

# **Evolutionary relationships of liverworts with a special focus on the order Porellales and the family Lejeuneaceae**

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Academic dissertation

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*Cover photo*

Open sporophyte capsule of *Jubula pennsylvanica*.

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To Per, Anja, and Anton.

This thesis is based on the following original articles referred to in the text by their Roman numerals:

**I** He-Nygrén, X., I. Ahonen, A. Juslén, D. Glenny, and S. Piippo. 2004. Phylogeny of liverworts — beyond a leaf and a thallus. Pp. 87-118 in B. Goffinet, V. Hollowell & R. Magill (editors), *Molecular Systematics of Bryophytes. Monographs in Systematic Botany from the Missouri Botanical Garden, Volume 98.*  
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**III** Ahonen, I., J. Muona, and S. Piippo. 2003. Inferring the Phylogeny of the Lejeuneaceae (Jungermanniopsida): A First Appraisal of Molecular Data. *The Bryologist* 106: 297-308.  
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**IV** Ahonen, I. 2005. The taxonomical position of the genus *Nipponolejeunea* (Steph.) Hattori. *Journal of the Hattori Botanical Laboratory* 99: 319-342 (in press).  
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**V** Ahonen, I., A. Sass-Gyarmati, and T. Pócs. 2005. Molecular, morphological, and taxonomic evaluation of the *Ptychanthus striatus* - complex. *Acta Botanica Hungarica* 47 (in press).  
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**VI** Ahonen, I. 2004. Indels and direct optimization. Manuscript.  
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## Contributions

The following table shows the main contributions of authors to studies I-VI.

	<b>I</b>	<b>II</b>	<b>III</b>	<b>IV</b>	<b>V</b>	<b>VI</b>
Original idea	XH, IA	IA	IA, SP	IA	TP	IA
Molecular data	XH, IA, AJ	IA	IA	IA	IA	IA
Morphology	-	-	-	IA	AS, TP	-
<b>Analyses:</b>						
Direct optimization (POY)	IA, XH	IA	JM	IA	IA	IA
Nona	IA	-	-	IA	IA	IA
Paup	-	-	-	-		IA
MrBayes	-	-	-	IA	IA	-
Manuscript preparation	XH, IA, AJ, DG	IA	IA, JM	IA	IA, TP, AS	IA

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## Introduction

### Origin of liverworts

Liverworts are one of three groups of bryophytes, the other two being mosses and hornworts. While the evolutionary relationships of these bryophytes remain unclear, they are commonly considered to be among the oldest land plant lineages. The first land plant fossils, spores, dating back almost 500 million years, resemble spores of present-day liverworts (Gray 1985, 1991, Kenrick and Crane 1997, Wellman and Gray 2000, Wellman *et al.* 2003). Liverworts lack many features found in other land plants such as true stomata, abscisic acid, the ability to conjugate auxin to amide and ester compounds, RNA editing in chloroplasts, certain group II introns in the mitochondrial genome, and one of the gene *ycf3* introns in the chloroplast genome (Mishler and Churchill 1984, Stzein *et al.* 1995, Malek *et al.* 1996, Crandall-Stotler and Stotler 2000, Renzaglia *et al.* 2000, Pruchner *et al.* 2001, Kugita *et al.* 2003, Dombrowska and Qiu 2004, Kelch *et al.* 2004, Groth-Malonek and Knoop 2005, Groth-Malonek *et al.* 2005). Together with results of some phylogenetic studies (Mishler and Churchill 1984, Bremer *et al.* 1987, Waters *et al.* 1992, Lewis *et al.* 1997, Kugita *et al.* 2003, Kelch *et al.* 2004), this suggests that liverworts were the first group of plants to diverge after land colonization. This scenario appears also in Goremykin and Hellwig (2005), in their analysis of amino-acid sequences of 57 protein-coding genes common to 17 chloroplast genomes of land plants, although they discard it as being due to compositional bias in the liverwort

sequences. However, many studies have reached different conclusions, with hornworts being the sister-group to all the other land plants (Mishler *et al.* 1994, Hedderson *et al.* 1996, Malek *et al.* 1996, Beckert *et al.* 1999, Nishiyama and Kato 1999, Nickrent *et al.* 2000, Renzaglia *et al.* 2000, He-Nyngren *et al.* 2005), or at least to liverworts and mosses with bryophytes together forming a monophyletic sister group to tracheophytes (Garbary *et al.* 1993, Nishiyama *et al.* 2004, Goremykin and Hellwig 2005). The liverworts were even considered poly- or paraphyletic (Bopp and Capesius 1996, Kawai and Otsuka 2004).

