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2009
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http://hdl.handle.net/10138/24019
Olsson , S , Buchbender , V , Enroth , J , Huttunen , S , Hedenäs , L & Quandt , D 2009 , ' Evolution of the Neckeraceae (Bryophyta): resolving the backbone phylogeny ' , Systematics and Biodiversity , vol 7 , no. 4 , pp. 419-432 .

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Systematics and Biodiversity **7** (4): 419–432
doi:10.1017/S1477200009990132

Issued 1 December 2009
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submitted November 2008
accepted July 2009

Evolution of the Neckeraceae (Bryophyta): resolving the backbone phylogeny

Abstract Earlier phylogenetic studies, including species belonging to the Neckeraceae, have indicated that this pleurocarpous moss family shares a strongly supported sister group relationship with the Lembophyllaceae, but the family delimitation of the former needs adjustment. To test the monophyly of the Neckeraceae, as well as to redefine the family circumscription and to pinpoint its phylogenetic position in a larger context, a phylogenetic study based on molecular data was carried out. Sequence data were compiled, combining data from all three genomes: nuclear ITS1 and 2, plastid *trnS-rps4-trnT-trnL-trnF* and *rpl16*, and mitochondrial *nad5* intron. The Neckeraceae have sometimes been divided into the two families, Neckeraceae and Thamnobryaceae, a division rejected here. Both parsimony and Bayesian analyses of molecular data revealed that the family concept of the Neckeraceae needs several further adjustments, such as the exclusion of some individual species and smaller genera as well as the inclusion of the Leptodontaceae. Within the family three well-supported clades (A, B and C) can be distinguished. Members of clade A are mainly non-Asiatic and nontropical. Most species have a weak costa and immersed capsules with reduced peristomes (mainly *Neckera* spp.) and the teeth at the leaf margins are usually unicellular. Clade B members are also mainly non-Asiatic. They are typically fairly robust, distinctly stipitate, having a single, at least relatively strong costa, long setae (capsules exserted), and the peristomes are well developed or only somewhat reduced. Members of clade C are essentially Asiatic and tropical. The species of this clade usually have a strong costa and a long seta, the seta often being mammillose in its upper part. The peristome types in this clade are mixed, since both reduced and unreduced types are found. Several neckeraceous genera that were recognised on a morphological basis are polyphyletic (e.g. *Neckera*, *Homalia*, *Thamnobryum*, *Porotrichum*). Ancestral state reconstructions revealed that currently used diagnostic traits, such as the leaf asymmetry and costa strength are highly homoplastic. Similarly, the reconstructions revealed that the 'reduced' sporophyte features have evolved independently in each of the three clades.

Key words Pleurocarpous mosses, homoplasy, morphological delimitation, peristome, taxonomy, ancestral character state reconstruction

Introduction

Although morphological characters provide important insights into the evolution of organisms, coding and weighting of morphological characters, as well as homology assumptions, might be biased by the investigators' evolutionary ideas or interpreted in the frame of a prevalent phylogenetic concept and might therefore be misleading (see Scotland *et al*. 2003). In 'cryptic' organisms such as bryophytes the problem is even more evident, as morphological variation is rather limited and therefore provides only shallow evidence for phylogenetic relationships at several taxonomic levels. In such cases, phylogenetic reconstructions, based on DNA sequence data, are the only option to infer a robust evolutionary concept. With the increasing ease of generating sequence data, solid phylogenetic studies, based on DNA sequences, become more and more feasible, even in recent or fast radiations and allow the independent testing of hypotheses of morphological evolution, e.g. via the reconstruction of ancestral character states. However, we believe that in order to test and develop concepts of

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morphological evolution, it is inevitable to collect and to search for 'new' morphological data in addition to the advancing molecular phylogenetic approaches. Although the aforementioned as well as the following reasoning is true for many groups of organisms, we will concentrate the arguments to the group we studied, i.e. pleurocarpous mosses.

There are several problems involved with morphology-based phylogenetic analyses of pleurocarpous moss relationships. Numerous characters can, in principle, be used if they are correctly understood and interpreted, but the often reduced morphology and abundant convergence implies homology problems. Thus, in many cases only a limited number of characters add to the phylogenetic signal. The simple structures observed in mosses limit the number of potentially useful morphological characters for phylogenetic analyses. Therefore, in pleurocarpous mosses at family level, for example, only about 50 to 100 morphological characters can be used (Hedenäs, 1995, 1997; Pedersen & Hedenäs, 2002; Vanderpoorten *et al.*, 2002b; Huttunen & Ignatov, 2004). Several previous studies have shown that morphological characters can be misleading with a high degree of convergent evolution even at the genus and species levels (Hedenäs, 2001; Huttunen & Ignatov, 2004; Vanderpoorten *et al.*, 2002a, 2002b). Also, morphological reduction has occurred several times in different moss lineages (e.g. Frey, 1981). Therefore, the identification of relevant characters to be used in pleurocarpous moss classification is crucial, and a failure to do this would result in an incorrect phylogenetic placement based on morphology (Hedenäs, 1995). Sporophytic characters have traditionally been considered the most important criteria in moss classification at all taxonomic levels. Sporophytes are, however, subjected to environmental pressures as are the gametophytes (Hedenäs, 2001, 2002), and sporophytic characters can be as homoplastic and therefore misleading in moss classifications as gametophyte characters at and above the family level (Buck, 1991). A good example of parallel evolution of sporophytic characters was shown in a study by Huttunen *et al*. (2004), who concluded that structural reduction in the sporophytes has independently taken place in the Brachytheciaceae in several lineages representing all four subfamilies.

The moss family that is studied here, the Neckeraceae, belong to the pleurocarpous mosses, i.e. 'the Core Pleurocarps' as defined by Bell *et al*. (2007). They form a monophylum, which consists of typically perennial mosses with creeping stems and abundant lateral branches. In pleurocarpous mosses, the archegonium and thus also sporophyte development is restricted to the apices of short, specialised lateral branches, in contrast to most other mosses, where archegonia and sporophytes develop terminally on the main axis (acrocarpous) or on major branches (cladocarpous).

The pleurocarps comprise approximately 5000 species, which corresponds to about half of all mosses (Buck & Goffinet, 2000). Traditionally, pleurocarpous mosses have been divided into the orders Hookeriales, Leucodontales (or Isobryales) and Hypnales, with the Neckeraceae belonging to the Leucodontales (Brotherus, 1925). Buck and Vitt (1986) defined the Hypnales as mainly terricolous species with an unreduced peristome (i.e. 'perfect' or 'well-developed'), and the Leucodontales were defined by a reduced peristome. Most likely this grouping does, however, not correspond to natural relationships, but is due to convergent peristome evolution in several lineages (e.g. Buck & Crum, 1990; Buck, 1991). Supported by molecular analyses, the separation of the Leucodontales was therefore rejected (Buck *et al*., 2000; Tsubota *et al*., 2002); thus the Neckeraceae are currently treated within the Hypnales (Goffinet & Buck, 2004). The Hypnales have probably radiated relatively recently and rapidly, as indicated by the short branch lengths in the backbone phylogeny (Buck *et al*., 2000) and low DNA sequence variation (Vanderpoorten *et al.*, 2002a; Shaw *et al.*, 2003). Due to these problems, the phylogenetic relationships among the Hypnalean families are extremely difficult to reconstruct and remain largely unresolved (Buck *et al*., 2000; Shaw *et al*., 2003). More analyses are needed to provide reliable answers addressing the evolution of the Hypnales. Although few sister-group relationships are resolved among the Hypnales, previous analyses highly support a close relationship between the Neckeraceae and the Lembophyllaceae (Olsson *et al.*, 2009; Quandt *et al.*, 2009), even if the current circumscription of the Neckeraceae is challenged (Buck *et al.*, 2000; Tsubota *et al.*, 2002; Ignatov *et al.*, 2007; Olsson *et al.*, 2009).

