S.I (Gottschelioideae)	54.68 (37.56–143.67)	122.73 (70.63–194.64)	69.19 (47.45–94.78)	124.45 (84.49–211.58)
S.II	46.13 (30.7–98.46)	97.53 (53.41–129.59)	48.96 (36.98–65.08)	96.43 (60.71–137.91)
Anastrophyllum	33.00 (18.03–65.07)	74.85 (30.63–87.99)	37.77 (21.35–44.5)	65.82 (36.43–94.92)
Sphenolobus	24.02 (8.72–47.97)	39.8 (14.29–67.05)	20.77 (8.93–35.48)	49.75 (17.67–70.98)
Barbilophozia (calibration node C*)	30.42 (12–56.45)	57.43 (20.01–79)	18.04 (13.45–41.05)	**51.76*** (35–81.28)
Neoorthocaulis	22.64 (7.63–49.54)	50.81 (14.18–68.89)	30.62 (9.72–36.49)	56.1 (18.45–74)
Tetralophozia/Plicanthus	28.2 (15.32–57.23)	50.91 (26.55–75.92)	29.16 (18.21–38.69)	64.48 (31.58–80.76)
S.III (Lophozioideae)	57.17 (28.02–102.92)	115.92 (51.13–136.52)	54.17 (35.28–70.12)	108.06 (59.76–148)
Tritomaria	19.29 (8.46–46.38)	49.29 (14.85–66.88)	18.23 (9.49–35.5)	37.38 (18.79–75.39)
Lophoziopsis/Lophozia	52.58 (23.27–89.07)	106.09 (42.07–118.2)	51.99 (26.8–60.28)	72.96 (46.92–125.88)
S.IV (Scapanioideae)	54.17 (35.58–110.37)	109.51 (65.37–145.03)	62.93 (48.53–71.33)	166.05 (75.81–166.05)
Diplophyllum	30.96 (9.29–45.93)	44.98 (16.57–63.92)	16.47 (11.4–35.58)	48.57 (21.58–72.05)
Scapania (calibration node D1*)	24.39 (18.16–58.84)	76.08 (38.69–78.05)	**36.46*** (35–40.67)	65.96 (42.27–84.42)
Scapania subg. *Scapania* (calibration node D2*)	20.92 (14.25–45.96)	**62.72*** (35–62.72)	27.25 (20.85–34.48)	**53.78*** (35–64.17)

Mean ages of the families, the calibration nodes and selected genera in different analyses in Ma, 95 % HPD indicated in parentheses. The asterisks indicate the age constraints

Results

Divergence time estimates using a single age constraint with different prior-models for the Cephaloziineae

The three analyses (1.A, 2.A, and 3.A; Tables 2, 3, 4) provide different age estimates for Cephaloziineae (node A*; Figs. 1, 2). The partitioned dataset with a uniform prior recovers the oldest estimate with a node age of 208.68 Ma and a confidence interval of 158.01–371.77 Ma (Table 2; Fig. 3). The age of node A fluctuates strongly in different runs with the same settings (trees not shown) and the confidence intervals are quite broad (Fig. 3).

The analyses with normal constraints produce younger ages and different runs do not show the fluctuation seen in analysis 1.A (Tables 2, 3, 4; Fig. 3). In analysis 2.A node A* is estimated to 171.84 Ma (confidence interval 153.03–185.76 Ma) and in analysis 3.A it is estimated to 180.01 Ma (confidence interval 153.88–185.96 Ma). Unpartitioned and partitioned analyses with a normal distributed basal calibration show rather narrow confidence intervals, in contrast to the results in analysis 1.A.

Table 3 Results of the analyses using age constraints (bold face) with normally distributed prior for the Cephaloziineae (A) and uniformly distributed minimum age priors for the fossil constraints (B*, C*, D1*, D2*)