What was the ancestral liverwort like? According to the view advocated strongly by Schuster (1966, 1979, 1984, 2000, 2002), the ancestral liverworts were erect, leafless, radial, and branched, an idea adopted originally from Wettstein (1903-1908). However, Grolle (1969) claimed there are no truly radial liverworts besides *Takakia*, which was at the time still considered to be a liverwort but has subsequently been shown to be a moss (Smith and Davison 1993, Gradstein 2005). Grolle argues that the dorsiventral form is more primitive, an idea originating with Leitgeb (1874-1881). In some later phylogenetic studies, thalloid liverworts have indeed been placed more basally than leafy liverworts (Malek *et al.* 1996, Samigullin *et al.* 1998, Beckert *et al.* 1999, Crandall-Stotler and Stotler 2000, Davis 2004), and Davis (2004) presents the hypothesis that the first liverworts may have been simple thalloids (see also Schofield 1985). However, leafy Calobryales *Cambo.* ex Hamlin and Treubiales *Schljak.* also appear in a sister-position to the rest of the liverworts in some phylogenies, giving

support to Schuster's view (Garbary *et al.* 1993, Forrest and Crandall-Stotler 2004, Crandall-Stotler *et al.* 2005, Heinrichs *et al.* 2005). According to Crandall-Stotler *et al.* (2005), on the other hand, prostrate bilateral leafy growth form may be ancestral. Thus, only after establishing the evolutionary order of the major bryophyte groups will we be able to state with certainty the growth form of the first liverwort.

The erect or thallose growth form is probably related to the form and division of the apical cell (Renzaglia *et al.* 2000). Charales, the algae regarded as the sister group of land plants (Karol *et al.* 2001), are filamentous and branched. Their apical cell produces daughter cells only at its lower surface (Graham *et al.* 2000, <http://www.greenbac.org/tree.html>). The mosses, which in some analyses have appeared as a sister group of liverworts (Garbary *et al.* 1993, Mishler *et al.* 1994, Malek *et al.* 1996, Nishiyama and Kato 1999, Renzaglia *et al.* 2000), have mostly a tetrahedral apical cell which divides from three cutting faces and produces an erect gametophyte. Thalloid hornworts have a four-sided apical cell, resembling in this respect many thalloid liverworts. Changes in apical cell and body plan may, in turn, be regulated by auxin, possibly via MADS box genes (Cooke *et al.* 2003). MADS box is a domain of conserved sequences, which are important in developmental processes.

The sporophytes of bryophytes are less variable in their form than their gametophytes and are always composed of a foot penetrating the gametophyte tissue, a capsule containing the spores, and often an unbranched stalk between them. Although all bryophyte groups have developed their own special features, e.g. the liverwort

sporophyte being more ephemeral and simpler in structure than the moss sporophyte, sporophytes do not seem to serve as a basis for deducing large-scale evolutionary trends, unlike the gametophytes. In addition, the features of the sporophyte apical and other meristematic cells are less well known than those of gametophyte apical cells. Renzaglia *et al.* (2000) postulated that the original bryophyte sporophyte was a small mass of cells with a fertile internal region, with all bryophyte groups independently developing their own special sporophyte features. However, no extant liverwort group has a sporophyte resembling this allegedly original sporophyte form. Actually, in liverworts, sporophytes with a massive foot, seta (the stalk), and many-stratose capsule wall have traditionally been considered primitive (Schuster 1966, 1981).

### **Liverwort synapomorphies**

Possibly the best-known liverwort synapomorphy, lacking in both "green algae" and the other land plants, is the presence of oil-bodies. Oil-bodies are peculiar membrane-bound cell organelles that consist of ethereal terpenoid oils suspended in a carbohydrate- or protein-rich matrix (Crandall-Stotler and Stotler 2000). They are derived from endoplasmic reticulum in meristematic cells (Duckett and Ligrone 1995). The function of oil-bodies is not known, but they may protect the plant from herbivores or ultraviolet radiation. However, only about 90% of the liverworts have oil-bodies. The oil-bodies may thus have disappeared many times during the course of evolution, and they are not present



in e.g. Blasiaceae H. Klinggr., Cephaloziaceae Mig., and Lophoziaceae Cavers. Obviously, though, oil-bodies are likely to serve some important function since they have been preserved in most liverworts.

The liverworts are the only land plant group that mainly utilizes the growth hormone lunularic acid instead of abscisic acid (ABA), which is found in other land plant groups and in those green algae that are supposed to be closest to them, namely Coleochaetales and Charales (Karol *et al.* 2001, Yoshikawa *et al.* 2002). However, there is some evidence for ABA even in liverworts (Hartung *et al.* 1987). In higher plants, abscisic acid plays a role in stomatal closure, leaf abscission, and seed dormancy. The role of lunularic acid in liverworts may be similar, growth inhibition and dormancy induction (Pryce and Kent 1971). According to Yoshikawa *et al.* (2002) lunularic acid could be an older molecule than ABA, because the latter needs molecular oxygen for its biosynthesis.

The liverwort sporophyte generation develops completely inside the gametophyte, and only when the spores are mature does the stalk bearing the capsule, i.e. the seta, elongate lifting the capsule above the gametophyte. In the other land plants, the sporophyte either extends during the early organological stage above the gametophyte tissue, as in mosses, or is completely independent, as in vascular plants. In hornworts, the reproductive tissues are internalized in mucilage-filled spaces inside the gametophyte (Renzaglia *et al.* 2000). The confinement of the sporophyte inside the gametophyte may have been an advantageous adaptation to the harsh environments of the late Silurian and Devonian.

