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Multi-gene phylogeny supports single origin of jungermannioid perigynium

Xiaolan He-Nygrén

Botanical Museum, Finnish Museum of Natural History, P.O. Box 7, FI-00014 University of Helsinki, Finland

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Within the leafy liverworts, the evolution of the perigynium, the stem-derived structure that protects the developing sporophytes, has been understood as merely a parallelism, and families that have this feature have been placed to various suborders. The present study suggests a single origin of the perigynium in the leafy liverworts and the presence of perigynium being a synapomorphy for the suborder Jungermanniineae. The phylogenetic analyses were conducted using thirty-four leafy liverworts including twelve genera bearing a perigynium, and sequence data for *rbc*L, *rps*4, *trn*L-F cpDNA and 26S nrDNA. Within the Jungermanniineae three monophyletic lineages are recognized: the Acrobolbaceae lineage, the Trichotemnomaceae–Balantiopsidaceae lineage, and the lineage consisting of Jungermanniaceae and its closest related Gymnomitriaceae, Delavayellaceae, Geocalycaceae *s. stricto*, Antheliaceae, Calypogeiaceae and Gyrothyraceae. The long branches of the latter three families indicate that they are more isolated from a common jungermannioid ancestor. The family Jungermanniaceae is resolved as paraphyletic and its circumscription and relationships require further study.

Key words: homology, Jungermanniineae, leafy liverworts, morphological innovation, perigynium, phylogeny, systematics

Introduction

The leafy liverworts, including 4000–6000 species, are the most speciose and morphologically diverse group of the liverworts. The immediate distinction of the leafy liverworts from rest of the liverworts is the development of a leaf, two or three ranks of which are inserted on the stem; and a perianth, which is formed by the fusion of two to three leaves surrounding the archegonia protecting the developing sporophyte. The substrata colonized by this group of species vary from soil and/or rock surface to tree trunks and even to living leaves; the species occur in greatest diversity and abundance in the humid subtropics and tropics. Because of their remarkable variations in morphology and great profusion of structure, comprehending evolution and taxonomy of the leafy liverworts has been difficult and challenging. Schuster (1984) listed abundant examples of presumable parallelism and homoplasy in this group and stated

that problems induced by parallelism and the malleability of ontogenetic patterns hinder any attempt to construct a phylogenetically-sound classification. Nevertheless, leafy liverworts have been classified in as many as 15 suborders or orders based on morphological observations (Schljakov 1972, Schuster 1972, 1984, 2000, 2002) representing independent evolutionary lines, but the evolutionary relationships between these groups have largely remained obscure. There are no morphological characters providing clear-cut distinction between them. The above-mentioned assumption on leafy liverwort evolution by Schuster and other authors has long been accepted and followed by hepaticologists until the recent outcome of the phylogenetic studies on leafy liverworts using multiple gene sequence data (Davis 2004, He-Nygrén *et al*. 2004, Forrest *et al*. 2006) and using combined multiple gene sequences and morphology (He-Nygrén *et al*. 2006). In these studies, novel, and well resolved phylogenetic relationships of major groups of leafy liverworts were provided; they do not support previous assumptions on leafy liverwort evolution. Their results imply that our current understanding of many morphological characters should be re-examined.

In He-Nygrén *et al*. (2004), leafy liverworts were suggested to be the most derived lineage among the liverworts. Except *Pleurozia*, a very isolated leafy lineage, two major clades were recognized. Based on the result derived from more extensive data, He-Nygrén *et al*. (2006) classified the leafy liverworts into three orders, Pleuroziales, Porellales, and Jungermanniales. The Jungermanniales are classified to four suborders, Perssoniellineae, Cephaloziineae, Jungermanniineae and Lophocoleineae. Their result on the Jungermanniineae is striking, since it suggests a close affinity between various families that were previously thought unrelated. The families of the Jungermanniineae in the analysis (He-Nygrén *et al*. 2006) are Jungermanniaceae, Delavayellaceae, Gyrothyraceae, Acrobolbaceae, Gymnomitriaceae, Antheliaceae, Calypogeiaceae, Trichotemnomataceae, and Balantiopsidaceae. Among them, Jungermanniaceae, Delavayellaceae and Gymnomitriaceae are the only families that belong to the traditionally recognized Jungermanniineae (Schuster 1984). Other families have been assigned to various suborders (*see* Schuster 1972, 1984, Crandall-Stotler & Stotler 2000). However, it is noteworthy that the Jungermanniineae comprise by all the leafy families that have a perigynium, the stem tissue-derived protective structure of the developing sporophyte. Only two families, Antheliaceae and Delavayellaceae of the Jungermanniineae lack this feature. The study of Hentschel *et al*. (2006) based on single gene also suggests the union of families Jungermanniaceae, Geocalycaceae, Calypogeiaceae, Balantiopsidaceae, Trichotemnomaceae and Acrobolbaceae. These results have brought up questions as whether the character perigynium in the Jungermanniineae represents a single origin. Previously, the occurrence of perigynium in different families had been understood as merely a parallelism. In the present study, the monophyly of the perigynium and the circumscription of the Jungermanniineae will be addressed.