Partitioned analyses: normal constraint for the Cephaloziinae/uniform minimum age constraint for the fossils				
	2.A (Fig. 1) tmrca (A) = 171.1 ± 8.3	2.B tmrca (A) = 171.1 ± 8.3 tmrca (D2) = 35.0–100.0	2.C tmrca (A) = 171.1 ± 8.3 tmrca (D1) = 35.0–100.0	2.D (Fig. 2) tmrca (A) = 171.1 ± 8.3 tmrca (B) = 35.0–100.0 tmrca (C) = 35.0–100.0 tmrca (D2) = 35.0–100.0
Cephaloziineae (calibration node A)	**171.84*** (153.03–185.76)	**191.76*** (164.59–194.25)	**180.62*** (161.2–191.97)	**182.81*** (167.97–197.89)
C.0 (Cephaloziaceae)	143.66 (126.56–172.08)	165.38 (137.88–178.57)	158.87 (133.22–175.08)	147.71 (141.27–181.54)
C.I	69.1 (44.22–93.35)	72.02 (51.73–104.85)	84.71 (50.19–100.25)	62.42 (55.47–111.1)
C.II	86.42 (71.09–112.27)	115.21 (83.6–123.37)	95.13 (79.98–119.4)	91.67 (86.22–126.61)
AD.0 (Adelanthaceae)	84.43 (64.73–124.49)	109.18 (81.3–143.46)	106.72 (76.72–136.78)	142.92 (88.27–151.95)
AD.I (Adelanthoideae)	57.03 (33.17–70.93)	58.59 (41.89–85.59)	65.33 (38.41–79.13)	64.19 (43.8–91.02)
AD.II (Jamesonielloideae)	44.02 (39.81–73.36)	62.87 (49.68–87.63)	79.42 (45.31–81.19)	75.46 (53.78–94.38)
Syzygiella	42.36 (35.31–64.3)	59.73 (44.38–77.75)	59.69 (42.39–73.6)	68.05 (49.28–84.12)
CE.0 (Cephaloziellaceae)	40.29 (28.11–56.19)	51.46 (35.82–68.48)	56.02 (33.29–62.69)	69.89 (48.53–77.97)
calibration node B*	14.31 (10.5–25.94)	21.54 (13.58–32.45)	16.16 (12.09–29.69)	**41.86*** (35–43.06)
S.0 (Scapaniaceae s. l.)	76.78 (58.28–99.64)	94.73 (77.36–117.06)	87.68 (71.97–110.95)	108.11 (85.28–126.32)
S.I (Gottschelioideae)	65.8 (39.85–85.08)	82.57 (52.41–104.17)	65.75 (48.65–96)	94.14 (56.89–110.67)
S.II	39.35 (30.52–56.23)	74.57 (39.37–68.85)	63.56 (36.34–64.06)	61.3 (43.39–73.92)
Anastrophyllum	27.95 (18.89–39.35)	48.09 (22.47–46.79)	44.69 (21.57–44.47)	37.89 (23.83–48.93)
Sphenolobus	10.8 (7.51–31.37)	18.64 (9.35–39.9)	21.67 (9.42–36.21)	40.38 (12.66–49.44)
Barbilophozia (calibration node C*)	17.22 (11.64–36.81)	23.65 (14.2–45.73)	24.68 (13.64–41.62)	**52.2*** (35–54.38)
Neoorthocaulis	17.52 (7.92–32.14)	28.96 (10.7–39.53)	28.46 (9.86–36.66)	32.18 (11.13–44.6)
Tetralophozia/Plicanthus	21.37 (15.22–33.97)	29.3 (19.44–41.89)	31.45 (17.68–37.79)	35.69 (22–46.8)
S.III (Lophozioideae)	35.02 (29.38–60.51)	56.88 (37.23–75.25)	55.32 (34.7–69.19)	67.02 (41.27–81.51)
Tritomaria	16.56 (8.16–30.79)	39.28 (10.45–38.9)	37.75 (9.73–36.04)	20.96 (10.28–41.48)
Lophoziopsis/Lophozia	31.84 (23.72–52.52)	50.71 (29.14–65.39)	49.74 (27.5–60.49)	61.77 (31.72–70.99)
S.IV (Scapanioideae)	42.68 (35.52–62.31)	71.32 (51.64–77.47)	55.22 (48.62–71.25)	68.38 (54.56–82.29)
Diplophyllum	18.22 (9.41–29.26)	29.12 (12.02–39.2)	22.87 (11.55–36.07)	21.76 (13–42.38)
Scapania (calibration node D1*)	21.09 (18.89–34.13)	41.77 (36.02–46.42)	**36.56*** (35–40.7)	47.36 (36.73–48.82)
Scapania subg. *Scapania* (calibration node D2*)	18.7 (15.05–27.95)	**36.33*** (35–39.02)	35.27 (21.14–34.66)	**42.74*** (35–42.74)

Mean ages of the families, the calibration nodes and selected genera in different analyses in Ma, 95 % HPD indicated in parentheses. The asterisks indicate the age constraints

Clades with assignable amber inclusions (calibration nodes B*, C*, D1* and D2*; Figs. 1, 2) are estimated younger than the fossils indicate. The mean age of the node B* within the Cephaloziellaceae ranges between 25.17 Ma in analysis 1.A, 14.31 Ma in analysis 2.A and 22.66 Ma in analysis 3.A (Tables 2, 3, 4). The mean age of node C* (crown group *Barbilophozia*) ranges between 30.42 Ma (analysis 1.A), 17.22 Ma (analysis 2.A) and 14.11 Ma (analysis 3.A). Node D1* (crown group *Scapania*) shows a similar pattern; the mean ages are 24.39 Ma (analysis 1.A), 21.09 Ma (analysis 2.A) and 28.25 Ma (analysis 3.A), while the age of node D2* (crown group *S.* subg. *Scapania*) ranges between 20.92 Ma (analysis 1.A), 18.7 Ma (analysis 2.A) and 22.71 Ma (analysis 3.A).

Divergence time estimates using different models of root node constraints and different fossil calibrations

Additional uniform minimum age constraints have a strong impact on the divergence time estimates (analyses 1–3.B) with a constraint for node D2* (crown group *Scapania* subg. *Scapania*); analyses 1–3.C with a constraint for node