According to Crosby *et al.* (1999), the Neckeraceae consists of 211 species. Enroth (1994a) and Olsson *et al*. (2009, in press) suggest that the species number of the family is somewhat lower, around 200. The family has a wide geographic distribution, comprising largely tropical (*Neckeropsis*, *Pinnatella*, *Himantocladium*, *Porotrichodendron*), as well as predominantly temperate (*Homalia*, *Neckera*, *Thamnobryum*) genera. The species are mainly epiphytic or epilithic, although some aquatic (rheophytic, i.e. growing in flowing water) species belong here as well. Members of the family are generally recognised by their usually large, glossy plants that have creeping stolons bearing very small leaves and tufts of rhizoids (Enroth, 1989), and more or less frondose (rarely dendroid) stems with or without distinct stipes. The leaf cells are almost always smooth, relatively short and firm-walled, and the marginal cells are typically quadrate to short-rectangular in few to several rows (Enroth, 1994a). The sporophyte features are variable but usually fairly consistent within genera. For example, the peristomes may be perfect (as in *Thamnobryum*, *Homalia*, *Pendulothecium*), slightly reduced (*Porotrichodendron*) or strongly reduced (*Pinnatella*, *Neckeropsis*, *Homaliodendron*, *Neckera*).

This study, where our main focus is the delimitation of the Neckeraceae, is based on molecular data from all three genomes. It includes most of the genera in the family and is the first modern comprehensive family-level study on the Neckeraceae. Phylogenetic studies based on morphological data have not been frequent either. Enroth (1994a) and Hyvönen and Enroth (1994) are the only published studies, but they focused solely on the genus *Pinnatella*. We tested the monophyly of the Neckeraceae and evaluated its position in the pleurocarpous moss phylogeny, with a representative set of taxa from the Neckeraceae and its sister family Lembophyllaceae, as well as from other potentially closely related taxa. In addition to resolving the main patterns of relationships among the Neckeraceae and

their relatives, we explored the morphological character evolution using Bayesian ancestral state reconstruction methods. We also shed light on some distinctive phytogeographic patterns among the Neckeraceae.

Materials and methods

Taxon sampling and molecular markers

Seventy-three taxa from 47 different genera were included in the analysis. Thirty-eight members representing the Neckeraceae, Thamnobryaceae and Leptodontaceae, as well as supposedly neckeraceous species (according to Buck & Goffinet, 2000) were included in the sampling. In addition, nine representatives of the Lembophyllaceae (according to Quandt *et al*., 2009), and 24 outgroup species from several Hypnalean families as well as the Hookeriaceae were sampled. The selection of species was based on earlier treatments of the Neckeraceae (compare Table 2), as well as previous analyses by Olsson *et al*. (2009). Samples were sequenced for four genomic regions: the nuclear ribosomal ITS1 and 2, a mitochondrial group I intron residing in *nad5* (and part of the gene) as well as two plastid regions: *rpl16* and *trnS-trnF*. The *trnS-trnF* area includes the fast evolving protein coding gene *rps4*, four intergenic spacers (*trnS-rps4* IGS, *rps4-trnT* IGS, *trnT-trnL* IGS and *trnL-trnF* IGS), the *trnL* intron as well as four tRNAs genes (*trnS*, *trnT*, *trnL* and *trnF*). Species sampled, together with voucher information and EMBL or GenBank accession numbers, are listed in Appendix 1, which is available as 'Supplementary data' on Cambridge Journals Online: http://www.journals.cup.org/abstract_S1477200009990132

DNA isolation, PCR amplification and sequencing

DNA was extracted using the DNeasy® Plant Mini Kit from Qiagen (Qiagen GmbH, Germany) following the manufacturer's protocol. Cleaning and grinding of plants prior to extraction followed Olsson *et al*. (2009). Amplification of the ITS1–5.8S-ITS2 as well as the *trnS-trnF* region followed Olsson *et al*. (2009) and Hernández–Maqueda *et al*. (2008), respectively. Whereas *rpl16* was amplified using the primer F71 (Jordan *et al*., 1996, GCT ATG CTT AGT GTG TGA CTC GTT) and rpl16R (this paper: designed for pleurocarpous mosses; GTA ATC CAA GCT GGT TCA AGT GC; Olsson, 2009) using a standard PCR setup with the following thermal cycles: 35 (95 °C 30 s, 56 °C 60 s, 68 °C 90 s) and a final extension of 4 min at 68 °C. *Nad5* was amplified using the strategy and primers of Buchbender and Quandt (in press). Gel cleaned PCR products were sequenced by Macrogen Inc., South Korea (www.macrogen.com). Sequences were edited manually with PhyDE® v0.995 (Müller *et al*., 2005) and primer sequences eliminated. All sequences are deposited in EMBL.

Alignment, sequence analyses and phylogenetic reconstructions

Alignment of sequence data was done manually with PhyDE® v0.995 using the alignments of Olsson *et al*. (2009) as scaffold and applying the alignment and hotspot definition approach described in Olsson *et al*. (2009). The known inversion in front of *trnF* was positionally separated in the alignment (Quandt & Stech, 2004), and included in the phylogenetic analyses as reverse complement in order to gain information from substitutions as discussed in Quandt *et al*. (2003). Alignments are available from the authors on request. A ready-to-use nexus file containing the sequence alignment with an automatically generated binary indel matrix appended based on the simple indel coding approach of Simmons and Ochoterena (2000) was generated using the computer program SeqState (Müller, 2005). Command files for using the parsimony ratchet (Nixon, 1999) were generated using the programme PRAP2 (Müller, 2007) applying the default settings, and executed in PAUP 4.0b10 (Swofford, 2002). Heuristic bootstrap searches under parsimony were performed with 1000 replicates.

Bayesian analyses were performed with MrBayes v3.1.2, applying the GTR+Γ+I model for the sequence data and the restriction site model for the binary indel partition. To allow for possibly deviating substitution matrices for the different regions, the data set was divided into four sequence data partitions (partition 1: *trnS-trnF* (plastid); partition 2: *rpl16* (plastid)*;* partition 3: ITS1 and 2 (nuclear); partition 4: *nad5* (mitochondrial)). Partition 5 contained the indel matrix. Different matrices were applied to each of the partitions, with model parameters being sampled independently. The a priori probabilities supplied were those specified in the default settings of the programme. Posterior probability (PP) distributions of trees were created using the Metropolis-coupled Markov chain Monte Carlo (MCMCMC) method and the following search strategies suggested by Huelsenbeck *et al*. (2001, 2002). Ten runs with four chains (1×10^6 generations each) were run simultaneously, with the temperature of the single heated chain set to 0.5. Chains were sampled every 10 generations and the respective trees written to a tree file. The program Tracer v1.4 (Rambaut & Drummond, 2007) was used to calculate the burning point and to examine the log likelihoods, ensuring that the runs were in the stationary phase. Calculations of the consensus tree and of the posterior probability of clades were performed based upon the trees sampled after the chains converged (at generation 25 000). Consensus topologies and support values from the different methodological approaches were compiled and drawn using TreeGraph (Müller & Müller, 2004).