Material and methods

Taxon sampling

Thirty-four taxa of leafy liverworts were chosen as exemplars. All the 14 species that constitute the Jungermanniineae in He-Nygrén *et al*. (2006) were included in the present study, and are marked with asterisks in Fig. 1. Eleven additional ingroup species from five families were added. They are *Jungermannia crenuliformis*, *J. exsertifolia*, and *Nardia scalaris* (Jungermanniaceae); *Gymnomitrion* sp. (Gymnomitriaceae); *Balantiopsis diplophylla*, *Isotachis lyallii*, *I. multiceps*, and *Neesioscyphus bicuspidatus* (Balantiopsidaceae); *Lethocolea glossophylla* (Acrobolbaceae); and *Harpanthus scutatus* (Geocalyceceae). *Harpanthus* was included in the present study because it bears a perigynium. Other families which possess also perigynia, Arnelliaceae (*Arnellia* and *Southbya*) and Jackiellaceae (*Jackiella)* were unfortunately not able to be included in the analysis. *Mylia taylorii*, representing Mylioideae of Jungermanniaceae (Grolle 1963) or Myliaceae in the suborder Myliineae (Engel & Braggins 2005), was included in the present analysis in order to test further its systematic position. *Mylia* is a genus that lacks perigynium

but is resolved as sister to a group of species possessing a perigynium in Hentschel *et al*. (2006) based on *rbc*L sequences. Nine species of leafy liverworts were selected as outgroup taxa according to He-Nygrén *et al*. (2006), two of which are from Schistochilales, and seven from Cephaloziineae. Details on voucher specimens are given in Table 1.

Morphological character

Character perigynium is scored as present or absent in the analysis. It was examined by the author based on specimens available at H, and also according to previous studies (Schuster 1966, 1969, 1974, 1984, 1996, Gradstein & Pinheiro da Costa 2003).

DNA markers and sequences

Sequence data for *rbc*L, *rps*4 and *trn*L-F cpDNA and 26S nrDNA were utilized in the present study. Novel sequences were generated for *rbc*L and *trn*L-F cpDNA of *Neesioscyphus bicuspidatus*; the rest were obtained respectively from previous studies of He-Nygrén *et al*. (2004, 2006), and from the GenBank. The protocols for DNA extraction, PCR, and sequencing followed the methods used by the author in He-Nygrén *et al*. (2004). All 34 taxa represent data from at least two of the four sequenced regions. The amount of missing data and sequence length variation within the data sets is given below.

*rbc*L 1035 base pairs of the large subunit of the ribulose bisphospate carboxylase (*rbc*L) gene were used. The amount of missing data was 23.5%. There is no length variation in *rbc*L sequences.

*rps*4 This gene codes for the chloroplast ribosomal protein S4. Only the obtained *rps*4 gene of 573 base pairs were included in the analyses whereas the *rps*4 primers (Cox & Hedderson 1999) isolate a longer region including a region that contains from 5´ end incomplete *rps*4 gene as well as part of the intergenic spacer between *rps*4 and the gene for serine transfer RNA. The amount of missing data was 6%. There is no sequence length variation in the *rps*4 sequences.

*trn*L–*trn*F The data set consisted of two exons and the intron of partial sequence of the leucine transfer $RNA_{(UAA)}$ and a partial sequence of phenylalanine $tRNA_{(GAA)}$. The gene areas of P6 and P8 of intron and intergenic spacer were excluded in the analysis because certain elements in these regions are not homologous but have independent origins (*see* Stech *et al*. 2003, Quandt *et al*. 2004). Recognition of P6 and P8 regions was based on presentations of secondary structures by Stech *et al*. (2003) and Quandt *et al*. (2004). The amount of missing data was about 17%.

26S In the data set of nuclear large subunit ribosomal DNA sequences the amount of missing data was approximately 28%. The sequence lengths vary from 1037–1045 bp.

Phylogenetic analyses

Two optimality criteria, parsimony and Bayesian inference of phylogenetic analysis were used in the present study. The sequences from each gene were aligned using DIALIGN2 (Morgenstern 1999). 2941 equally weighted nucleotide characters from the four gene fragments were simultaneously analyzed. The parsimony analysis was performed using NONA 2.0 (Goloboff 1998), run within Winclada (Beta) 0.99 (Nixon 1999). Gaps were treated as missing data. Tree search options of hold 30000, hold/20, mult*1000 were used. In order to estimate clade support on a cladogram, Bremer support (Bremer 1988, 1994) values were calculated by successively increasing the number of tree held to avoid an over-estimation of support values. The morphological characters studied here were optimized using Winclada.

The Bayesian analysis was run using MrBayes version 3.0B4 (Huelsenbeck & Ronquist 2001, Ronquist 2004). Searches were based on 1 000 000 generations with four MCMC chains, which were sampled at every 100th generation, starting from random trees. The log-likelihood scores were plotted against generation

Table 1. The GenBank accession numbers for the sequences used in the phylogenetic analysis. The accession numbers for novel sequences are shown in italics. The species that constitute the Jungermanniineae in He-Nygrén *et al.* (2006) are marked with asterisk. The voucher specimens for novel sequences herein, as well as references for sequences taken from the GenBank are listed under vouchers. Liverworts are arranged to orders and suborders according to He-Nygrén *et al.* (2006) classification.