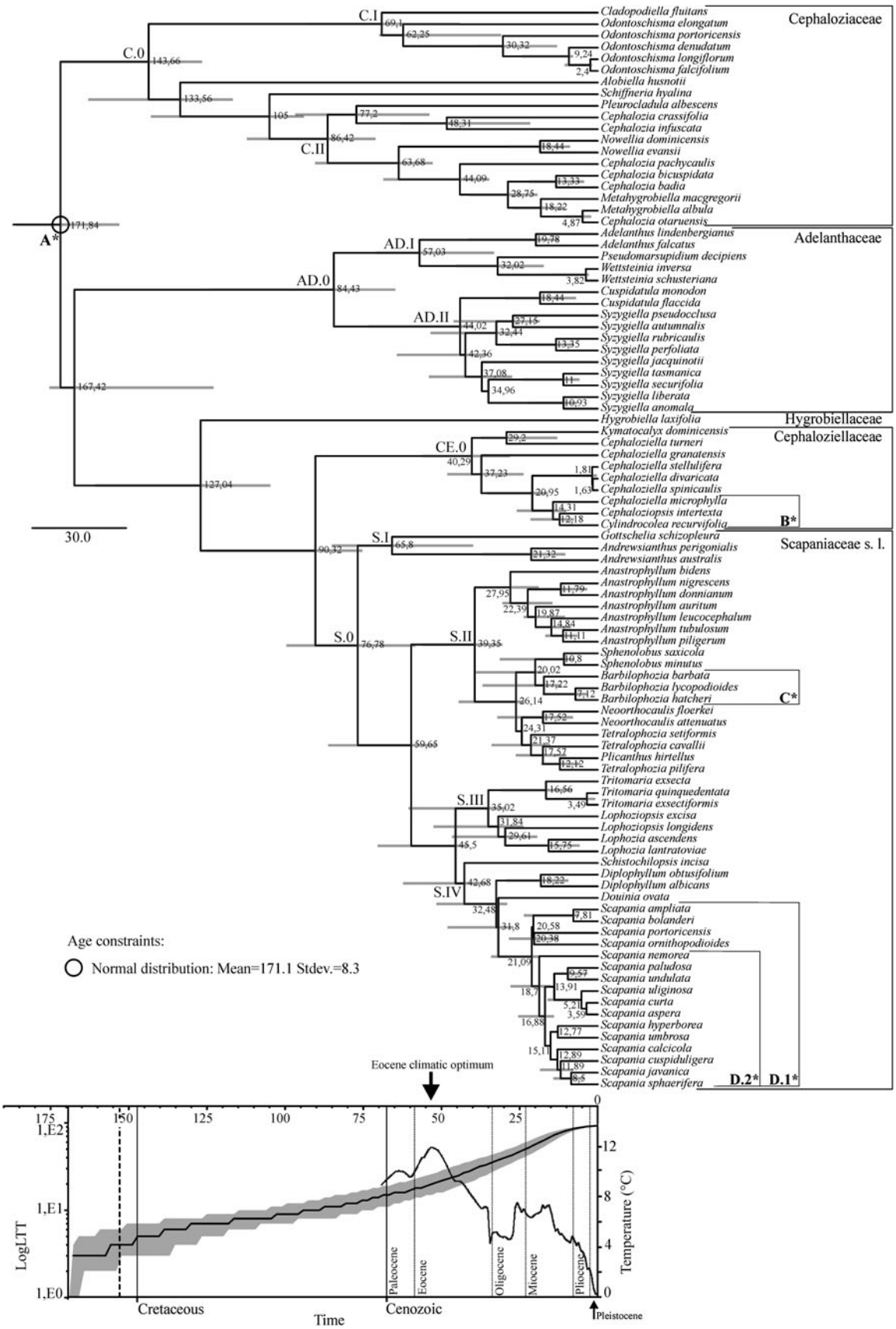
Table 4 Results of the analyses using age constraints (bold face) with normal distributed prior for the Cephaloziineae (A) and uniform distributed minimum age priors for the fossil constraints (B*, C*, D1*, D2*)

Unpartitioned analyses: normal constraint for the basal node/uniform minimum age constraint for the fossils				
	3.A tmrca (A) = 171.1 ± 8.3	3.B tmrca (A) = 171.1 ± 8.3 tmrca (D2) = 35.0–100.0	3.C tmrca (A) = 171.1 ± 8.3 tmrca (D1) = 35.0–100.0	3.D tmrca (A) = 171.1 ± 8.3 tmrca (B) = 35.0–100.0 tmrca (C) = 35.0–100.0 tmrca (D2) = 35.0–100.0
Cephaloziineae (calibration node A)	**180.01*** (153.88–185.96)	**182.36*** (162.34–192.41)	**180.99*** (159.85–190.54)	**178.74*** (164.46–195.22)
C.0 (Cephaloziaceae)	151.91 (118.83–169.41)	157.08 (130.7–176.26)	165.16 (128.17–174.09)	158.59 (134.74–178.93)
C.I	60.34 (38.71–93.54)	55.45 (46.66–103.9)	72.44 (43.64–100.52)	96.43 (48.82–110.25)
C.II	92.25 (71.01–115.19)	101.29 (78.85–125.01)	108.15 (76.6–120.38)	80.02 (81.68–127.78)
AD.0 (Adelanthaceae)	109.86 (65.6–136.78)	97.07 (80.86–152.59)	82.42 (75.32–145.84)	116.81 (89.2–158.35)
AD.I (Adelanthoideae)	59.11 (31.27–76.49)	74.33 (38.67–90.91)	48.31 (35.83–84.93)	71.18 (39.48–97.67)
AD.II (Jamesonielloideae)	90.4 (38.84–79.69)	61.64 (48.62–95.13)	64.79 (46.58–89.09)	67.37 (53.55–102.21)
Syzygiella	61.48 (35.65–69.86)	56.55 (42.15–81.7)	61.64 (41.01–77.02)	59.53 (47.04–88.56)
CE.0 (Cephaloziellaceae)	42 (30.4–64.52)	48.14 (38.92–78.19)	48.24 (34.85–70.6)	66.79 (51.78–88.79)
calibration node B*	22.66 (9.98–27.85)	17.22 (11.64–32.61)	25.97 (11.54–31.27)	**37.89*** (35–45.62)
S.0 (Scapaniaceae s. l.)	82.46 (62.82–108.08)	89.78 (78.04–122.03)	94.29 (73.69–115.58)	100.76 (84.85–129.59)
S.I (Gottschelioideae)	58.23 (42.38–92.3)	81.22 (51.32–107.84)	85.97 (47.39–100.27)	97.96 (53.05–114.85)
S.II	54.79 (30.99–59.4)	46.48 (39.19–70.24)	46.89 (36.55–66.24)	50.12 (44.31–76.47)
Anastrophyllum	35.41 (17.13–39.5)	21.54 (21.58–47.35)	29.91 (20.07–44.35)	35.57 (17.73–39.22)
Sphenolobus	16.43 (6.86–35.79)	23.35 (8.21–45.31)	27.26 (7.56–40.1)	39.02 (22.63–48.98)
Barbilophozia (calibration node C*)	14.11 (10.67–41.01)	38.13 (13.23–51.73)	24.45 (12.47–47.25)	**35.34*** (35–58.38)
Neoorthocaulis	21.39 (8.79–34.92)	18.02 (11.09–43.37)	26.61 (9.03–39.03)	11.25 (11.06–46.6)
Tetralophozia/Plicanthus	17.44 (15.08–37.38)	19.82 (18.71–45.12)	28.01 (18.21–41.48)	28.85 (20.92–49.47)
S.III (Lophozioideae)	40.06 (29.03–63.97)	64.6 (37.66–78.58)	58.58 (35.1–73.2)	67.5 (39.08–83.5)
Tritomaria	27.57 (7.8–34.97)	18.52 (9.37–42.77)	19.86 (9.95–40.3)	16.41 (10.73–47.16)
Lophoziopsis/Lophozia	38.84 (22.68–55.57)	57.48 (28.7–69.45)	56.56 (27.27–64)	61.56 (28.7–73.48)
S.IV (Scapanioideae)	54.2 (36.62–66.24)	66.04 (51.92–79.69)	51.36 (47.98–73.52)	67.6 (54.59–85.21)
Diplophyllum	26.66 (9.24–33.32)	14.32 (12.62–43.91)	20.15 (11.75–39.28)	32.04 (11.9–45.12)
Scapania (calibration node D1*)	28.25 (19.54–37)	43.98 (36.51–48.36)	**38.56*** (35–42.69)	41.49 (36.93–50.67)
Scapania subg. *Scapania* (calibration node D2*)	22.71 (15.05–29.75)	**36.46*** (35–40.49)	29.71 (22.34–36.76)	**36.55*** (35–41.69)