Morphological data and ancestral state reconstruction

The morphological information for characters that are often discussed in connection with taxonomical delimitation of the Neckeraceae was compiled by the authors. The scored data are based both on the specimens used for molecular sampling and on additional material, since the specimens in the molecular study did not always include all characters (e.g. sporophytes). Moreover, morphological scoring based on several vouchers better reflects the infra-specific variation. When no herbarium material was available (in S or H) or it was inadequate, literature sources were used. Specimen information for taxa not included in the molecular study is presented in Appendix 2, which is available as 'Supplementary data' on Cambridge

No.	Position	Region	No.	Position	Region
H1	743–745	*rps4-trnT* IGS	H14	2920–2923	*rpl16* intron
H2	870–877	*rps4-trnT* IGS	H15	2941–2945	*rpl16* intron
H3	914–915	*rps4-trnT* IGS	H16	3311–3116	*rpl16* intron
H4	933–939	*rps4-trnT* IGS	H17	3325–3329	*rpl16* intron
H5	985–1001	*rps4-trnT* IGS	H18	3397–3400	*rpl16* intron
H6	1029–1031	*rps4-trnT* IGS	H19	3501–3506	ITS 1
H7	1096–1098	*rps4-trnT* IGS	H20	3569–3573	ITS 1
H8	1176–1179	*rps4-trnT* IGS	H21	4025–4031	ITS 1
H9	1469–1481	*trnT-trnL* IGS	H22	4299–4307	ITS 1
H10	1507–1510	*trnT-trnL* IGS	H23	4436–4439	ITS 1
H11	1651–1656	*trnT-trnL* IGS	H24	4460–4462	ITS 1
H12	1692–1710	*trnT-trnL* IGS	H25	4483–4487	ITS 1
I1 §	2255–2261	*trnL-trnF IGS*	H26	4659–4664	ITS 2
H13	2534–2541	*rpl16* intron	H27	4871–5131	ITS 2

Table 1 Location, i.e. absolute position in the combined data set and corresponding region of mutational hotspots (H), including the observed inversion (I). § Location of the inversion is given with respect to the corrected and analysed matrix (i.e. the inversion is included as reverse complement).

Journals Online: http://www.journals.cup.org/abstract_S1477200009990132. Appendix 3, which is available as 'Supplementary data' on Cambridge Journals Online: http://www.journals.cup.org/abstract_S1477200009990132 provides the characters that were scored as well as the resulting data matrix. Capsule orientation is described in relation to the axis of the seta, according to the terminology recently introduced by Hedenäs (2006). The longitudinal axis of an *orthotropous* capsule is parallel with the seta and its mouth points in the distal direction, *homotropous* is between orthotropous and orthogonal, the axis of an *orthogonal* capsule is perpendicular to that of the seta, *reclinate* is between orthogonal and antitropous, and the axis of an *antitropous* capsule is parallel with the seta and its mouth points towards the seta base.

The evolutionary history of each morphological character was reconstructed by determining the posterior probability with which each character state occurred in the ancestral species. We used the Markov chain model implemented in BayesTraits to estimate the posterior probability distributions of ancestral states at every node of the tree (Pagel & Meade, 2004). The method takes into account the effect of phylogenetic uncertainty by using a Bayesian posterior tree sample in estimating the ancestral states. Using a perl script (written by Kai Müller, available from www.bioinf.web) 500 trees were randomly sampled among 1 000 010 trees from MrBayes analyses. Outgroups were excluded from ancestral state reconstructions. Trees were pruned with PAUP 4.0b10 (Swofford, 2002) leaving the 45 ingroup taxa for the analyses (see Figs 2–4). In BayesTraits the rate at which parameters get changed ('ratedev'), was set at the beginning of each run so that the acceptance rate of the proposed changes globally ranges between 20 and 40%. A uniform distribution with a range of 0–100 was used as prior. Rate coefficients and ancestral character states were sampled every 500 generations to ensure independence from successive samplings. The chain was run for 5 050 000 generations. In order to circumvent issues associated with the fact that not all of the trees necessarily contain the internal nodes of interest, reconstructions were performed using a 'most recent common ancestor' approach that identifies, for each tree, the most recent common ancestor to a group of species and reconstructs the state at the node, then combines this information across trees (Pagel & Meade, 2004).

Results

Phylogenetic analyses

The original alignment contained 6847 characters (3384 plastid, 2248 nuclear and 1215 mitochondrial). Twenty-seven hotspots with poly-mononucleotid repeats were recognised following Olsson *et al*. (2009) and excluded from the analyses (Table 1). The resulting data matrix (with the inversion included as reverse complement) used for the phylogenetic analyses contained 6417 nucleotide characters, of which 5138 (80%) were constant, 1279 (20%) were variable and 664 (10%) parsimony informative. After coding and including the 796 indels (209 plastid, 569 nuclear and 18 mitochondrial) the resulting matrix contained 7213 characters (5141 constant (71%), 2072 (29%) variable, 944 (13%) parsimony informative). The parsimony analysis including indel coding retained four most parsimonious trees (MPT, length 4355, CI = 0.549, RI = 0.638), while the analysis excluding indels retained 35 MPTs (length 3091, CI = 0.517, RI = 0.637).

The MrBayes trees from both analyses (with and without indel coding) are well resolved and highly supported with no incongruence. No supported topological conflicts between the strict consensus trees from the parsimony analyses and the majority rule trees from Bayesian analyses were observed. Therefore, only the MrBayes tree based on the analyses including indel coding is illustrated in Fig. 1, complemented with information from the parsimony analyses. Throughout