Another putative synapomorphy is the elaters, which are spring-like structures in liverwort capsules that enhance spore release, even though other land plants, like hornworts and pteridophytes, have elaters related to spore dispersal. Only the elaters of hornworts resemble those of liverworts (Crandall-Stotler and Stotler 2000). The homology of liverwort and hornwort elaters is uncertain, although both Kenrick and Crane (1997) and Crandall-Stotler and Stotler (2000) treat them as homologous. Both arise by the division of an archesporic cell into two sister cells, one of which gives rise to a spore mother cell, the other to the elaters. However, the plane of this division is different in liverworts and hornworts, and the liverwort elaters are generally unicellular, and the hornwort elaters multicellular (Crandall-Stotler and Stotler 2000).

### Major groups of liverworts

The first liverwort classification is found already in Linné's *Species Plantarum* (1753): Linné placed all leafy liverworts known at the time in the genus *Jungermannia*. In addition, he distinguished simple thalloid liverworts and complex thalloid liverworts. Although the internal taxonomy of these three groups has been greatly refined by later students (e.g. de Jussieu 1789, Endlicher 1841, Gottsche *et al.* 1844-7, Schiffner 1893, 1895, Evans 1939, Schuster 1958, 1966, 1984, 1992, Schljakov 1972, Crandall-Stotler and Stotler 2000, Forrest and Crandall-Stotler 2004, 2005, Crandall-Stotler *et al.* 2005, Heinrichs *et al.* 2005, He-Nyngren *et al.* 2005, see also the Historical outlines in I), the simple

thalloids, complex thalloids, and leafy liverworts are still today recognized in the liverwort systematics. In the recent comprehensive classification by Crandall-Stotler and Stotler (2000), complex thalloid liverworts are ranked as class Marchantiopsida Stotl. and Stotl.-Crand., while simple thalloids and leafy liverworts are considered as subclasses Metzgeriidae Barthol. Began and Jungermanniidae Engl. emend. Stotler and Stotl.-Crand., respectively, in the class Jungermanniopsida Stotler and Stotl.-Crandall. The liverworts as a whole are ranked as phylum Marchantiophyta.

The thallus of the complex thalloids is organized into different cell layers with different functions such as air chambers, photosynthetic tissue, rhizoids, and scales. The complex thalloids have been divided into several groups, e.g. Sphaerocarpales Cavers, Monocleales R. M. Schust., and Marchantiales Limpr., on the basis of the thallus and sexual organ structure. Simple thalloids, in contrast, have a thallus differentiated at most into a simple midrib and leaf-like wings or scales (e.g. *Pellia* Raddi, *Fossombronia*, Raddi). The leafy liverworts, comprising almost 85% of all liverworts, have a stem and leaves, which are in most cases organized into three rows.

In addition to these traditional groups, Schuster (1958, 1966, 1979, 1984) placed the order Calobryales containing radial, leafy Haplomitrium and Takakia (later shown to be a moss, Smith and Davidson 1993) first in his class Hepaticae, and Stotler and Crandall-Stotler (1977) established the order Haplomitriales Bold ex R. Stotl. et B. Stotl. with the same contents and taxonomic position. Schljakov (1972) established the order Treubiales, and Stech and Frey (2001)

the class Blasiopsida Stech and W. Frey. Both Haplomitriales and Treubiales are leafy but with many simple thalloid features. Blasiopsida is simple thalloid but distinguished from other simple thalloids by two rows of ventral scales and ventral auricles filled with cyanobacterial colonies.

In Schuster's classification (1972), leafy liverworts came after Calobryales, followed by Treubiales and simple thalloids, with complex thalloids being the last and most derived group. Crandall-Stotler and Stotler (2000) placed the complex thalloids first in their classification, followed by simple thalloids and leafy liverworts. They merged Calobryales, Treubiales, and Blasiopsida with simple thalloids in Metzgeriidae. Their classification was the first one based on phylogenetic analysis, they used 61 morphological characters and 34 liverwort species.

In the past decade, with the development of phylogenetic methods as well as sequencing techniques, a boom of land plant and bryophyte phylogenies based on morphology or DNA sequence level information has appeared (e.g. Mishler and Churchill 1984, Garbary *et al.* 1993, Mishler *et al.* 1994, Malek *et al.* 1996, Hedderson *et al.* 1996, Lewis *et al.* 1997, Samigullin *et al.* 1998, Beckert *et al.* 1999, Nishiyama and Kato 1999, Crandall-Stotler and Stotler 2000, Renzaglia *et al.* 2000, Samigullin *et al.* 2002, Davis 2004, Forrest and Crandall-Stotler 2004, 2005, Stech and Frey 2004, Crandall-Stotler *et al.* 2005, Heinrichs *et al.* 2005, He-Nyngren *et al.* 2005, Study I). Especially in the latest analyses including a large number of liverworts, a pattern begins to appear. The complex thalloids and the rest of the liverworts are separated as distinct clades, and many of the phylogenies suggest

a close relationship between the simple thalloids and the leafy liverworts (Lewis *et al.* 1997, Beckert *et al.* 1999, Davis 2004, Forrest and Crandall-Stotler 2004, 2005, Stech and Frey 2004, Heinrichs *et al.* 2005, Crandall-Stotler *et al.* 2005, He-Nyngren *et al.* 2005, Study I). *Treubia* and *Haplomitrium* (from Treubiales and Haplomitriales, respectively) form a sister clade to the remainder of the liverworts in Forrest and Crandall-Stotler (2004) and (2005), in Crandall-Stotler *et al.* (2005), in Heinrichs *et al.* (2005), and in He-Nyngren *et al.* (2005, unpublished).