Taxon	Genbank accession number				Voucher
	*rbc*L	*rps*4	*trn*L-F	26S	
Ingroup					
**Acrobolbus ciliatus*	DQ026579	DQ026596	DQ026613		He-Nygrén *et al.* 2006
**Anthelia julacea*	DQ026581	DQ026599	DQ026616		He-Nygrén *et al.* 2006
				AY608196	Davis 2004
**Balantiopsis cancellata*	AY462286	AY462340	AY463545		He-Nygrén *et al.* 2004
				DQ026524	He-Nygrén *et al.* 2006
Balantiopsis diplophylla		AY608047		AY608199	Davis 2004
**Calypogeia integristipula*	AY462290	AY462344	AY463550		He-Nygrén *et al.* 2004
				DQ026529	He-Nygrén *et al.* 2006
**Calypogeia muelleriana*	U87065				Lewis *et al.* 1997
		AY608052		AY608203	Davis 2004
			AY463551		He-Nygrén *et al.* 2004
**Delavayella serrata*	DQ026583	DQ026601	DQ026618		He-Nygrén *et al.* 2006
**Gymnomitrion concinnatum*		AY608065		AY608215	Davis 2004
			AF519202		*Yatsentyuk* et al. *2004*
Gymnomitrion sp.		AY462352	AY466440		He-Nygrén *et al.* 2004
**Gyrothyra underwoodiana*	DQ026584	DQ026602	DQ026619		He-Nygrén *et al.* 2006
Harpanthus scutatus		AY608069		AY608217	Davis 2004
**Isotachis armata*	DQ026585			DQ026540	He-Nygrén *et al.* 2006
		AY462358	AY463561		He-Nygrén *et al.* 2004
**Isotachis humectata*	AY462302	AY462359	AY463561		He-Nygrén *et al.* 2004
				DQ026541	He-Nygrén *et al.* 2006
Isotachis lyallii	AY608032	AY608073	AY608130	AY608221	Davis 2004
Isotachis multiceps	AY507407	AY507449	AY507537		Forrest & Crandall-Stotler 2004
Jungermannia exsertifolia		AY608077		AY608225	Davis 2004
Jungermannia crenuliformis		AY608078		AY608226	Davis 2004
**Jungermannia leiantha*	AY149838		AY149857		He-Nygrén & Piippo 2003
		AY462361			He-Nygrén *et al.* 2004
Lethocolea glossophylla		AY608084		AY608230	Davis 2004
**Marsupidium latifolium*	AY608034	AY608088	AY608134	AY608233	Davis 2004
Mylia taylorii	DQ312506				Hentschel *et al.* 2006
			AY327779		Yatsentyuk *et al.* 2004
**Nardia compressa*	DQ026587	DQ026603	DQ026621		He-Nygrén *et al.* 2006
Nardia scalaris		AY608092		AY608236	Davis 2004
Neesioscyphus bicuspidatus	*DQ354978*		*DQ354977*		Costa Rica, *Dauphin 2042* (MO)
**Trichotemnoma corrugatum*	AY462333	AY462399	AY463591		He-Nygrén *et al.* 2004
				DQ026574	He-Nygrén *et al.* 2006
Outgroup					
Schistochilales					
Schistochila appendiculata	AY462328	AY462394	AY463596		He-Nygrén *et al.* 2004
				DQ026566	He-Nygrén *et al.* 2006
Schistochila laminigera	AY462329	AY462395	AY463586		He-Nygrén *et al.* 2004
				DQ026567	He-Nygrén *et al.* 2006
Cephaloziineae					
Adelanthus bisetulus	DQ026580	DQ026597	DQ026614		He-Nygrén *et al.* 2006
Adelanthus lindenbergianus	AY462285		AY463544		He-Nygrén *et al.* 2004
		AY608042		AY608193	Forrest & Crandall-Stotler 2004
Chaetophyllopsis whiteleggei	AY462292	AY462346	AY463553		He-Nygrén *et al.* 2004
				DQ026530	He-Nygrén *et al.* 2006
Chandonanthus sp.	AY462293	AY462347	AY463554		He-Nygrén *et al.* 2004
				DQ026531	He-Nygrén *et al.* 2006
Lophozia ventricosa	AY462312	AY462369	AY463572		He-Nygrén *et al.* 2004
				DQ026552	He-Nygrén *et al.* 2006
Syzygiella geminifolia	DQ026591	DQ026607	DQ026624	DQ026568	He-Nygrén *et al.* 2006
Wettsteinia schusteriana	DQ026593	DQ026610	DQ026626	DQ026577	He-Nygrén *et al.* 2006