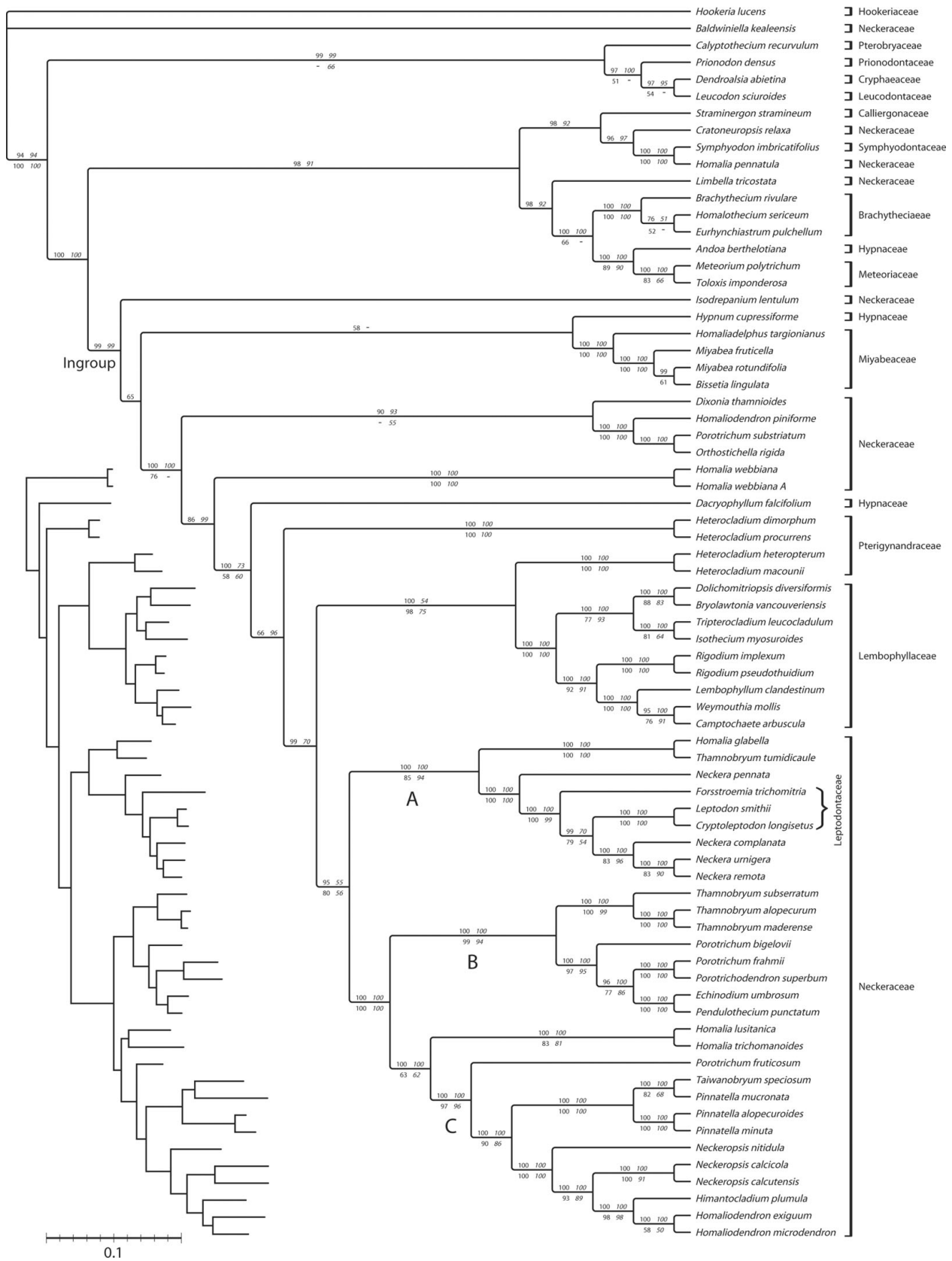


Figure 1 Majority consensus of trees sampled after stationarity in the Bayesian analysis of the matrix including indels. Values along the branches indicate posterior probabilities (above the branches) and bootstrap support values from the parsimony analyses (below). The first value corresponds to the analyses with the indel coding matrix included in the analyses. A miniature picture of the consensus tree is depicted to show the branch lengths.

the text posterior probabilities (PP) are listed first followed by the bootstrap support (BS) values. Values resulting from analyses with the SIC-matrix included precede the values from analyses without an indel coding approach. Thus support values from the different analyses will be referred to in the text following this scheme (PPsic/PP/BSsic/BS).

The Neckeraceae in its current circumscription is resolved as polyphyletic. Some taxa are actually resolved among outgroup taxa, such as *Baldwiniella kealeensis* and *Homalia pennatula*. The latter retains a close relation to *Symphyodon imbricatifolius*, with maximal support. The ingroup contains the Lembophyllaceae, the polyphyletically resolved *Heterocladium*, the Miyabeaceae, the polyphyletically resolved Neckeraceae as well as two representatives of the Hypnaceae. Among the ingroup taxa *Isodrepanium lentulum* branches off first, well outside the Neckeraceae. The position of *Hypnum cupressiforme*, grouping together with the Miyabeaceae has only weak support, while the Miyabeaceae receives full support regardless of the analysis method used. Some of the species currently placed in the Neckeraceae are forming a separate well supported cluster outside the Lembophyllaceae – Neckeraceae clade. This clade, known as the OPP-clade (Quandt *et al*., 2009), contains members of *Orthostichella*, *Porotrichum* plus *Dixonia orientalis*, and *Homaliodendron piniforme*. The position of *Dixonia* has only moderate support (90/93/55/-), but the rest of the clade gets maximal support. *Homalia webbiana*, like *Dacryophyllum falcifolium*, are closely related to this clade but branching off separately. The genus *Heterocladium* is resolved as polyphyletic, forming two pairs of species: *H. dimorphum* and *H. procurrens* cluster together with maximal support, as well as *H. heteropterum* and *H. macounii*. The latter clade seems to be more closely related to the Lembophyllaceae than the first one, with good support for its position from the analysis including indel coding (100/54/98/75). The monophyly of the Lembophyllaceae is fully supported with all analysis methods, and the Lembophyllaceae being the sister group of the Neckeraceae reaches high statistical support, albeit only regarding Bayesian statistics. The Neckeraceae *sensu stricto* are divided into three distinct clades: clade A with *Neckera* as the main genus, clade B including *Thamnobryum* and its allies, and clade C with *Pinnatella* and *Neckeropsis* as the prominent genera. Some genera, e.g. *Neckera*, *Porotrichum* and *Homalia* are strongly polyphyletic while others, such as *Pinnatella* and *Thamnobryum* form well-supported clades including, however, only a part of the species, thus not being monophyletic.

Ancestral state reconstructions of morphological characters

Ancestral state reconstructions revealed that the ancestor of the Lembophyllaceae – Neckeraceae clade (node I) had symmetric leaves (with posterior probability of 0.41 ± 0.16; Fig. 2), costa absent to weak (0.56 ± 0.18; Fig. 3), a perfect peristome (0.83 ± 0.12; Fig. 4), a seta that was more that 9 mm long (0.69 ± 0.18) and an orthogonal or widely homotropous capsule (0.44 ± 0.16). The ancestor of all Neckeraceae species (at node II) differed from it by having clearly asymmetric leaves (with posterior probability of 0.56 ± 0.14), an orthotropous to homotropous capsule (0.59 ± 0.16) and, with almost the same posterior probability, a strong (0.43 ± 0.11) or absent to weak (0.35 ± 0.13) costa. Within Neckeraceae the asymmetric leaves, strong costa, and perfect peristome are lost four times in different lineages. A short seta has evolved twice (in the clades A and C), and an orthotropous to homotropous capsule has been lost twice (in clade B as well as in the *Homalia lusitanica – H. trichomanoides* clade).

Discussion

Phylogenetic position of the Neckeraceae

Our study supports a close relationship between the Neckeraceae and the Lembophyllaceae, as suggested by, for example, Quandt *et al*. (2000, 2009) and Stech *et al*. (2008). Already Brotherus (1925) placed the Neckeraceae close to the Lembophyllaceae in the order Isobryales (= Leucodontales) (see also Robinson, 1975). The exact position of the Neckeraceae/Lembophyllaceae clade among the pleurocarpous mosses still remains to be established, but the merging of the data into a broad study that is in preparation (cf. Buchbender *et al*., 2006) and includes representatives covering all pleurocarpous moss families will give further insight into this question.