Mean ages of the families, the calibration nodes and selected genera in different analyses in Ma, 95 % HPD indicated in parentheses. The asterisks indicate the age constraints

D1* (crown group *Scapania*); analyses 1–3.D with constraints for nodes D2*, B* [*Cylindrocolea* plus *Cephaloziopsis intertexta* (Gottsche) R.M.Schust. and *Cephalozia microphylla* Steph.] and C* (crown group *Barbilophozia*). The results are summarized in Tables 2, 3, 4.

The analysis 1.B yields older node ages than analysis 1.C. If the age constraint is placed in *Scapania* subg. *Scapania* the genus becomes older than if the constraint is placed in *Scapania* s. l. (76.08 vs. 36.46 Ma; Table 2). This difference does not only affect *Scapania*, but also other nodes. The Cephaloziaceae (C.0) have an estimated age of 396.08 Ma in 1.B and of 151.58 Ma in 1.C, the Adelanthaceae (AD.0) have an estimated age of 235.2 Ma in 1.B and 106.69 Ma in 1.C, the Cephaloziellaceae (CE.0) have an estimated age of 95.23 Ma in 1.B and 45.11 Ma in 1.C, and the Scapaniaceae s. l. (S.0) have an estimated age of 156.22 Ma in 1.B and 94.5 Ma in 1.C (Fig. 3; see Table 2 for the ages of other groups). The oldest node ages are estimated in analysis 1.D with all fossil constraints applied. Here, the Cephaloziaceae (C.0) have an age of 349.05 Ma, the Adelanthaceae (AD.0) of 209.89 Ma, the Cephaloziellaceae (CE.0) of 90.31 Ma, and the


Cladopodiella fluitans
Odontoschisma elongatum
Odontoschisma portoricensis
Odontoschisma denudatum
Odontoschisma longiflorum
Odontoschisma falcifolium
Alobiella husnotii
Schiffneria hyalina
Pleurocladula albescens
Cephalozia crassifolia
Cephalozia infuscata
Nowellia dominicensis
Nowellia evansii
Cephalozia pachycaulis
Cephalozia bicuspidata
Cephalozia badia
Metahygrobiella macgregorii
Metahygrobiella albula
Cephalozia otaruensis
Adelanthus lindenbergianus
Adelanthus falcatus
Pseudomarsupidium decipiens
Wettsteinia inversa
Wettsteinia schusteriana
Cuspidatula monodon
Cuspidatula flaccida
Syzygiella pseudocclusa
Syzygiella autumnalis
Syzygiella rubricaulis
Syzygiella perfoliata
Syzygiella jacquinotii
Syzygiella tasmanica
Syzygiella securifolia
Syzygiella liberata
Syzygiella anomala
Hygrobiella laxifolia
Kymatocalyx dominicensis
Cephaloziella turneri
Cephaloziella granatensis
Cephaloziella stellulifera
Cephaloziella divaricata
Cephaloziella spinicaulis
Cephaloziella microphylla
Cephaloziopsis intertexta
Cylindrocolea recurvifolia
Gottschelia schizopleura
Andrewsianthus perigonialis
Andrewsianthus australis
Anastrophyllum bidens
Anastrophyllum nigrescens
Anastrophyllum donnianum
Anastrophyllum auritum
Anastrophyllum leucocephalum
Anastrophyllum tubulosum
Anastrophyllum piligerum
Sphenolobus saxicola
Sphenolobus minutus
Barbilophozia barbata
Barbilophozia lycopodioides
Barbilophozia hatcheri
Neoorthocaulis floerkei
Neoorthocaulis attenuatus
Tetralophozia setiformis
Tetralophozia cavallii
Plicanthus hirtellus
Tetralophozia pilifera
Tritomaria exsecta
Tritomaria quinquedentata
Tritomaria exsectiformis
Lophoziopsis excisa
Lophoziopsis longidens
Lophozia ascendens
Lophozia lantratoviae
Schistochilopsis incisa
Diplophyllum obtusifolium
Diplophyllum albicans
Douinia ovata
Scapania ampliata
Scapania bolanderi
Scapania portoricensis
Scapania ornithopodioides
Scapania nemorea
Scapania paludosa
Scapania undulata
Scapania uliginosa
Scapania curta
Scapania aspera
Scapania hyperborea
Scapania umbrosa
Scapania calcicola
Scapania cuspiduligera
Scapania javanica
Scapania sphaerifera
Cephaloziaceae
Adelanthaceae
Hygrobiellaceae
Cephaloziellaceae
Scapaniaceae s. l.
A*
B*
C*
D.2*
D.1*
C.0
C.I
C.II
AD.0
AD.I
AD.II
CE.0
S.0
S.I
S.II
S.III
S.IV
30.0
Age constraints:
Normal distribution: Mean=171.1 Stdev.=8.3
Eocene climatic optimum
LogLTT
Temperature (°C)
Time
Cretaceous
Cenozoic
Paleocene
Eocene
Oligocene
Miocene
Pliocene
Pleistocene

◀ **Fig. 1** Chronogram for Cephaloziineae: here, only a basal constraint (*A** normal distribution: 171.1 ± 8.3 Ma) was used to estimate divergence times; the nodes for possible age constraints (B*, C*,D1*, D2*) are indicated in *brackets*. Lineage through time plots are indicated below the time scale

Scapaniaceae s. l. (S.0) of 174.53 Ma (Fig. 3; see Table 2 for the ages of other groups).