The internal relationships of leafy liverworts also remain unclear. Schuster's (e.g. 1979) and Crandall-Stotler's and Stotler's (2000) classifications treat many groups in very different ways, e.g. Schuster places Ptilidiineae Schuster among the fairly derived leafy liverworts, while Crandall-Stotler and Stotler place the group in the basal position in their leafy liverwort classification. However, a feature common to both of these classifications is that the isophyllous leafy liverworts are mainly considered primitive. According to Schuster (1966, 1984, 1996a), the original form of leafy liverworts would probably have been isophyllous. In both classifications, the suborder Porellineae Schust. or order Porellales (R. M. Schust.) Schljakov emend. Stotler and Stotl.-Crand. is considered derived, and is regarded by Schuster (1984) as the summit of liverwort evolution. A totally different picture is revealed in Davis (2004), Heinrichs *et al.* (2005), He-Nyngren *et al.* (2005), and Study I. In those studies, leafy liverworts are divided into two sister clades, one containing Porellales, Radulales, and Lepidolaenineae, and the other the rest of the leafy liverworts. Heinrichs *et al.*

(2005) calls these clades Jungermanniales and Porellales. Isophyllous liverworts are scattered, being derived rather than primitive. The results of Forrest and Crandall-Stotler (2004) and (2005) reflect the same evolutionary order, although they include substantially fewer representatives of the leafy liverworts in their studies, none of them from Lepidolaenineae or Radulales.

### Fossil data

Except for the spore fossils of the Ordovician and Silurian Periods, the fossil records do not unambiguously support the view of liverworts as the oldest land plant group. The first unequivocal liverwort plant body fossils are about 370-350 million years old, from the Devonian and Carboniferous Periods, when the vascular plants had already formed forests (Schuster 1966, Krassilov and Schuster 1984, Oostendorp 1987, Kenrick and Crane 1997). These fossils resemble present-day simple thalloids, suggesting that simple thalloids might be the liverwort group that appeared first. The unequivocal complex thalloid and leafy liverwort fossils appear in the fossil record at about the same time in Permian, (Oostendorp 1987) and they become more abundant during the Jurassic Period about 200 Ma BP (Schuster 1966, Krassilov and Schuster 1984, Oostendorp 1987).

Some Cretaceous (about 100 Ma BP) leafy liverworts may have an affinity to the Jungermanniales and *Schistochila* (Krassilov and Schuster 1984). One Mesozoic fossil, *Cheirorhiza* Krassilov, resembles the present day Porellinae R.M Schust. with its complicate-bilobed leaves and smaller ventral leaf lobes (Krassilov and Schuster

1984, Schuster and Janssens 1989).

However, the leafy liverwort fossils become unambiguous in their affinity as recently as in the Eocene 60-50 Ma BP. From about 40 Ma BP, at the turn of the Eocene and Oligocene Epochs, abundant well-preserved fossils have been discovered in Baltic and Dominican amber. Genera that have been recognized with certainty are *Cephalozia* (Dumort.) Dumort., *Radula* Dumort., *Porella* L., *Frullania* Raddi, *Jungermannia* L., *Nipponolejeunea* S. Hatt., *Lejeunea* Lib., *Cheilolejeunea* (Spruce) Schiffn., *Mastigolejeunea* (Spruce) Schiffn., and *Trocholejeunea* Schiffn. (Schuster 1966, Grolle 1981, 1982, 1984a, 1998, Grolle and May 2004). The fossil *Nipponolejeunea europaea* Grolle is even suggested to be conspecific with the extant *N. subalpina* Horik. (Grolle 1981). Equally old fossils have been found in Dominican amber containing, in addition to *Frullania*, *Radula*, and *Bazzania* Gray, many members of Lejeuneaceae (*Archilejeunea* (Spruce) Schiffn., *Blepharolejeunea* S. W. Arnell, *Bryopteris* (Nees) Lindenb., *Ceratolejeunea* (Spruce) Schiffn., *Cyclolejeunea* A. Evans, *Cyrtolejeunea* A. Evans, *Drepanolejeunea* (Spruce) Schiffn., *Lejeunea*, *Leucolejeunea* A. Evans, *Lopholejeunea* (Spruce) Schiffn., *Marchesinia* Gray, *Mastigolejeunea*, *Neurolejeunea* (Spruce) Schiffn., *Prionolejeunea* (Spruce) Schiffn., *Stictolejeunea* (Spruce) Schiffn.) (Grolle 1984b, 1993, Gradstein 1993). It is noteworthy that no fossil record exists of the isophyllous leafy liverworts (Krassilov and Schuster 1984), which Schuster (e.g. 1996a) suggested to be the original form of the leafy liverworts.

## Porellales

Schuster accomplished in 1953 his first comprehensive classification of liverworts with incorporated evolutionary order. In this context, he also established the suborder Porellineae. He placed Porellineae last in his order Jungermanniales H. Klinggr., i.e. leafy liverworts. Originally, Porellineae contained five families: Porellaceae Cavers, (Dumort) K. Müll., Goebeliellaceae Verd., Frullaniaceae Lorch, Radulaceae (Dumort.) K. Müll., and Lejeuneaceae. Schuster (1958) later removed Radulaceae to a suborder of its own, Radulineae R. M. Schust., because the family is morphologically quite different from the other families in Porellineae. Schljakov (1972) elevated Porellineae to the order Porellales (R. M. Schust.) Schljakov, including Radulaceae. Crandall-Stotler and Stotler (2000), in turn, excluded Radulaceae from their Porellales (R. M. Schust.) Schljakov emend. Stotler and Stotl.-Crand, but added the family Bryopteridaceae Stotler. For a more detailed discussion about the taxonomical history of Porellales and the morphological features used in the classifications, see the Introduction of Study II.