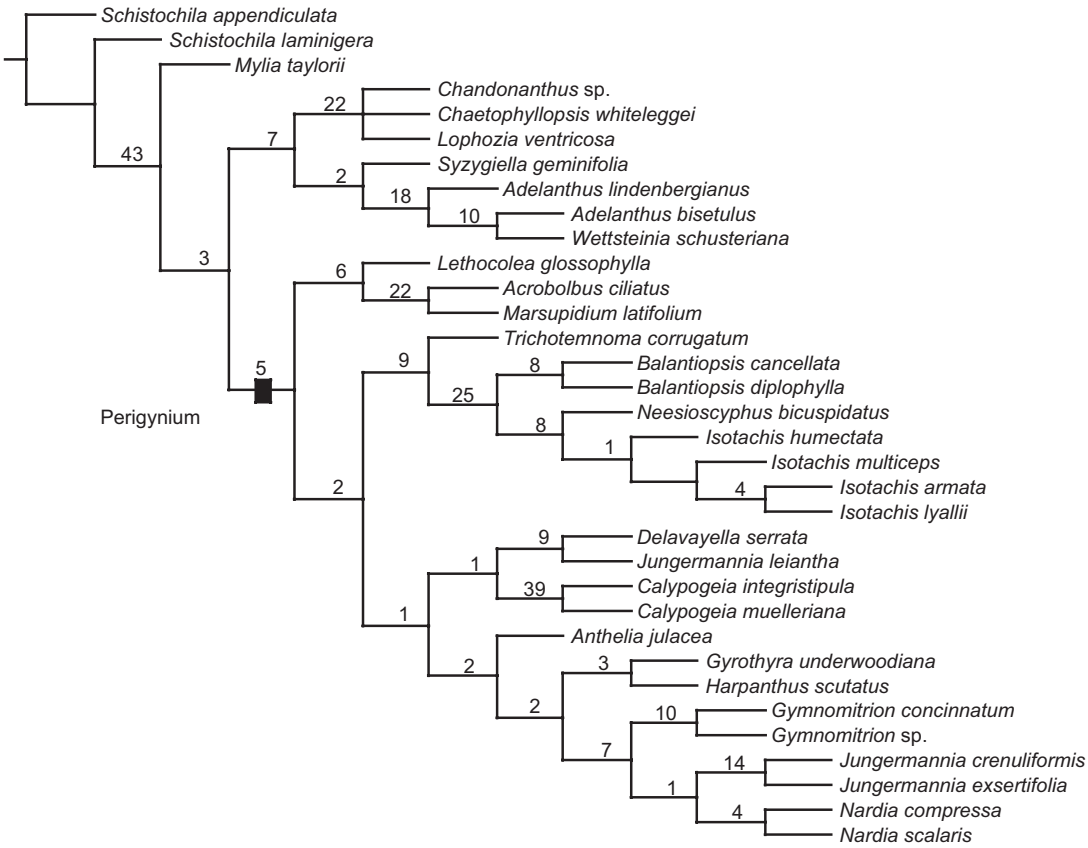

Fig. 1. Parsimony analysis using NONA of all data combined. Strict consensus of two most parsimonious trees, length = 1984 steps. Bremer support values indicated above nodes.

time, and stationarity of Markov chains was assumed when log-likelihood values reached a stable equilibrium. All sample points prior to stationarity were discarded as burn-in values, and the remaining points were used to generate a 50% majority consensus tree. The analysis was performed using a GTR model with distribution of rates across the sites (nst = 6 rates = gamma), and by applying substitution model 4×4 for the molecular data (nucmodel = 4×4). The latter is the standard model of DNA substitution with four states. All site patterns had a possibility of being sampled (coding = all). The prior assumption of rate heterogeneity across the data partitions was set at variable (ratepr = variable). The heating of the chains was adjusted to get the acceptance rates for the swaps between chains to 10%–70%, as recommended in the MrBayes tutorial (temp = 0.005000) (Ronquist & Huelsenbeck 2004). The prior assumptions for transition/transversion rate ratio, the state frequences, gamma shape parameter for among site variation, the substitution rates of the GTR model, and the synonymous/nonsynonymous rate ratio were unlinked between data sets (unlink tratio = (all) statefreq = (all) shape = (all) revmat = (all) omega = (all) ratemultiplier = (all)).

Results

Parsimony analyses

A combined analysis of the four gene fragments resulted in two most parsimonious trees with lengths of 1984 steps (540 phylogenetically informative base positions), consistency index of 0.52, and retention index of 0.60 (Fig. 1). The topologies of the two trees remain the same for the ingroup taxa. The ingroup taxa are formed