In analyses 2.B/3.B Cephaloziaceae (C.0) have an age of 165.38/157.08 Ma and in analyses 2.C/3.C of 158.87/165.16 Ma. Adelanthaceae (AD.0) are estimated to 109.18/97.07 Ma in analyses 2.B/3.B and 106.72/82.42 Ma in analyses 2.C/3.C; Cephaloziellaceae (CE.0) are estimated to 51.46/48.14 Ma in analyses 2.B/3.B and 56.02/48.24 Ma in analyses 2.C/3.C. The Scapaniaceae s. l. crown group (S.0) has an age of 94.73/89.78 Ma in analyses 2.B/3.B and 87.68/94.29 Ma in analyses 2.C/3.C (Fig. 3; see Tables 3, 4 for the ages of other nodes). Node ages in analyses 2.B/3.B and 2.C/3.C are more similar than in analyses 1.B/1.C. The nodes B*, C*, D1* and D2* are often estimated younger than the fossils indicate.

In analyses 2.D (Fig. 2)/3.D with all fossil constraints applied the Cephaloziaceae (C.0) have an estimated age of 147.71/158.59 Ma, the Adelanthaceae (AD.0) have an age of 142.92/116.81 Ma, the Cephaloziellaceae (CE.0) of 69.89/66.79 Ma, and the Scapaniaceae s. l. (S.0) of 108.11/100.76 Ma (Fig. 3; see Table 3, 4 for the ages of other groups).

Partitioned versus non-partitioned analyses and lineages through time plots

There are no significant differences between the partitioned and the unpartitioned analyses (Fig. 3; Tables 2, 3, 4). Lineage through time plots (Figs. 1, 2) of all analyses showed evidence for nearly constant diversification rates.