Schuster (1980) considered these anisophyllous epiphytes with modified ventral lobes of the leaf and no ventral branching as the culmination of liverwort evolution. However, Porellales are not the only epiphytes among the liverworts. Epiphytes are found also among the genera *Schistochila* Dumort., *Plagiochila* (Dumort.) Dumort., *Nowellia* Mitt., *Metzgeria* Raddi, and *Pleurozia* Dumort., and especially in Lepidolaenineae R. M. Schust. (see below). Nevertheless, Porellales is the largest taxonomic unit in which all genera exhibit

this feature. According to Schuster (1984), epiphytism may be an adaptation to escape the competition on the forest floor, although this seems not fully plausible when looking at present-day tropical forests, which actually have very few bryophytes growing on the forest floor (Sinikka Piippo, pers. comm.). Epiphytism is also generally considered an adaptation of forest plants (not only bryophytes) to gain more light. Some members of Porellales, especially the large subfamily Lejeuneoideae Herzog of the Lejeuneaceae, have even become epiphyllous, growing on angiosperm leaves. These findings led Schuster (1980) to hypothesize that the evolution of Porellales was connected to the radiation of angiosperms. Sure enough, the first Porellales fossils found are 40 Ma old (Schuster 1966, Grolle 1981, 1982, 1984a, b, 1993, 1998, Gradstein 1993). By that time, angiosperms had already flourished, having started their explosive radiation some 50 Ma earlier.

All members of the Porellales have a modified ventral lobe of the leaf known as a lobule. This lobule is, however, morphologically different in every family assigned to the Porellales, and thus far, no studies have been conducted on the ontogeny and possible homology of the lobules. Porellaceae have (mostly) a non-swollen lobule, which is not attached to the stem. Goebeliellaceae have two horn-shaped lobules that are open from their upper end. They are also not attached to the stem. Frullaniaceae have a helmet-shaped lobule, formed as the central cells of the ventral lobe swell. The lobule opens downwards and is attached to the stem and leaf very narrowly. In Lejeuneaceae, the ventral lobe is turned against the dorsal lobe and is attached to the

stem by a long insertion line. The central cells of the lobule also swell in the Lejeuneaceae lobule, but in the opposite direction than in Frullaniaceae, forming a pocket-like structure. In Radulaceae, the lobule resembles that of Lejeuneaceae, but the central cells are often not swollen, and the lobule margin lacks the teeth and undulations found in Lejeuneaceae. However, lobules are also found in other liverworts, e.g. in *Pleurozia* and especially in Lepidolaenineae. The role of the lobule is unclear. It has been claimed to act in water retention (Goebel 1905, Schuster 1984) – epiphytes would doubtless dry easily, and most species that have a lobule are epiphytes. However, the experiments of Blomquist (1929) called into question the efficacy of lobules in water retention. He noticed that the lobules are filled with water only for as long as the plant is covered with water. When the water surrounding the plant evaporates, the capillary force pulls the water out even from the lobules.

The lobules could serve some nutritional purpose instead. Zelinka (1886) was first to propose that the liverworts and *Rotifera*, frequently found in water sacs, could have a symbiotic relationship. M. Puterbaugh and her research group (2003) ([http://www.pitt.edu/~severson/ainh/ecological\\_research.htm](http://www.pitt.edu/~severson/ainh/ecological_research.htm)) have studied rotifers inhabiting lobules of *Frullania eboracensis*, and they too suggest that this relationship may not be simple commensalism. At least the waste products or decaying bodies of rotifers may be a significant source of nitrogen for the liverworts (Thiers 1988). Even nitrogen-fixing bacteria could be confined within the lobules. Indirect evidence for this is found in studies indicating that more nitrogen is fixed on

leaves with covering of epiphyllous liverworts, which often have water-sacs (Edmisten 1970). Merrifield and Inghan (1998) also found that bryophytes provide microhabitats for aquatic invertebrates, many of which are capable of anhydrobiosis. The lobules might thus provide a location for some sort of symbiotic or at least mutualistic relationship between micrometazoans or microorganisms and the plants. It seems possible that this role of lobules in nutrient supply could have developed to compensate for the loss of beneficial fungal associations in epiphytic habitats (Heinrichs *et al.* 2005, Selosse 2005).