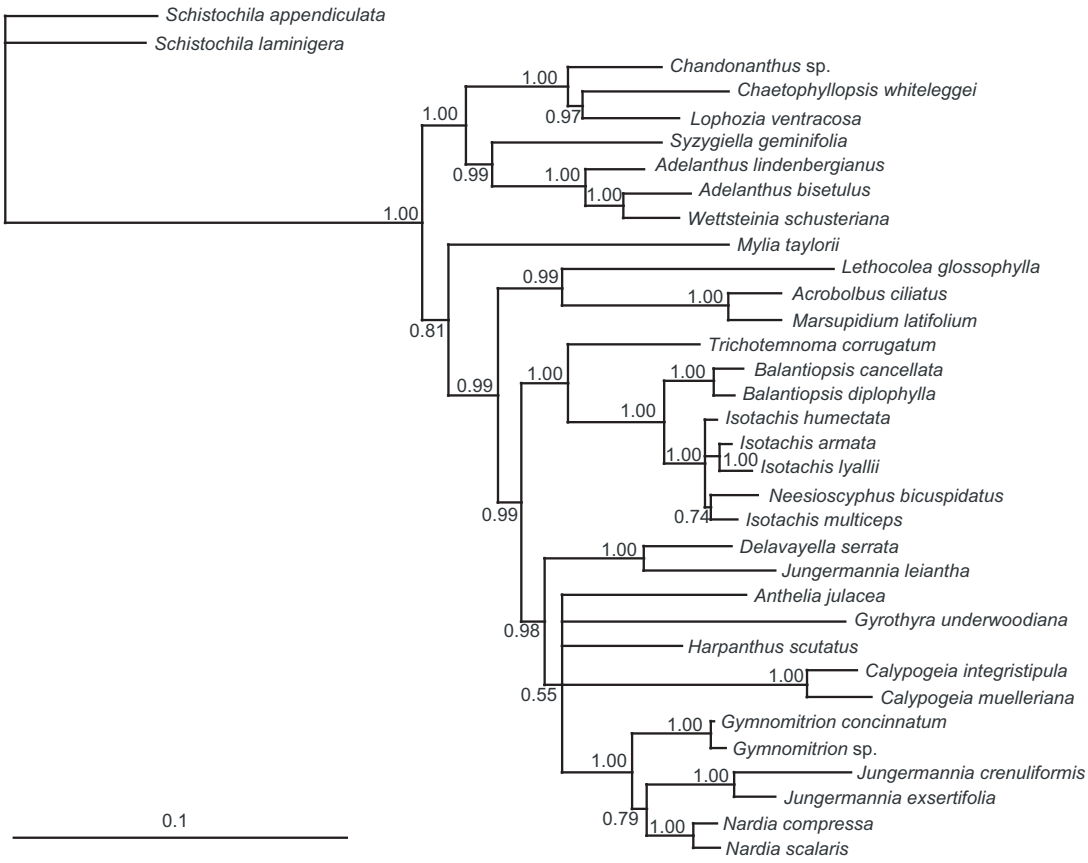

Fig. 2. Bayesian analysis using MrBayes of all data combined. The tree is a 50% majority consensus tree of the trees with best likelihood scores. Numbers indicate the posterior probability support values.

as monophyletic when *Mylia* is excluded. *Mylia* is resolved in a position between outgroup taxa *Schistochila* and the clade consisting of the rest outgroup species (Fig. 1). Acrobolbaceae, represented by *Lethocolea glossophylla*, *Acrobolbus ciliatus* and *Marsupidium latifolium*, form a monophyletic group and it is sister group to the clade consisting of two subclades. One subclade, with good support (BR = 9), contains Trichotemnomaceae and Balantiopsidaceae; another, with less support (BR = 1), the remaining ingroup species, which is called here the *Jungermannia* clade. Within the *Jungermannia* clade, *Delavayella* and *Jungermannia leiantha* are clustered together and form a sister group to *Calypogeia*, and they together form a sister to a clade consisting of *Anthelia*, *Gyrothyra–Harpanthus*, and *Gymnomitron–Jungermannia–Nardia* grades.

Bayesian inference

In the 50% majority consensus tree generated from the trees sampled (3159 trees) after the log-likelihood values reached a stable equilibrium (Fig. 2). The topology differs from the one resulted from the parsimony analysis mainly in the position of *Mylia*. Although *Mylia* in the MrBayes analysis forms sister group to the remaining ingroup species, no support is gained for the clade that subtends these species (posterior probabilities < 95). The rest of the ingroup species are supported as a monophyletic group, in which three major clades are recognized and supported as resolved in the NONA analysis. Within the *Jungermannia* clade, support is only found for the clade of *Delavayella* and *Jungermannia leiantha*, and the clade consisting *Gymnomitrion*, the rest two *Jungermannia* and *Nardia*

species. The relationships of *Anthelia*, *Gyrothyra* and *Harpanthus* remain unresolved.

Discussion

Systematic position of *Mylia*

Mylia encompasses four species with *M. taylorii* as the generitype. In early morphological studies, it was placed in Harpanthaceae (now in Geocalycaceae), Plagiochilaceae, and, more widely accepted, in the subfamily Mylioideae of Jungermanniaceae (*see* Engel & Braggins 2005). Characters of *Mylia*, i.e. the *plagiochiloid*, laterally compressed perianth, the 1-seriate antheridal stalk and the patterns of epidermal cell development of the capsule, however, contradict the circumscription of Jungermanniaceae. Recently, Engel and Braggins (2005) observed a unique ultrastructure of cell walls in *Mylia* and they placed the genus in an independent family, Myliaceae. Engel and Braggins (2005) proposed a new suborder for the leafy liverworts, Myliineae, to include Myliaceae and Trabeculaceae, a family that has similar cell wall features with Myliaceae.

Mylia taylorii, accepted as a member of Jungermanniaceae, has also been included in a few DNA sequence based studies (Schill *et al*. 2004, Yatsentyuk *et al*. 2004, Hentschel *et al*. 2006). In both Schill *et al*. (2004) based on combined *trn*L and *atp*B-*rbc*L regions and Yatsentyuk *et al*. (2004) based on *trn*L-F, *Mylia* is resolved as sister of the Cephaloziineae, however, it is sister to species of Jungermanniieae in Hentschel *et al*. (2006). In the present study, although results compiled from two different analytical methods resolve *Mylia* alternatively, i.e., sister to a group clustered both Cephaloziineae and Jungermanniineae in NONA analysis, and paraphyletic relationship to either Cephaloziineae or Jungermanniineae in MrBayes analysis, neither of them supports a close relationship between *Mylia* and Jungermanniineae. Therefore, this study corroborates the study of Engel and Braggins (2005) in removing *Mylia* from Jungermanniaceae and establishing its independent status at family level. The placement of *Mylia* in the parsimony analysis of this study suggests also that *Mylia* may well be recognized as Myliineae, in correspondence with Perssoniellineae, Cephalozinnineae, Jungermanniineae and Lophocoleineae within the order Jungermanniales (*see* He-Nygrén *et al*. 2006).

Perigynium origin

The analyses based on both parsimony and Bayesian inference produced a consistent phylogeny for the group put in question. The monophyly of the ingroup taxa in the present study is compatible with the circumscription of the Jungermanniineae by He-Nygrén *et al*. (2006). In addition, the alignment of *Harpanthus* of Geocalycaceae with other ingroup taxa extends the boundary of the Jungermanniineae. The positions of various families are different from those given in He-Nygrén *et al*. (2006), but the topologies of the present analysis bear better node support in major clades, most probably due to a higher density of taxon selection.