Discussion

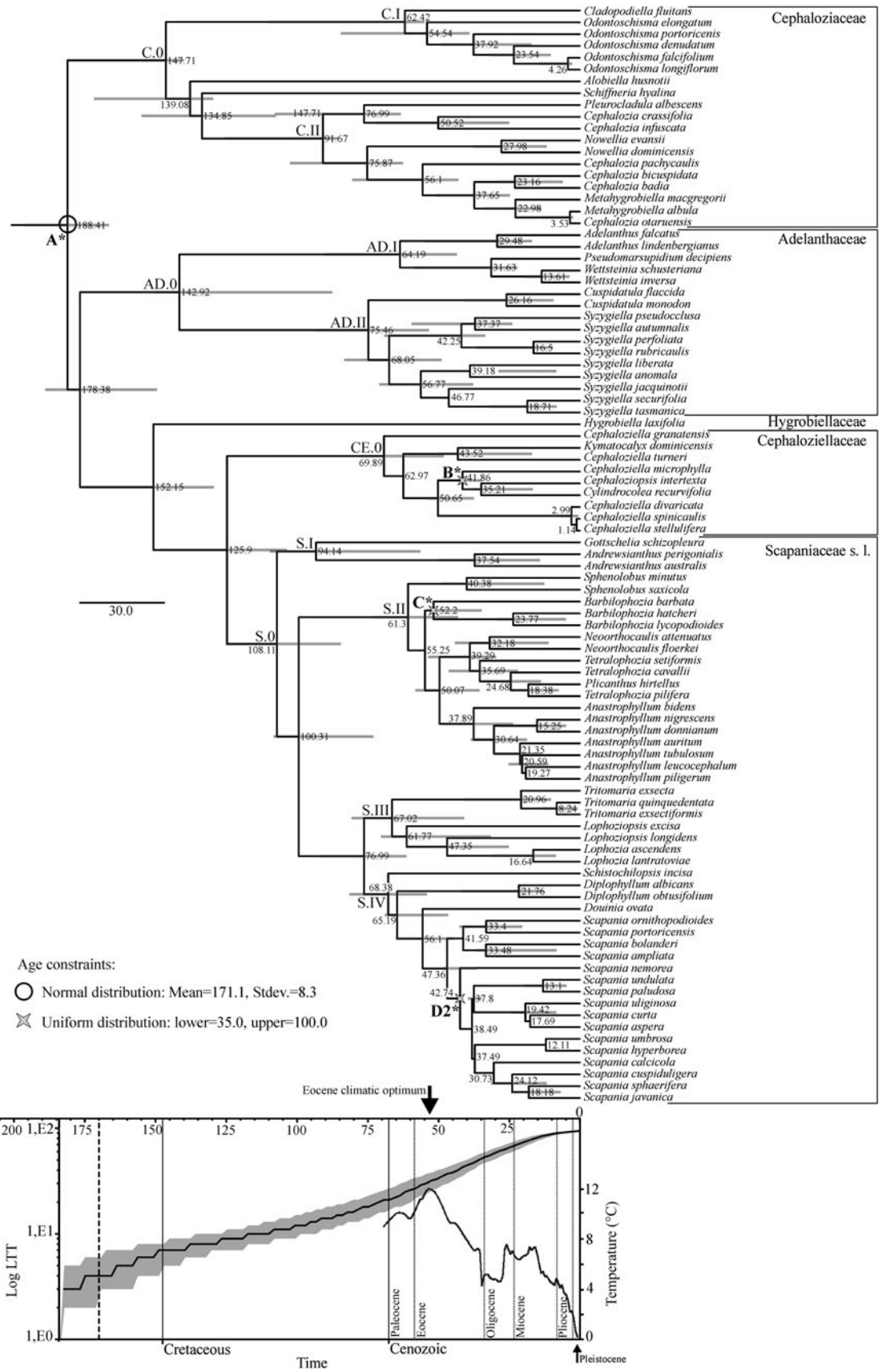
Uncertainty of assignment of fossils

The assignment of fossil taxa to nodes reconstructed using extant taxa proves to be a challenge of assumptions. The division of stem versus crown group assignments is among the most widely accepted but still poorly resolved issues (Rutschmann et al. 2007; Gernandt et al. 2008). The distinction is logically consistent but both estimates may create substantially different time frames in which macroevolutionary hypotheses such as the Gondwanan vicariance hypothesis (Raven and Axelrod 1974) are inferred. A far less addressed issue is the interpretation of the morphological characters used to assign the fossil to a particular node in the tree. Ideally, the character used represents an apomorphy, such as the lindsaeoid root anatomy recovered in Cretaceous fern fossils (Schneider and Kenrick 2001; Schneider et al. 2004). However, many fossils do not show characters that represent unambiguous apomorphic character states. The vast majority of characters preserved in fossils show a strong pattern of homoplasy as a result of factors such as exhaustion of morphological variation, frequent parallelism and fossil bias in the preservation of particular structures (e.g., Wagner 2000; Schneider 2007; Schneider et al. 2009). Thus, assignments are often based on the combination of characters of which each one separately is ambiguous.

Furthermore, assignments are often done without careful consideration of the evolution of characters preserved in the fossil under study. The use of plotting morphological characters onto phylogenetic trees based on the extant taxa may be a realistic approach to overcome these questions (Schneider et al. 2009). However, also this approach has its own limits because it assumes that the reconstruction of the character evolution using extant taxa alone may be congruent to the true evolution of this character through time including both extant and extinct taxa (e.g. Schneider 2007).

The fossil liverworts used in this study are excellent examples to illustrate this problem. There are many homoplasies in the relatively uniform vegetative morphology of liverworts (Crandall-Stotler et al. 2005; Hentschel et al. 2006; Heinrichs et al. 2007) and also the reproductive structures, which are generally better suited for evolutionary reconstruction, show sometimes signs of independent parallel evolution (Hentschel et al. 2006).

Furthermore, there are only few fossil liverworts with well-preserved female structures (Heinrichs et al. 2012c). *S. hoffeinsiana* is one of these rare amber fossils with perianth and sporophyte and, like mentioned above; it is not easy to assign it to an extant group. Like other members of Cephaloziineae the genus *Scapania* possesses a true perianth made up of adnate modified leaves. It is plicate or eplicate with both character states seen in early diverging *Scapania* lineages and the derived subg. *Scapania* (Heinrichs et al. 2012a). To cope with the tentativeness of the *S. hoffeinsiana* assignment we tested several possibilities, with great impact on the age of not only the genus *Scapania*, but also its sister groups (Figs. 1, 2, 3; Tables 2, 3, 4). Other fossil assignments are hampered by incongruence of morphology-based genus concepts and molecular phylogenies, as demonstrated by the paraphyly of the genus *Cephaloziella* with *Cylindrocolea*, *Cephaloziopsis* and *Kymatocalyx* nested in it (Figs. 1, 2). Consequently, deeper insights into the morphological evolution of the *Cephaloziella* lineage and the taxonomical value of certain character states require more comprehensive phylogenies with a dense sampling of species. These phylogenies will


Cephaloziaceae
Adelanthaceae
Hygrobiellaceae
Cephaloziellaceae
Scapaniaceae s. l.
Cladopodiella fluitans
Odontoschisma elongatum
Odontoschisma portoricenis
Odontoschisma denudatum
Odontoschisma falcifolium
Odontoschisma longiflorum
Alobiella husnotii
Schiffneria hyalina
Pleurocladula albescens
Cephalozia crassifolia
Cephalozia infuscata
Nowellia evansii
Nowellia dominicensis
Cephalozia pachycaulis
Cephalozia bicuspidata
Cephalozia badia
Metahygrobiella macgregorii
Metahygrobiella albula
Cephalozia otaruensis
Adelanthus falcatus
Adelanthus lindenbergianus
Pseudomarsupidium decipiens
Wettsteinia schusteriana
Wettsteinia inversa
Cuspidatula flaccida
Cuspidatula monodon
Syzygiella pseudocclusa
Syzygiella autumnalis
Syzygiella perfoliata
Syzygiella rubricaulis
Syzygiella liberata
Syzygiella anomala
Syzygiella jacquinotii
Syzygiella securifolia
Syzygiella tasmanica
Hygrobiella laxifolia
Cephaloziella granatensis
Kymatocalyx dominicensis
Cephaloziella turneri
Cephaloziella microphylla
Cephaloziopsis intertexta
Cylindrocolea recurvifolia
Cephaloziella divaricata
Cephaloziella spinicaulis
Cephaloziella stellulifera
Gottschelia schizopleura
Andrewsianthus perigonialis
Andrewsianthus australis
Sphenolobus minutus
Sphenolobus saxicola
Barbilophozia barbata
Barbilophozia hatcheri
Barbilophozia lycopodioides
Neoorthocaulis attenuatus
Neoorthocaulis floerkei
Tetralophozia setiformis
Tetralophozia cavallii
Plicanthus hirtellus
Tetralophozia pilifera
Anastrophyllum bidens
Anastrophyllum nigrescens
Anastrophyllum donnianum
Anastrophyllum auritum
Anastrophyllum tubulosum
Anastrophyllum leucocephalum
Anastrophyllum piligerum
Tritomaria exsecta
Tritomaria quinquedentata
Tritomaria exsectiformis
Lophoziopsis excisa
Lophoziopsis longidens
Lophozia ascendens
Lophozia lantratoviae
Schistochilopsis incisa
Diplophyllum albicans
Diplophyllum obtusifolium
Douinia ovata
Scapania ornithopodioides
Scapania portoricensis
Scapania bolanderi
Scapania ampliata
Scapania nemorea
Scapania undulata
Scapania paludosa
Scapania uliginosa
Scapania curta
Scapania aspera
Scapania umbrosa
Scapania hyperborea
Scapania calcicola
Scapania cuspiduligera
Scapania sphaerifera
Scapania javanica
C.I
C.0
C.II
A*
AD.I
AD.0
AD.II
CE.0
B*
S.I
S.II
C*
S.0
S.III
S.IV
D2*
30.0
Age constraints:
Normal distribution: Mean=171.1, Stdev.=8.3
Uniform distribution: lower=35.0, upper=100.0
Eocene climatic optimum
200 1,E2
175
150
125
100
75
50
25
0
1,E1
1,E0
Log LTT
12
8
4
0
Temperature (°C)
Paleocene
Eocene
Oligocene
Miocene
Pliocene
Cretaceous
Cenozoic
Pleistocene
Time