Even if recent molecular analyses have not challenged the close relationship between the Neckeraceae and Lembophyllaceae, the composition and taxonomy within both families have been less stable. In the Lembophyllaceae the generic composition has undergone drastic changes. Originally with just four genera (*Lembophyllum*, *Camptochaete*, *Dolichomitra*, *Isothecium*) (Brotherus, 1909) the family later on expanded to contain 12 genera (Fleischer 1906–1908, 1915–1923; Brotherus, 1925), and then drastically redefined to contain only *Lembophyllum* and *Camptochaete* (e.g. Andrews, 1952; Walther, 1983; Buck & Vitt, 1986; Crum, 1991). The latest revision based on molecular data (Quandt *et al*., 2009), however, nearly retrieved the 1925 concept of Brotherus although a clear morphological circumscription of the family is still lacking. Likewise our molecular data also challenge morphology-based classifications of the Neckeraceae. The Neckeraceae as treated by Brotherus (1925) contained 16 genera grouped into three subfamilies: Leptodontoideae, Neckeroideae and Thamnioideae (see Table 2). Walther (1983) accepted the division of the Neckeraceae into Leptodontoideae and Neckeroideae and recognised the Thamniaceae (later renamed Thamnobryaceae) as a separate family. The Leptodontaceae was erected by Schimper (1856), but it was generally not recognised until resurrected by Buck (1980) and employed by Buck and Vitt (1986). The division of the Neckeraceae must however be rejected, as the three clades that are resolved in the current analyses do not correspond to the subfamilies that Brotherus (1925) proposed. According to our results in Fig. 1 (compare Olsson *et al.*, 2009), the Neckeraceae include the species that have been previously placed in the Thamnobryaceae (Buck & Vitt, 1986) and in the Leptodontaceae (Schimper, 1856; Goffinet *et al.*, 2008). Brotherus' (1925) subfamilies Leptodontoideae, Neckeroideae and Thamnioideae are shown

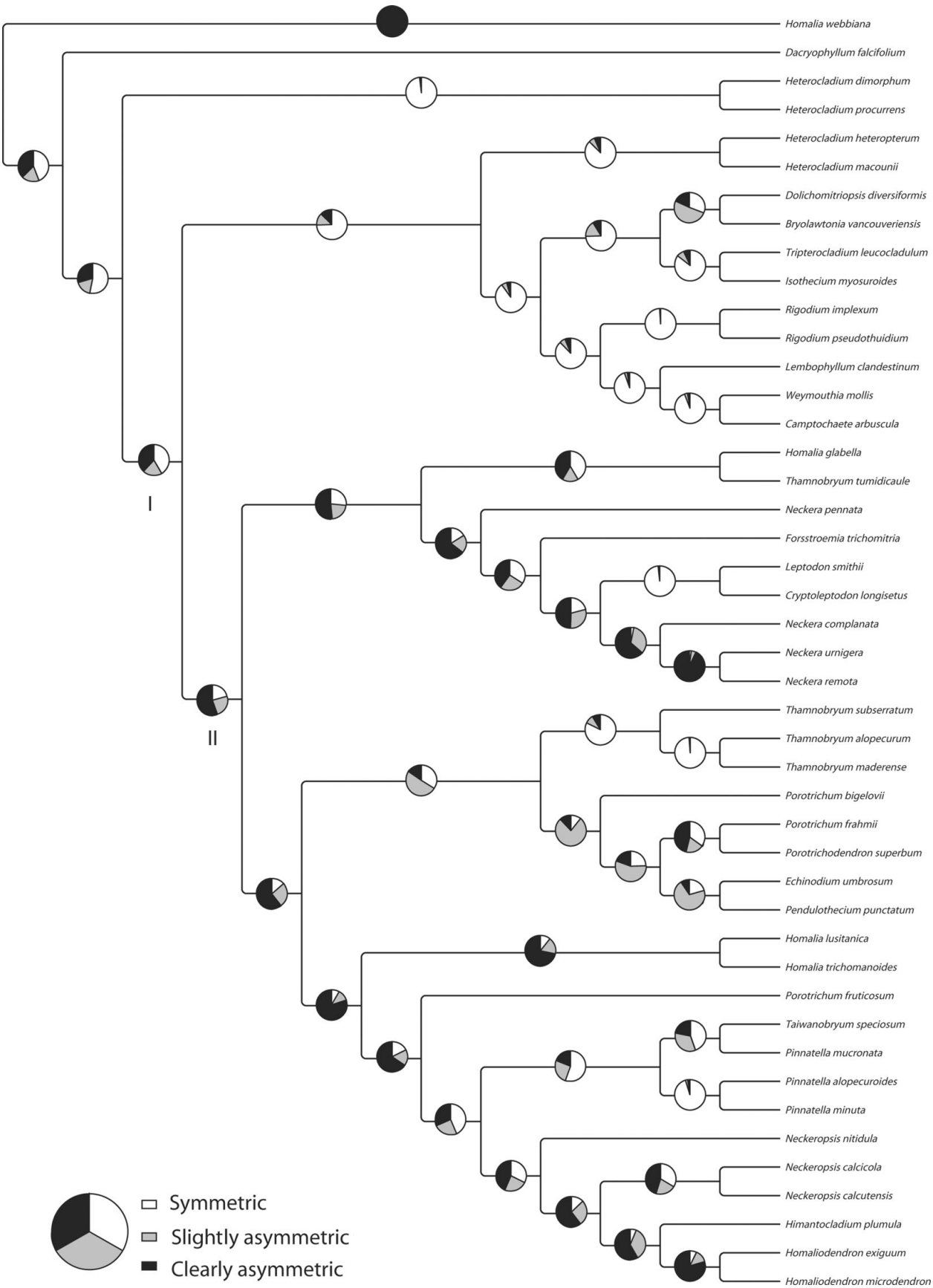


Figure 2 Ancestral character state reconstruction for leaf asymmetry among the ingroup. The circles plotted on the inferred Bayesian topology represent three states of leaf asymmetry: symmetric (white), slightly asymmetric (grey), clearly asymmetric (black).