The monophyly of the Jungermanniineae demonstrates close genetic affinities between the ingroup families. Morphologically, however, these families vary considerably from isophyllous, subisophyllous to anisophyllous growth forms, lateral-intercalary to ventral branchings, entire to lobed lateral leaves, and, presence or absence of underleaves and perianths. The variability of these characters, which are generally considered of taxonomic importance, has led taxonomists to arrange the families to different taxonomic categories. Consequently, the perigynium, occurring in these "unrelated" families Acrobolbaceae, Balantiopsidaceae, Calypogeiaceae, Geocalycaeceae (subfam. Geocalycoideae), Gymnomitriaceae, Gyrothyraceae and Jungermanniaceae, has been considered to have evolved independently for several times. The present study (Figs. 1 and 2), however, suggests that the presence of perigynium is a synapomorphy of the Jungermanniineae because it characterizes all three major clades and most of the subclades of the ingroup. Reversals of this character have likely occurred in Antheliaceae, Delavayellaceae, and *Jungermannia leiantha* and *J. exsertifolia* of Jungermanniaceae, where the perigynium is lacking. This result suggests

a single origin of perigynium in the leafy liverworts and that the occurrence of perigynium is an evolutionary innovation as suggested by its synapomorphic nature. Considering the systematic position of Jungermanniineae in the overall analysis of liverworts (*see* He-Nygrén *et al*. 2006), perigynium represents the most recent derived character that protects the developing sporophyte in leafy liverworts, compared with more ancient shoot calyptra and perianth.

Familial relationships in the Jungermanniineae

Acrobolbaceae, with six mostly southern hemisphere genera, are in both analyses resolved as the sister group to the rest of the ingroup species. Vegetatively, it resembles species of the Plagiochilaceae, but notably differs from the latter in presence of perigynium and lack of a perianth. Acrobolbaceae has been placed after Plagiochilaceae in the Geocalycineae (Schuster 1972, 1980, 1984) and assumed as probably derived from a common ancestor with the Plagiochilaceae (*see* Schuster 1980). This assumption remained unchallenged until very recently He-Nygrén *et al*. (2006) placed Acrobolbaceae to the Jungermanniineae. As shown in He-Nygrén *et al*. (2006), the similarities between the two families, for example, in production of microphyllous stolons, leaf modification, and formation of tapered and spicate androecia are analogous. Placing Acrobolbaceae in the Jungermanniineae is further supported by the present study. In Acrobolbaceae, the gametangia are terminal on leading, leafy axes, pendent and rhizoidous perigynium occurring at a right angle to the shoot apex, as in *Acrobolbus* and *Tylimanthus*. However, when shoots are tightly prostrate, the perigynium usually penetrates the substrate becoming subterranean, as in *Goebelobryum*, *Lethocolea*, and *Marpsupidium*. In this case, the perigynium is usually called a marsupium.

Trichotemnomaceae and Balantiopsidaceae form a well-supported sister group, as resolved in He-Nygrén *et al*. (2006). Schuster (1972) established the suborder Balantiopsidineae, including the families Balantiopsidaceae and Gyrothyraceae. The unifying characters of the families are the presence of perigynium, the bifid underleaves, and the spirally twisted capsule valves. As stated by Schuster, the chief reason for establishing the suborder lies in his belief that it may represent one of the major evolutionary lines of the leafy liverworts, because characters that were assumed for primitive liverworts at the time, such as triradial and isophyllous organization occur in Balantiopsidaceae. For this reason, some other characters of this suborder, such as the presence of spiral capsule valves, has also been assumed to have evolved very early in leafy liverworts (*see* Schuster 1972).

A close affinity between Balantiopsidaceae and Gyrothyraceae as suggested by Schuster is not supported by the present analysis; the Gyrothyraceae is shown rather to be close to families of the *Jungermannia* clade (Figs. 1 and 2) and it forms sister group with *Harpanthus* in the NONA analysis. Morphologically, the North American endemic *Gyrothyra* is a *Nardia*-like plant. Buch *et al*. (1937) placed *Gyrothyra* in Jungermanniaceae, and so did Müller (1951–58). Frye and Clark (1937–47) placed *Gyrothyra* in Ptilidaceae near *Isotachis* based on similar cylindrical capsule and spiral valves. Schuster (1955) erected the family Gyrothyraceae and placed it between Jungermanniaceae and Southbyaceae (now Arnelliaceae). Crandall-Stotler (1976) argued the placement of Gyrothyraceae in Balantiopsidineae, based on anatomical and developmental study of the sporophyte of *Gyrothyra*, and concluded that the Gyrothyraceae is more closely related to the Geocalycineae than to the Balantiopsidineae. The present parsimony analysis also suggests the close relationship between *Gyrothyra* and *Harpanthus*. However, the long branch of *Gyrothyra* and the unresolved relationship of the two genera within the Jungermanniineae based on Bayesian inference (Fig. 2) imply a genetic isolation of Gyrothyraceae from other families. Better resolution within the suborder may be achieved by adding more relevant taxa to a future study. The position of *Harpanthus* resolved in the present study together with the studies of He-Nygrén *et al*. (2004, 2006) suggest that the current family Geocalycaceae is paraphyletic, and its circumscription should be restricted only to the subfamily Geocalycoideae, which bears a perigynium. The characters,

which were used to ally Gyrothyraceae and the Geocalycineae, such as the large, haustorial sporophyte foot and generalized seta (*see* Crandall-Stotler 1976), have been demonstrated to be plesiomorphic in liverworts (He-Nygrén *et al*. 2004, 2006).