### **Relationships of Jubulaceae and Lejeuneaceae**

Jubulaceae Limpr. (Jubulaceae was called Frullaniaceae until Grolle in 1964 conserved the name Jubulaceae) and Lejeuneaceae of Porellales have been considered to be closely related. According to Buch (1936) and Arnell (1956), Frullaniaceae and Lejeuneaceae constituted the suborder Jubulineae Dumort of the order Jungermanniales acrogynae. Müller (1951-1958) placed Lejeuneaceae and Frullaniaceae into Jubuleae Nees of Jungermanniales. Grolle (1964) included both Lejeuneaceae and Frullaniaceae in his conserved Jubulaceae. Crandall-Stotler and Stotler (2000) divided Porellales into the suborders Porellineae with Porellaceae, and Jubulineae (Spruce) Müll. Frib. with Goebeliellaceae, Jubulaceae, Bryopteridaceae, and Lejeuneaceae. However, Goebeliellaceae is clearly distinguished from Jubulaceae and

Lejeuneaceae by its two horn-like leaf lobules, large but unicellular spores with nipple-like papillae, and polymorphic and highly differentiated leaf cells (Schuster 1965). Moreover, Gradstein (1975, 1987, 1994, 2001) merged Bryopteridaceae with the Lejeuneaceae. The division into Porellineae and Jubulineae finds support also in Davis (2004).

In Jubulaceae, the genus *Jubula* Dumort. in particular is considered to be closely related to Lejeuneaceae, and Mizutani (1961) included *Jubula* in Lejeuneaceae. They share, for example, the structure of the seta, with 16 outer and 4 inner cells (Schuster 1980). *Jubula* and *Frullania*, the other large genus of the Jubulaceae, on the other hand, share the structure of the lobule, bilobed underleaves, and the general features shared by all Porellales, including endosporic germination and *Frullania*-type branches. In the phylogenies presented by Wilson *et al.* (2004), Heinrichs *et al.* (2005), He-Nyngren *et al.* (2005) *Jubula* and Lejeuneaceae are sister groups. For a more detailed description of the taxonomical history of Jubulaceae and Lejeuneaceae, see the Introduction of the Study II.

### **Lejeuneaceae**

Lejeuneaceae is the largest liverwort family, with about 1000 species and 90 genera (Gradstein *et al.* 2003). The taxonomy of Lejeuneaceae has been controversial even at subfamily level (Herzog 1957, Mizutani 1961, 1985, Schuster 1963a, Gradstein 1975, 1994, 2001) ever since Casares-Gil (1919) formally recognized the family Lejeuneaceae. However, from the onset of Lejeuneaceae taxonomy, all authors have

distinguished between species with undivided underleaves, species with divided underleaves, and species without underleaves or with a double number of underleaves. The double number of underleaves means that there is an underleaf per dorsal leaf, not per dorsal leaf pair, as in most leafy liverworts. In the early classifications, the underleaf form and number were almost the only criteria used (Spruce 1884, Frye and Clark 1947, Herzog 1957). The two subfamilies accepted today, Ptychanthoideae Mizut. (most members with undivided underleaves) and Lejeuneoideae (most members with divided underleaves), still reflect this distinction, although the classification is now based on manifold gametophytic and sporophytic characters (e.g. Mizutani 1961, Gradstein 2001). However, some changes have taken place; Gradstein (1994) shifted the tribe Brachiolejeuneae Van Slageren & Berendsen, with undivided underleaves, into Lejeuneoideae based on their sporophytic characters. Furthermore, in his classification the subfamily Nipponolejeuneoideae R. M. Schust. & Kachroo, which possesses divided underleaves and transitional characters between *Jubula* (Jubulaceae) and Ptychanthoideae, constituted the tribe Nipponolejeuneae in Ptychanthoideae. The subfamily Cololejeuneoideae Herzog, with a double number of underleaves or no underleaves, was treated as the tribe Cololejeuneae Gradst. in Lejeuneoideae (Gradstein 2001). Gradstein *et al.* (2003) found the name Cololejeuneae invalid and replaced it with Calatholejeuneae R. M. Schust.

In addition to Ptychanthoideae, Lejeuneoideae, and Cololejeuneoideae, Schuster (1963a) distinguished small

subfamilies Tuyamaelloideae R. M. Schust., Metzgeriopsioideae R. M. Schust., and Myriocoleoideae R. M. Schust., as well as the above-mentioned Nipponolejeuneoideae, and Gradstein (1975) established the subfamily Bryopteridoideae (Stotl.) Gradst. These have subsequently all been merged with Lejeuneoideae and Ptychanthoideae (Gradstein 1975, Mizutani 1985). For a more detailed description of the taxonomical history of Lejeuneaceae and the morphological characters used to resolve the relationships, see the section entitled “History of subfamilial classification of Lejeuneaceae” in Study III.

The subfamily Ptychanthoideae has been considered as more primitive than Lejeuneoideae (Mizutani 1961, Schuster 1980, Gradstein 1991, 1992) because the members of Ptychanthoideae are more robust in texture and less specialized in their life strategy, being mostly saxicolous or corticolous. The Lejeuneoideae, in contrast, are more reduced in their morphology and mostly live in ephemeral habitats, e.g. as epiphylls on angiosperm leaves. The Calatholejeuneae are the most reduced in their morphology and are often epiphylls (Schuster 1980).