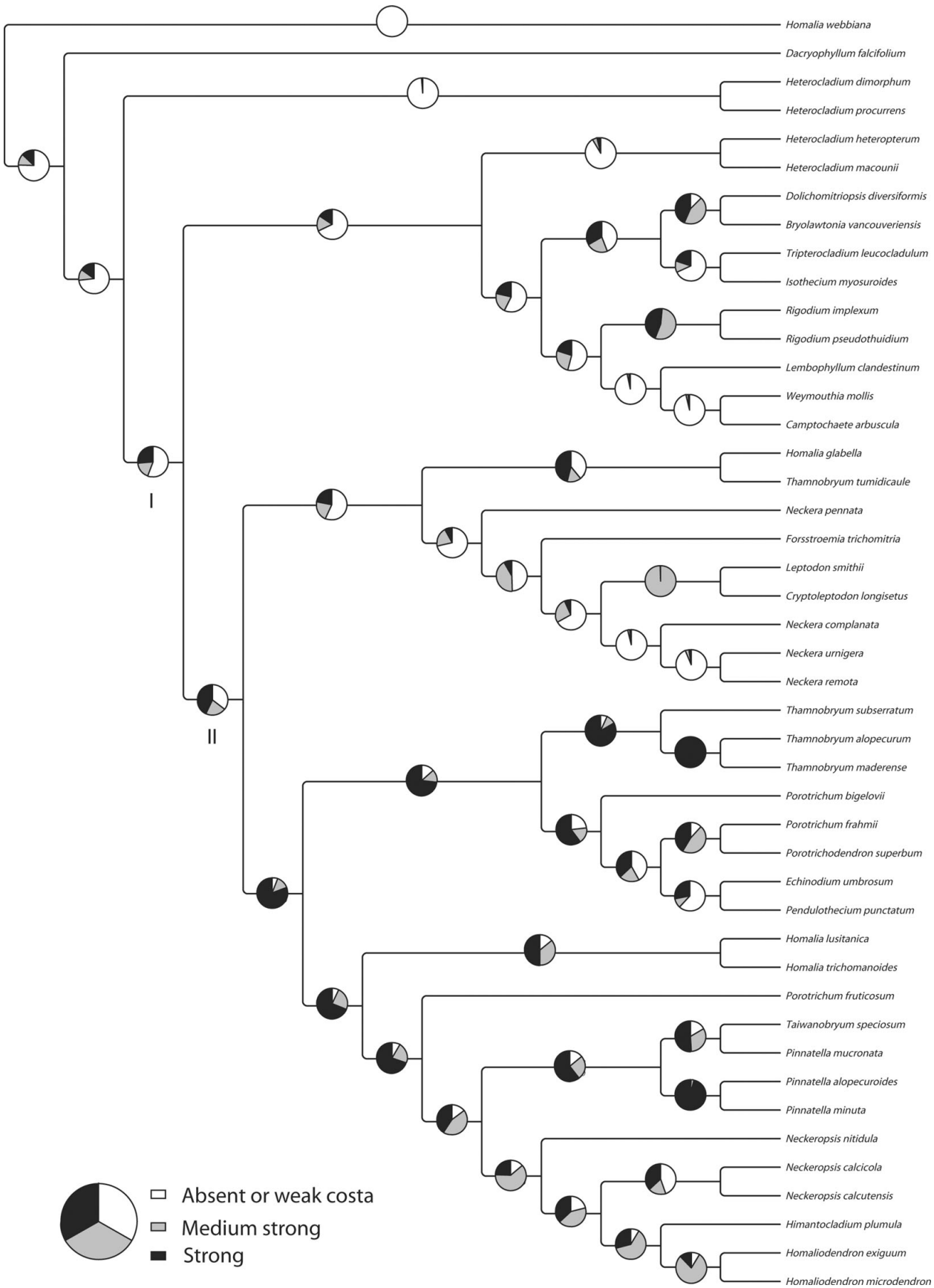


Figure 3 Ancestral character state reconstruction for strength of the leaf costa among the ingroup. The circles plotted on the inferred Bayesian topology represent three states of costa strength: absent or weak costa (white), medium strong costa (grey), strong costa (black).

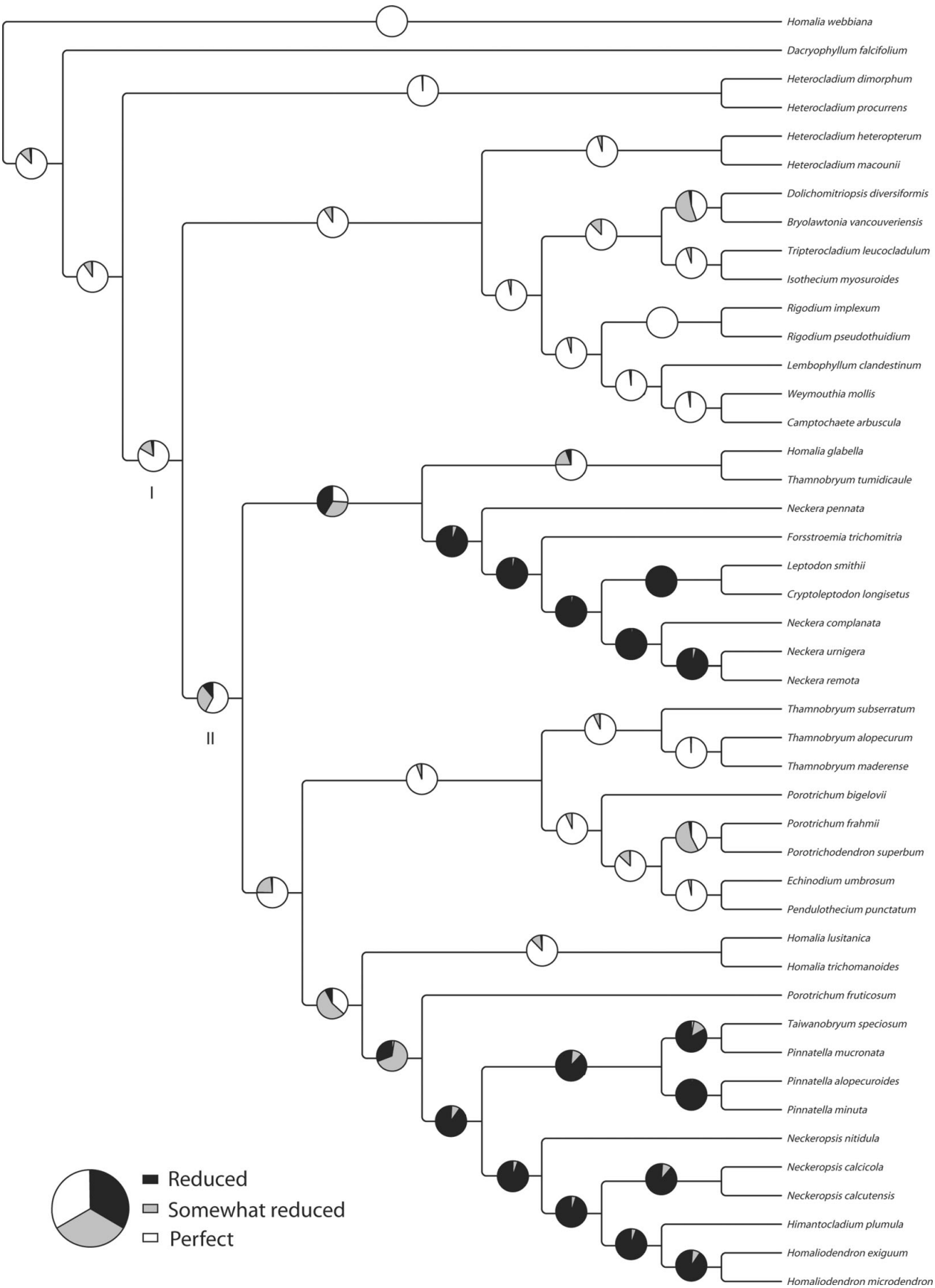


Figure 4 Ancestral character state reconstruction for peristome reduction among the ingroup. The circles plotted on the inferred Bayesian topology represent three states of peristome reduction: reduced (black), somewhat perfect (grey), perfect (white).