The present study also shows that the spiral dehiscence is likely a synapomorphy for the clade which comprises all the families of the Jungermanniineae except Acrobolbaceae, because this feature also presents in Calypogeiaceae in addition to its occurrence in *Gyrothyra* and Balantiopsidaceae. Outside the Jungermanniineae, spiral dehiscence also occurs in derived species of *Plagiochila* of the Lophocoleineae *sensu* He-Nygrén *et al*. (*see* Heinrichs 2002) and *Radula* of the Porellales (*see* Renner & Braggins 2005). This suggests multiple origins of this character in leafy liverworts. The evolution of spiral dehiscence after the split of Acrobolbaceae and rest of the species of the Jungermanniineae and in other derived species indicates that it is a derived trait in the leafy liverworts, thus negating the hypothesis that it was established at a very early point in evolution of leafy liverworts (Schuster 1972).

The long branches are also present in *Anthelia* and *Calypogeia* (Fig. 2), which signifies that these two groups have differentiated largely from their common ancestor and developed into the lineages of their own. Morphologically, the monogeneric family Antheliaceae has been considered as a highly isolated family with uncertain affinity, thus it has been treated as an autonomous suborder Antheliineae (Schuster 1972, Crandall-Stotler & Stotler 2000). A series of characters of *Anthelia*, for example, the transverse, suberect to erect, deeply bilobed leaves and underleaves, identical in sizes, lack of oil bodies, presence of coelocaule, and spherical capsule distance its connection with other families of leafy liverworts. A perigynium is absent in *Anthelia*, and its sporophyte is protected by a distinct perianth and also a coelocaule, an extreme form of the shoot calyptra. The feature may be related to producing maximal protection for the developing sporophyte in the extreme arctic and alpine environments where *Anthelia* grows. Within the Jungermanniineae, coelocaule also occurs in species of Gymnomitriaceae, which mostly grow in extremely exposed and windswept sites (Schuster 1996). Otherwise, the sporophytes of majority of the species of the family are protected by both a perigynium and a perianth, or solely by a perigynium.

The family Calypogeiaceae until recently (*see* He-Nygrén *et al*. 2006) was placed in the Lepidoziineae (Schuster 1966, 1972, Schljakov 1972, Crandall-Stotler & Stotler 2000). Although there are many morphological characters in Calypogeiaceae differing from the rest of the Lepidoziineae, the distinguishing features of the suborder have been emphasized on the restriction of the sexual organs to abbreviated branches, the general development of microphyllous stolons or flagella, and the "two-phase" development of the epidermal cells of the capsule (*see* Schuster 1969, 1972, 2000). The overall phylogenetic studies of liverworts (He-Nygrén *et al*. 2004, 2006), however, have revealed that these characters are homoplasious as they also occur in other groups of the leafy liverworts, and the Lepidoziineae are paraphyletic, with Calypogeiaceae grouped within the Jungermanniineae and the rest within the Lophocoleineae *sensu* He-Nygrén *et al*. (2006). The present study confirms the position of Calypogeiaceae resolved in He-Nygrén *et al*. (2006), and further points out the evolution of spiral dehiscence within the Jungermanniineae.

Jungermanniaceae are resolved as paraphyletic in the present analysis, as *Jungermannia leiantha* is the sister of *Delavayella serrata* of Delavayellaceae, and *Jungermannia exsertifolia* and *J. crenuliformis* form a sister group to *Nardia* species, a group together with species of *Gymnomitron* of Gymnomitriaceae further constituting a monophyletic group. The family Jungermanniaceae is one of the largest Holarctic families of liverworts with approximately nine genera and 150 species (Váňa 1996), the circumscription of the family, however, varies considerably according to different authors (e.g. Schuster 1970, 1984, Váňa 1996, Crandall-Stotler & Stotler 2000, Grolle & Long 2000), and consequently the number of the species varies. Schuster (1984) defined Jungermanniaceae as a broad taxonomic entity including also Lophoziaceae, Myliaceae and Mesoptychiaceae. However, most European authors treat Jungermanniaceae and Lophoziaceae as separate families (e.g. Grolle

1983, Váňa 1996, Grolle & Long 2000), which is supported by recent studies based on molecular data (He-Nygrén *et al*. 2004, 2006). The present study demonstrates further that the taxonomic circumscription of Jungermanniaceae should be re-examined because it is not a monophyletic group and because of the newly suggested affinities within the Jungermanniineae from the present study.

The relationships of Delavayellaceae were addressed recently by Schuster (1999) on the basis of morphological observations. He concluded that there is no clear evidence that would derive Delavayellaceae from a common ancestry with Scapaniaceae and Lophoziaceae, as previously assumed (*see* Schuster 1961), but he did not know where to place the family. The present analysis indicating a close relationship between *Delavayella serrata* and *Jungermannia leiantha* suggests that the Delavayellaceae may have evolved from a jungermannioid ancestor.

Based on the present data, the Jungermanniineae include the highest numbers of families within a suborder in leafy liverworts (*see* also He-Nygrén *et al*. 2006). However, characters other than the perigynium should also be examined in order to gain better understanding of character evolution within the suborder.