◀**Fig. 2** Chronogram for Cephaloziineae: In addition to a basal constraint (*A** normal distribution: 171.1 ± 8.3 Ma) uniform minimum age constraints (35.0–100.0 Ma) were used to calibrate nodes with amber fossils (indicated as *stars*). Lineage through time plots are indicated below the time scale

possibly allow for more reliable assignments of the related fossil *Cylindrocolea/Cephaloziella dimorpha* than currently possible.

Evaluating the impact of multiple constraints and character-rich models

Our results demonstrate the potential strong impact of fossil constraints, but also highlight the need to carefully assess the assignment of the fossil to prevent misleading results. We suggest a reciprocal illumination approach being the ideal method addressing this question by comparing carefully the age estimates obtained by analyses without and with the fossil constraint under question. For example, the omission of *C. dimorpha* and *L. kutscheri* resulted in a young age not only for the clades in question, but also for the rest of the tree.

Several clades were estimated much younger without the inclusion of the information from their fossil relatives. This result may indicate either problems with the interpretation of their taxonomic relationships or more likely limitations of the Bayesian methodology to estimate the age of these nodes.

Interestingly, the lineages through time plots of all analyses provide evidence for persistent diversification rates. The slower accumulation of species diversity towards the present is likely the result of the taxonomic sampling and therefore considered as an artefact. Thus, the reported issues concerning the absolute age estimate of some nodes had little impact on the reconstructed processes. However, this may be partly due to the lack of sensitivity of lineage through time plots. Other methods to infer diversification rates (e.g., Rabovsky 2010; Cusimano et al. 2012) may behave differently but this needs to be explored in future studies.

Towards the reconstruction of the evolutionary history of leafy liverworts

Despite the main objective of this study concerned the assessment of divergence times, the results provide also some new insights into the history of leafy liverworts. Previous studies on the divergence times of liverworts addressed the age of the main lineages (e.g., Heinrichs et al. 2007; Cooper et al. 2012), biogeographic hypotheses such as pseudo-Gondwana distributions (Hartmann et al. 2006; Heinrichs et al. 2009b), and the pattern of

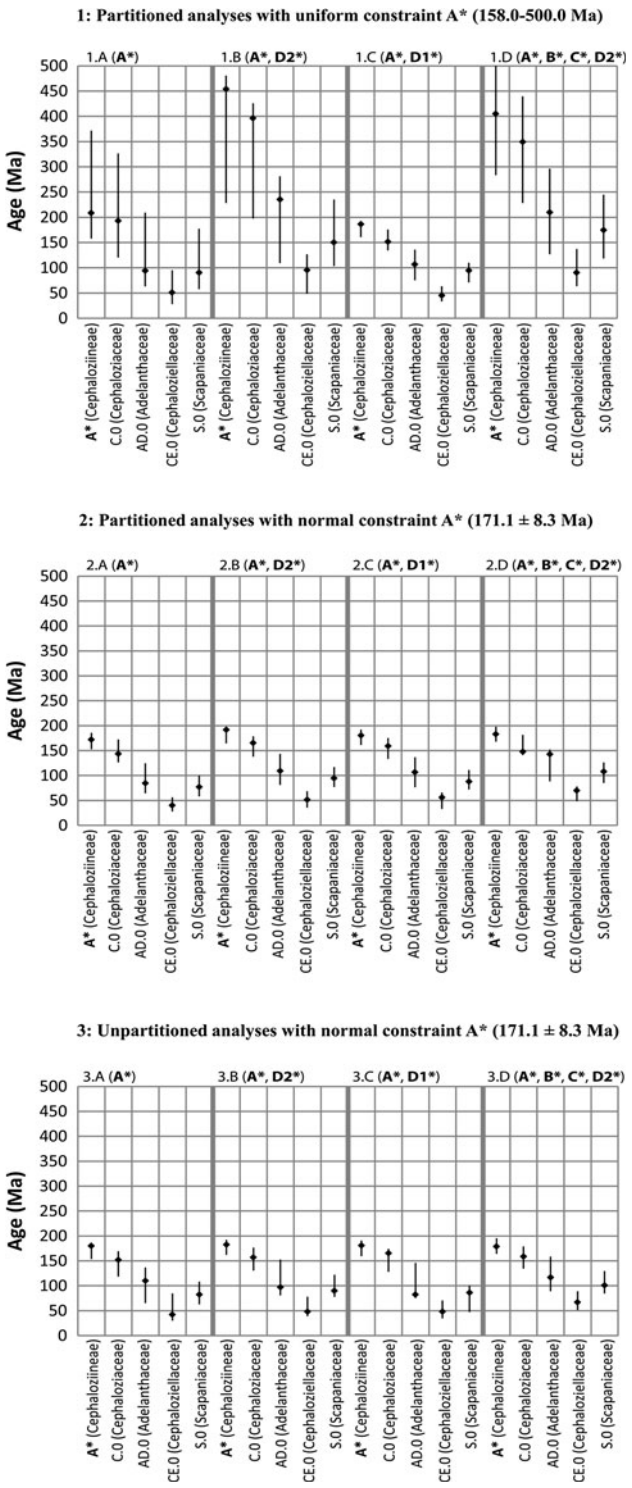


Fig. 3 Diagrams showing the means and confidence intervals of the crown group nodes A* (Cephaloziineae), C.0 (Cephaloziaceae), AD.0 (Adelanthaceae), CE.0 (Cephaloziellaceae) and S.0 (Scapaniaceae) for all three analysis chains