Brotherus (1925)	Vitt (1984)	Walther (1983)	Buck and Goffinet (2000)
Neckeraceae	**Neckeraceae**	**Neckeraceae**	**Leptodontaceae**
Leptodontoideae	*Baldwiniella*	**Leptodontoideae**	*Alsia*
Cryphidium	*Bissetia*	*Cryptoleptodon*	*Forsstroemia*
Leptodon	*Cryptoleptodon*	*Leptodon*	*Leptodon*
Cryptoleptodon	*Dolichomitra*	**Neckeroideae**	*Taiwanobryum*
Neckeroideae	*Handeliobryon*	*Homalia*	
Calyptothecium	*Himantocladium*	*Neckera*	**Neckeraceae**
Neckera	*Homalia*	*Metaneckera*	*Baldwiniella*
Neckeropsis	*Homaliadelphus*	*Neomacounia*	*Bissetia*
Bissetia	*Homaliodendron*	*Bissetia*	*Bryolawtonia*
Himantocladium	*Hydrocryphaea*	*Baldwiniella*	*Caduciella*
Baldwiniella	*Isodrepanium*	*Himantocladium*	*Crassiphyllum*
Homaliodendron	*Leptodon*	*Homaliadelphus*	*Cryptoleptodon*
Homalia	*Metaneckera*	*Homaliodendron*	*Curvicladium*
Thamnioideae	*Neckera*	*Neckeropsis*	*Dixonia*
Pinnatella	*Neckeropsis*		*Dolichomitra*
Handeliobryum	*Neomacounia*	**Thamniaceae**	*Handeliobryum*
Porotrichum	*Pinnatella*	*Porotrichum*	*Himantocladium*
Thamnium	*Porothamnium*	*Porothamnium*	*Homalia*
Porothamnium	*Porotrichodendron*	*Pinnatella*	*Homaliadelphus*
	Porotrichopsis	*Thamnobryum*	*Homaliodendron*
	Porotrichum	*Bestia*	*Hydrocryphaea*
		Handeliobryum	*Isodrepanium*
		Hydrocryphaea	*Metaneckera*
			Neckera
			Neckeropsis
			Neomacounia
			Noguchiodendron
			Pendulothecium
			Pinnatella
			Porothamnium
			Porotrichodendron
			Porotrichopsis
			Porotrichum
			Thamnobryum
			Touwia

Table 2 Overview of the different treatments of the Neckeraceae, including the Leptodontaceae and Thamnobryaceae (Thamniaceae). The treatment of the Neckeraceae by Goffinet and Buck (2004) is identical to Buck and Goffinet (2000), apart from the exclusion of *Porothamnium*. Buck and Vitt (1986) formally describe the Thamnobryaceae containing the dendroid Neckeraceae sensu Brotherus (1925) with cross-striolate exostomes (i.e. roughly the former subfamily Thamnioideae Broth.).

to be polyphyletic, since the clades in our analyses are composed of taxa belonging to at least two different subfamilies in his system.

Trends in morphological evolution and phytogeographic patterns

Enroth (1994a) presented some hypotheses of primitive vs. advanced character states within the Neckeraceae. He postulated that reduction was the 'key process' in the evolution, and that asymmetric leaves with a weak costa and fine dentation, irregular branching pattern, as well as a short seta with reduced peristome, would be advanced character states. Our results show that asymmetric leaves are ancestral in the Neckeraceae, but they support Enroth's (1994a) implied corollary that the ancestor of the Neckeraceae had a strong costa, long seta and perfect peristome. A notable observation is that for all these characters reduced states have evolved independently several times within the family. In each of the three main clades the same trends towards more specialised structures can be observed in the sporophyte evolution: from antitropous, orthogonal or homotropous capsules to orthotropous; from long setae to short; and from perfect peristomes to variably reduced. These trends are strongest in clade A and weakest in clade B. One plausible reason for such morphological character changes may be a shift to epiphytic habitats that were repeatedly and independently conquered in the three different clades within the Neckeraceae. In each clade the basal taxa

favour rock or soil as substrates, while the more advanced ones are mainly epiphytic. The clades are also geographically differentiated. Clade A includes mainly non-Asiatic members, like clade B, where the truly tropical taxa are almost exclusively restricted to South America, while clade C includes Asiatic and tropical members, except the basal *Homalia*, which is temperate, and *Pinnatella minuta*, which occurs in addition to India also in Africa and S America.

In many other pleurocarpous moss families, epiphytism is correlated with similar combinations of morphological character states (Hedenäs, 2001; Huttunen *et al*., 2004). Especially structures of the sporophyte generation appear prone to evolve adaptations to new environmental conditions (Hedenäs, 2001, 2002; Vanderpoorten *et al.*, 2002b; Huttunen *et al.*, 2004). It is clear that several morphological character states were independently acquired in the different Neckeraceae lineages, but further investigation is needed to unravel the evolutionary processes behind this. Factors that need to be studied further include both the genetic regulation of morphological characters and the evolutionary processes affecting morphology, including the role of habitat shifts in furthering character state changes. Although the primary factors promoting sporophytic reductions found in epiphytes are likely to affect spore dispersal, e.g. wind and humidity (Hedenäs, 2001), reduced reproductive costs involved in producing reduced sporophytes also need to be considered in this context. It was only recently shown experimentally that sporophyte production incurs a cost in terms of reduced future gametophytic growth also in bryophytes (Ehrlén *et al*., 2000), and one may thus speculate that small and simple sporophytes 'cost less' than large and elaborate ones to produce. If small sporophytes incur smaller reproductive costs than large ones they could potentially be advantageous in habitats where resources are limited, for example in epiphytic ones where low nutrient input or leaching may be problematic (cf. Smith, 1982; Nadkarni, 1984).

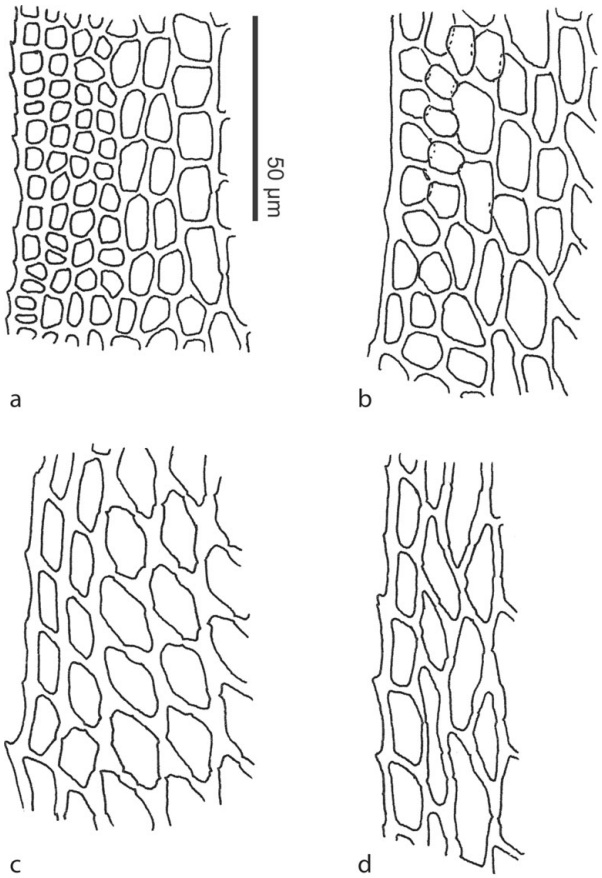


Figure 5 Variation in marginal leaf cells in the Neckeraceae. a. *Pinnatella alopecuroides* (redrawn from Enroth, 1994b, fig 8g). b. *Curvicladium kurzii* (redrawn from Enroth, 1993, fig. 1d). c. *Neckera neckeroides*(redrawn from Enroth & Tan, 1993, fig. 1d). d. *Neckera serrulatifolia* (redrawn from Enroth & Ji, 2007, fig 2f).