With the onset of phylogenetic methods attempts have been made to apply these methods to resolve relationships inside Lejeuneaceae. The first phylogenetic analysis was conducted by Gradstein (1994) using 26 morphological characters. Weis (2001) followed and used 36 sporophytic characters, then Gradstein *et al.* (2003) with 31 gametophytic, 18 sporophytic, and one chemical character. Later, a few molecular phylogenies have also been published, my own analyses (Studies II and III) using molecular data from three genomic regions

(*rbcL*, *trnL-trnF* and ITS2), Groth-Malonek *et al.* (2004) which is based on the nuclear ITS1-ITS2 region, and Wilson *et al.* (2004) and Heinrichs *et al.* (2005) based on *rbcL*. However, none of these studies have unequivocally been able to resolve the internal taxonomy of Lejeuneaceae. The morphological characters do not seem to provide adequate resolution to obtain a well-resolved topology (Weis 2001, Gradstein *et al.* 2003), and molecular studies have had insufficient numbers of representatives of the many genera and tribes of Lejeuneaceae (Study III, Groth-Malonek *et al.* 2003). Wilson *et al.* (2004) and Heinrichs *et al.* (2005) have conducted the most comprehensive studies thus far. However, even in these many relationships within Lejeuneaceae remain equivocal.

Lejeuneaceae is a subcosmopolitan family that is mainly concentrated in tropical and warm temperate areas. Both subfamilies are present in both the Old and New World, albeit with somewhat different compositions of genera and species (Gradstein 1987, 1991). Because Eurasia (Laurasia) separated from Gondwana already about 200 Ma BP and South America from Gondwanaland about 100-65 Ma BP, Lejeuneaceae would be extremely old had its members dispersed between Europe and South America by land. Moreover, many pantropical genera and species exist, so even they should have developed before the break-up of the Gondwana, if we do not assume long-distance dispersal. However, the oldest Lejeuneaceae fossils from Baltic (Europe) and Dominican (Caribbean) amber are only about 40 Ma old (Schuster 1966, Grolle 1981, 1982, 1984a, b, 1993, Gradstein 1993, Grolle and May 2004). Molecular studies are needed to confirm whether the transoceanic

species are indeed con-specific or cryptic species, as Gradstein (1987) suggested.

About 90% of the epiphylls in tropical rainforests belong to Lejeuneaceae (Lücking 1995, Zartman 2003). The ecological role of the Lejeuneaceae in rainforest ecosystems is thus likely to be great. Studies on the epiphytes in general and on bryophyte epiphytes have shown that they may have a role in, for example, water interception or nutrient retention (Bates 1992, Holscher *et al.* 2004). Forest fragmentation poses a threat to Lejeuneaceae, as well as other epiphylls (Zartman 2003), and epiphytes in general tend to be sensitive to air pollution (Sim-Sim *et al.* 2000). Also, because epiphyllous bryophytes seem to be more common in the understory than in the high canopy, they are particularly vulnerable to disturbance and forest destruction (Gradstein 1997).

### *Nipponolejeunea*

The genus *Nipponolejeunea* is morphologically intermediate between the genus *Jubula* of Jubulaceae and the subfamily Ptychanthoideae of Lejeuneaceae. It was placed in Lejeuneaceae mainly because of its Lejeuneaceae-type lobule (e.g. Schuster 1963a, Gradstein 1994, 2001). However, the ciliate leaves, divided underleaves, and some sporophytic characters resemble *Jubula*. Mizutani (1961) placed both *Jubula* and *Nipponolejeunea* in the subfamily Jubuloideae Schiffn. emend, regarding this subfamily as a part of Lejeuneaceae. Even novel phylogenetic studies have not been able to unequivocally resolve the position of *Nipponolejeunea*. Morphological characters place it as a sister



to the Lejeuneaceae (Weis 2001, Gradstein *et al.* 2003), while molecular data show a strong relationship with *Jubula* (Studies I - III, Wilson *et al.* 2004, Heinrichs *et al.* 2005). For more detailed description of the taxonomical history of the genus *Nipponolejeunea*, see the Introduction of the study IV.

If *Nipponolejeunea* proves to be closer to *Jubula* than to Lejeuneaceae, the presence of the true Lejeuneaceae-type lobule becomes difficult to explain. The explanation would require that this rather complicated structure had developed twice in the course of evolution, once in the Lejeuneaceae and again in the *Nipponolejeunea*, or that *Jubula* had somehow regained the *Frullania*-type lobule.

*Nipponolejeunea* is today confined to South-East Asian conifer forests, its species growing on bark and rocks, but its distribution was much wider before, as shown by a *Nipponolejeunea* fossil found in Eocene amber from Sweden (Grolle 1981). The cooling of the climate after the Eocene and the disappearance from Europe of the conifer rich highland forests, the type of habitat *Nipponolejeunea* is mostly confined to even today, may explain the reduction of its range.

### ***Ptychanthus* Nees and cryptic speciation**

The African and Asian–Oceanian populations of the widely distributed *Ptychanthus striatus* (Lehm. and Lindenb.) Nees of the family Lejeuneaceae consistently differ in the appearance of their oil-bodies (Kis and Pócs 1997). Thus far, no other morphological differences have been found, suggesting that the taxon *P. striatus*

could contain cryptic or nearly cryptic species. Molecular-level differences have not been studied earlier. For a more detailed description of the taxonomical history of the *P. striatus* taxon and its morphology, see the Introduction of Study V.