divergences of liverworts. The latter studies reported evidence for the conservation of diversification rates in liverworts for the most of the Mesozoic and the Cenozoic.

Nearly constant diversification rates of liverworts were found by both extensive analyses of a species-based sampling of the derived leafy liverwort family Lejeuneaceae (Wilson et al. 2007) and a family-based sampling of liverworts (Fiz-Palacios et al. 2011). These reports are remarkable given the trend of strong fluctuations of species richness through time recovered in studies on the fossil record of vascular plants (Niklas et al. 1983; Lidgard and Crane 1988; Benton 1995; Lupia et al. 1999) and in divergence time analyses in angiosperms, gymnosperms, ferns, and mosses (Schneider et al. 2004, 2010a, b; Janssen et al. 2008; Schuettpelz and Pryer 2009; Shaw et al. 2010; Crisp and Cook 2011; Eiserhardt et al. 2011; Nagalingum et al. 2011; Smith et al. 2011). The Cenozoic is characterized not only by strong modifications of the continental surfaces such as the rise of the Himalaya (Wang et al. 2012) but also by significant climatic events such as the Eocene climate optimum, the late Eocene and early Oligocene cooling and the Miocene warming. None of these events appears to have significantly influenced the diversification rates of the predominantly tropical Lejeuneaceae (Wilson et al. 2007).

With the exception of the mainly tropical Adelanthaceae, the Cephaloziineae are more abundant in temperate and subarctic regions than in the inner tropics. The predominant occurrence of Cephaloziineae in temperate regions possessing more unstable climates strongly indicated this family as a candidate of a leafy liverwort lineage with fluctuations in diversification rates in response to climatic fluctuations. Nevertheless, the recovered pattern for Cephaloziineae is consistent with the hypothesis of rather consistent diversification rates in liverworts, as already demonstrated for the Lejeuneaceae family. However, the reported evidence may be inaccurate because the present study includes less than 20 % of the extant species diversity of Cephaloziineae. The limited sample may led to an oversampling of deep nodes and thus reduce the sensitivity of the analyses to detect changes in the diversification rates. The same holds true for the Lejeuneaceae study (Wilson et al. 2007). Thus, the current hypothesis of constant diversification rates within the leafy liverworts may be a result of sampling bias (Cusimano et al. 2012). Follow-up studies will thus need to address issues concerning the impact of taxon sampling and phylogenetic robustness, and should aim at a more comprehensive taxon sampling. These studies need to consider the limitations of analyses based solely on the extant species in the recovery of changes in speciation and extinction rates (Marshall 2008; Rabovsky 2010). The bias in studies using exclusively extant taxa may result in revisionist estimates of the evolutionary history of these lineages (Tarver and Donoghue 2011). Hence, studies are required testing the molecular dating-based hypothesis using the fossil record.

State-of-the-art and perspectives

Reconstructing the divergence times of liverworts is hampered by our limited knowledge on their early evolutionary history (Clarke et al. 2011; Kenrick et al. 2012), their sparse fossil record (Taylor et al. 2009) and uncertainties with regard to the age of many fossils (Iturralde-Vincent and MacPhee 1996). Our results are consistent with the previous studies reporting evidence that the usage of multiple-constraints requires careful consideration because of the difficulties in predicting the impact of each individual constrain in Bayesian analyses (Inoue et al. 2010). However, more empirical studies are required to elucidate the robustness versus sensitivity of these methods as required to develop clear guidelines for their usage.

The deviations of divergence times in our different analyses provide a note of caution regarding interpretations of chronograms produced for lineages without or with a sparse fossil record. The present state-of-the art already allows for balancing different macroevolutionary scenarios, such as vicariance or dispersal, but explicit correlations of diversification events of bryophytes with climatic fluctuations or orogeneses are still premature and deserve more reliable chronograms. Advancements in our knowledge of the fossil record are highly desirable to arrive at more reliable conclusions on their historical biogeography (Heinrichs et al. 2009a).

In recent years, the number of well-studied liverwort fossils has remarkably increased, especially for the Cenozoic. Integrating this enhanced fossil record in studies on the diversification of liverworts through time may enable us to overcome the limitations of extant taxa only approaches, especially the question of overlooked species turnovers and lineage replacements. The suggested integration of amber fossils into a phylogenetic framework may also enable us to overcome the core problem of the liverwort fossil record, the discontinuous occurrence of amber deposits in the Phanerozoic. Because the majority of liverwort fossils represents amber inclusions, the fossil record of this group is biased in several aspects: (1) fragmented in time periods with or without known amber deposits, (2) preservation of taxa occurring in habitats dominated by seed plants producing resins that are finally deposited as amber, and (3) climate conditions favouring the formation of forests producing huge amounts of resin exudates.

Improving analytical methods, increasing taxon sampling, and integration of a more comprehensive fossil record will likely lead to more reliable chronograms and allow for scrutinizing current hypotheses on a constant diversification of liverworts through time. These studies will allow to relate the liverwort diversification to the diversification of angiosperms, and will provide evidence

for or against changes of liverwort lineages during the numerous rearrangements of terrestrial ecosystems in the late Mesozoic and early Cenozoic.

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