Morphological delimitation of the Neckeraceae

Although the monophyly of the Neckeraceae has been shown to be doubtful in its current circumscription (Buck *et al.*, 2000; Tsubota *et al.*, 2002; Olsson *et al.*, 2009) our present results allow us to retain a monophyletic family after the exclusion of several taxa (see below). Our morphological studies revealed two new morphological characters that aid in family level delimitation especially between the Neckeraceae and the Lembophyllaceae. Characters currently used to define the Neckeraceae as well as its sister group, the Lembophyllaceae, are not exclusive or discontinuous, hindering a clear morphological circumscription of both families. The Lembophyllaceae sensu Quandt *et al*. (2009) comprises a morphologically highly heterogeneous group of mainly epilithic or epiphytic plants with creeping stolons and often frondose stems bearing usually concave leaves. As a rule of thumb the Neckeraceae and Lembophyllaceae differ in their arrangement of leaves on the shoots. In the Neckeraceae the shoots are mostly complanate, whereas in the Lembophyllaceae they are usually terete, with the leaves being most often loosely appressed. In addition, the families differ in their habitat preferences; the Neckeraceae are most diverse in tropical environments, whereas the Lembophyllaceae are essentially found in temperate climates. According to our observations, all members of the Neckeraceae have at least 1–2 marginal cell rows (Fig. 5) that are at least partly composed of quadrate to rectangular cells shorter than the corresponding inner laminal cells, even when the leaf cells are generally elongate. In the Lembophyllaceae such a clearly differentiated leaf margin is not commonly present. *Baldwiniella kealeensis* and *Isodrepanium lentulum*, which according to our analyses do not belong in the Neckeraceae, lack such marginal cells. Furthermore, these two species share a bipolarity of character states in the two generations (cf. Enroth, 1994a): both have a distinctly advanced, '*Neckera*-like' gametophyte combined with a primitive type of sporophyte (long seta, homotropous capsules, cross-striolate lower exostome outsides and high basal membranes). Clearly, they have been placed in the Neckeraceae due to a superficial gametophytic resemblance to that family – fairly large, glossy plants with undulate and asymmetric leaves and a short, weak costa.

Another character state typical for the Neckeraceae seems to be a consistent lack of dwarf males. Such males have been found in most of the Lembophyllaceae genera (Tangney, 2006;

Buchbender, 2009) and they have also been found in *Homaliadelphus* and *Bissetia*, which have been placed in the Neckeraceae before but actually form with *Miyabea* a distinct family Miyabeaceae (Olsson *et al.*, 2009).

The genera included in the Neckeraceae in this analysis based on molecular data are different from those in the more traditional classifications of the Neckeraceae (Brotherus, 1925; Enroth, 1994a). According to the current classification by Goffinet *et al.* (2008), there are 28 genera in the Neckeraceae, but no comprehensive genus-level revision of the family has been made. Recent studies based on a wider taxon sampling have already reduced the number of genera included. For example, *Homaliadelphus* and *Bissetia* belong to the newly erected family Miyabeaceae, and *Limbella tricostata* belongs near the Meteoriaceae and Brachytheciaceae (Olsson *et al.*, 2009). The problematic nomenclature of the genus *Limbella* is discussed in some more detail in Olsson *et al.* (2009) and needs clarification, since the generic name was treated in the Amblystegiaceae by Goffinet *et al.* (2008), but in fact there are two taxonomic entities with the same name. On the other hand, Tsubota *et al*. (2002) provided evidence that members of the four genera (*Alsia*, *Forsstroemia*, *Leptodon*, *Taiwanobryum*) treated in Leptodontaceae by Goffinet *et al.* (2008) belong to the Neckeraceae. In addition, our results point out that more changes are needed in the delimitation and contents of several genera. Below is a commentary on the genera that were earlier included in the Neckeraceae by some authors, but which are excluded from it in the present study.

Systematic changes

The families Thamnobryaceae and Leptodontaceae become synonyms of the Neckeraceae. Furthermore, several taxa are excluded from the Neckeraceae, as follows (cf. Figure 1).

Baldwiniella kealeensis is an endemic of the Hawaiian archipelago. The exact relationships of the unispecific *Baldwiniella* need further elaboration, but it is clearly not at all closely related to the Neckeraceae, where it was originally placed (Fleischer, 1905).

Bryolawtonia vancouveriensis is another unispecific genus, from the California-Oregon district, and previously known as *Porotrichum vancouveriensis* and *Bestia vancouveriensis* (see Norris & Enroth, 1990). It belongs in the Lembophyllaceae where it fits well together with e.g. *Isothecium*.

Homalia pennatula was previously placed in the genus *Symphyodon* (Symphyodontaceae), but He and Enroth (1995) and He (1997) treated it in *Homalia*. Their decision was based on overall gametophyte similarity to other *Homalia* species (leaf shape, irregularly serrulate upper leaf margins). However, the sporophytes are unknown and the sequence information as well as several morphological characters (variable costae and linear, projecting median leaf cells), support a placement in *Symphyodon*.

Homalia webbiana (see He, 1997) and *Dacryophyllum falcifolium* (see Ireland, 2004; Kellman & Shevock, 2006) as well as the genus *Heterocladium* (see Gardiner *et al*., 2005) do not belong in the Neckeraceae. Their accurate position among the pleurocarpous mosses remains to be solved in further studies. As Gardiner *et al*. (2005) already showed, the genus *Heterocladium* is polyphyletic, since two of the species (*H. heteropterum* and *H. macounii*) nest within or reside as a sister group to the Lembophyllaceae while the other two (*H. dimorphum* and *H. procurrens*) do not.

Homaliadelphus and *Bissetia* appear together with *Miyabea* in a clade having strong support from both morphological and sequence data, supporting the results from Olsson *et al*. (2009).

The clade known as the OPP clade (Quandt *et al*. 2009), where *Homaliodendron piniforme* belongs, together with *Dixonia orientalis*, *Porotrichum substriatum* and *Orthostichella* (see also Allen & Magill, 2007) is supported by this study but will not be discussed further, since it will be treated in a forthcoming paper.

The unispecific genus *Isodrepanium*, placed in the Neckeraceae by nearly all authors (e.g. Goffinet & Buck, 2004), from Central and South America apparently does not belong to the Neckeraceae and with the present taxon sampling it seems to represent a separate evolutionary lineage.

Limbella includes two species: *L. tricostata* from Hawaii and *L. fryei* from Oregon, excluding *L. bartlettii* (cf. Olsson *et al*., 2009 for a more detailed discussion). They are big, stipitate and morphologically rather similar to *Thamnobryum sensu stricto* species and *Handeliobryum*, growing on shady, often even wet places (sometimes in running water), on ground, stones and tree bases. The peristome is a perfect hypnoid one. *Limbella* was placed in the Thamnobryaceae by Ochyra (1987), who emphasised a close relationship with *Thamnobryum*. However, in our current analyses as well as in previous studies (Olsson *et al*., 2009) it is located outside the Neckeraceae and close to the Brachytheciaceae and Meteoriaceae, where it seems to fit well according to morphology.

Acknowledgements

SO acknowledges financial support by the Helsingin Sanomat Centennial Foundation and the Research Foundation of the University of Helsinki. Furthermore, the authors received support from two researcher exchange grants by Finnish Academy/DAAD (JE, DQ, VB, SO) and DAAD/STINT (VB, LH, SH, SO, DQ), which is highly acknowledged. Research was funded by the Deutsche Forschungsgemeinschaft (DFG QU 153/3–1, 153/3–2) and SYNTHESYS (VB, JE, SO), which is financed by the European Community Research Infrastructure Action under the FP6 'Structuring the European Research Area' Programme (http://www.synthesys.info). In addition, we thank two anonymous reviewers for their highly appreciated comments.

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