Gametophytic tissue evolved protection of the developing sporophyte in leafy liverworts

Maturation of the sporophyte in liverworts takes place entirely within the confines of the gametophytic tissues, thus the protection provided by the gametophyte for the developing sporophyte can be essential to reproductive success. Gametophytic tissue evolved protection of the developing sporophyte in leafy liverworts can be inferred in three ways: the sporophytes are protected by shoot calyptra; by perianth; or by perigynium. Shoot calyptra is usually described as a multistratose or distally thin protective covering derived from the archegonial venter and peripheral axial or thallus tissue, so that the cell proliferation occurs in a ring-like zone immediately peripheral to the fertilized archegonium. Shoot calyptra is usually fleshy, rigid and forming a solitary cover. It occurs throughout major liverwort lineages, and has been suggested as a synapomorphy for liverworts (*see* He-Nygrén *et al*. 2006). In leafy liverworts shoot calyptra is included in or exserts the perianth and it can be massive and act as a sole protective device. Unfertilized archegonia are usually observed on the upper surface of a shoot calyptra. Perianth is derived from leaf tissue and formed by fusion of a row of leaves that are nearest to and surround the archegonia and it is usually tubular and unistratose. It is recognized as a synapomorphy for the leafy liverworts. Perigynium is a fleshy, multistratose tube-like sheath shielding the archegonia and developing sporophyte, formed by the proliferation of axial tissue under both perianth rudiments and bract rudiments. Inside the perigynium, clavate papilliform slime-producing cells usually fill up the space between the perigynium wall and the sporophyte, as in *Calypogeia*. Therefore, structurally, perigynium may be more effective in protecting the developing embryo from desiccation than a perianth. In some genera, for example in *Arnellia*, a pendent perigynium is accompanied by a rudimentary perianth and a shoot calyptra, which seems that the development of one type of protective device does not necessarily preclude formation of other devices.

Formation of protective devices derived from the gametophytic tissue and evolutionary trends and transformations between these devices have been speculated (Schuster 1966, 1984, 1996). Schuster presumed that both genetic and environmental factors were involved in the post-fertilization changes, but presented no evidence. The phylogenetic study of He-Nygrén *et al*. (2006) has provided evolutionary sequences of shoot calyptra and perianth, and the present analysis further clarifies the evolution of perigynium, indicating that perigynium is the most recent derived structure in sporophyte protection. The present study implies that the genetic control in forming each protective structure is probably different so that there is no switch from one structure to another. Thus, assuming that a well developed perigynium is responsible for reduction or loss of a perianth (*see* Schuster 1984) in some species is not supported by this study. The extant leafy liverworts with a single protective structure

or with various combinations of the protective systems may indicate their adaptive strategies that enable the group to cope with the constraints of terrestrial existence, especially desiccation. This leads to the plausible interpretation that a perigynium is not an ultimate protective structure of the developing sporophyte in liverworts, but rather its evolvement manifests a historical process accompanied by ecological and physiological demands.

The mechanism of the development of perigynium is poorly known. It has been presumed that the cells of the shoot tip are still meristematic at the time of maturation of archegonia or the young sporophyte may secrete hormonal substance which stimulates cell division and elongation (Schuster 1966). However, liverwort sporophytes never develop an apical meristem capable of producing additional organs (Cooke *et al*. 2003). Only seta elongation has been observed to be regulated by endogenous auxin (Schnepf *et al*. 1979, Thomas 1980, Poli *et al*. 2003). Gametophytes of liverworts exhibit various auxin responses (Cooke *et al*. 2002), but auxin regulation of developmental processes in liverworts has not been characterized and understood. Further research effort devoted on the abovementioned subject would provide new information for considering the evolutionary origins of relevant morphological structures, as well as for interpreting phylogenetic relationships of the liverworts.

Ecological implications to the formation of perigynium

No direct evidence derived from ecological perspective has been observed as related to the formation of perigynium. One would assume that the ecological and environmental stimuli could be difficult to detect because they could be derived over a historical process and interacted with genetic forces. However, the morphology and the substrates of the Jungermanniineae, and, if known, the seasonality or periodicity of the events related to reproduction and growth in the life cycle, may provide hints on the formation of perigynium. Within the Jungermanniineae, synapomorphies other than the occurrence of perigynium are not found, but most of the species are strongly anisophyllous, prostrating on the substrates, and have thin cell walls. They are mostly terrestrial, typically on mineral substrates, mineral soil, and shaded or exposed rocks. The thin-walled nature would require the species of the Jungermanniineae to have a moderately constant supply of water. The prostrate growth on the ground may favor the species to reach sufficient water when there is rainfall, especially in the northern hemisphere where humidity is much lower than in the tropics. The thick-layered perigynium can endure better from water loss and act as a container for storing water.

Schuster (1966) stated that protective devices are most fully elaborated in the leafy liverworts which more often inhabit sites where periodic desiccation occurs. However, information on reproductive events of the Jungermanniineae, for example, the duration of the development of the gametangia and the sporophyte, is lacking. Recently published, the excellent phenological study on the boreal liverwort *Lophozia silvicola* by Laaka-Lindberg (2005) provided valuable knowledge on the reproduction, which may to some extent be comparable to other leafy liverworts. Laaka-Lindberg observed that from fertilization to spore dehiscence it would take over two growing seasons; and that maturation of archegonia is significantly correlated with rainfall and humidity. This result implies that the protective structures contributed from the gametophytes must have been essential in the reproductive events accompanying the whole process of the sporophyte maturation, and that they have played an important role in preventing the sporophytes from desiccation. For the species of the Jungermanniineae, largely Holarctic, with a distinct seasonality, this role would be more significant.

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