Cryptic speciation is a concept that has been adopted along with the use of molecular methods in studies of intraspecific phylogenies. It refers to cases where two species or populations are indistinguishable or nearly indistinguishable morphologically but are clearly distinguished at the nucleotide, amino acid, or protein level. Cases of cryptic speciation have been found in practically all organism groups, including several species of bryophytes (Shaw 2000a, 2001, McDaniel and Shaw 2003, Vanderpoorten *et al.* 2003, Werner and Guerra 2004). Cryptic speciation has been identified in several liverworts; *Conocephalum conicum* (L.) Dum., *Marchantia polymorpha* L., *Reboulia hemisphaerica* (L.) Raddi, *Riccia dictyospora* Howe, and *Aneura pinguis* (L.) Dum., and sibling species *Porella platyphylla* (L.) Pfeiff. and *P. platyphylloidea* Schwein, and haploid *Pellia epiphylla* (L.) Corda and diploid *P. borealis* Lorb. (Shaw 2001). Cryptic bryophyte species may be either allopatric or sympatric (Shaw 2001), having either genetic or geographic reproductive barriers.

### **Lepidolaenineae**

The suborder Lepidolaenineae was established by Schuster (1972). In his classification he placed it just before the suborder Porellineae (Porellales sensu Crandall-Stotler and Stotler 2000) and

postulated that Lepidolaenineae might share an ancestral form with the Porellineae (Schuster 1966, 1980, 1984, 1992). His Lepidolaenineae included families Lepidolaenaceae Nakai (with subfamilies Lepidolaenoideae Nakai, Neotrichocoleoideae (Inoue) R. M. Schust., and Trichocoleopsidoideae R. M. Schust.) and Jubulopsidaceae (Hamlin) Schust. (with only genus *Jubulopsis* R. M. Schust.). Crandall-Stotler and Stotler (2000), however, placed the Lepidolaenineae in a basal position in their classification of leafy liverworts, in the order Lepicoleales Stotler and Stotl.-Crand., with families Lepidolaenaceae, Neotrichocoleaceae Inoue, Trichocoleaceae Nakai, and Jubulopsidaceae (Hamlin) R. M. Schust. These classifications thus differed essentially in the presence of Trichocoleaceae in the classification of Crandall-Stotler and Stotler (note that genus *Trichocoleopsis* from the subfamily Trichocolopsidoideae of Schuster was included in the family Lepidolaenaceae). Trichocoleaceae differed from the rest of the suborder by lacking a lobule and having exosporous germination. Otherwise, all members of Lepidolaenineae have ciliate, many-lobed leaves, a coelocaul (although *Gackstroemia magellanica* (Lamarck) Trevisan also has a reduced perianth), and a stylus that is often rather large. They also have Frullaniaceae-type helmet-shaped lobules, except for the genus *Trichocoleopsis*, which has a Lejeuneaceae-type lobule. Schuster's (1966, 1984, 1992) suggestion that Lepidolaenineae could be, or at least could resemble, the ancestors of Porellineae thus seems acceptable. Especially the peculiar genus *Jubulopsis* with its single species *Jubulopsis novae-zelandiae* (E. A. Hodgs. and S. W. Arnell)

R. M. Schust. has been given as an intermediate between Porellaleneae and Lepidolaenineae (Hodgson 1965, Grolle 1966, Hattori 1972, Schuster 1970, 1996b), and it has been placed either in the family Jubulaceae of Porellineae (Hodgson 1965, Schuster 1970, Hattori 1972) or in Lepidolaenineae (Grolle 1966, Hamlin 1973, Schuster 1996b).

The reason for *Jubulopsis* eventually being assigned to Lepidolaenineae is mainly because it has a coleocaul like the Lepidolaenineae, instead of a perianth (Hamlin 1973). A coleocaul is a hollow outgrowth from a leafy liverwort stem that surrounds the archegonium, although sometimes it is formed also partly of the archegonial tissue. A perianth, by contrast, is formed from the female bracts. However, many characters, such as *Frullania*-type lobules, *Frullania*-type branching, endosporic spore germination with *Frullania*-type sporeling development, presence of a stylus, and epiphytism, unite Lepidolaenineae with Porellales. The relationship between Porellales and Lepidolaenineae is investigated in Study I.

### Phylogenetic analysis

Phylogenetic analyses that operate directly on discrete character data, and not on pairwise distances, can be divided into two major groups; those based on parsimony and those based on the probabilistic approach (maximum likelihood, ML). Algorithms used in parsimony analyses search for the tree topology that requires the minimum number of transformations, e.g. changes in morphological characters, nucleotide substitutions, or amino acids. ML

incorporates the use of specific models of probability of nucleotide transformations in DNA (or even models of change in morphological characters, Lewis 2001). In analyses based on ML, attempts are made to calculate the probability that the model used and the hypothesized history (particular tree topology) would give rise to the observed data set.

Recently Bayesian inference (BI) implemented in the program MrBayes (Huelsenbeck and Ronquist 2001, Huelsenbeck *et al.* 2001) has also become very popular in testing phylogenetic hypotheses. Both BI and ML are based on the use of explicit models of evolution. However, in BI the Markov Chain Monte Carlo (MCMC) technique and Bayesian posterior probabilities are used to guide the tree searching process. BI also takes into account the prior probabilities of the distribution of the parameters in the analysis.

The basic requirements for phylogenetic analysis are that the members of the group of organisms studied are related by descent from a common ancestor, a bifurcating pattern of speciation is present, and transformations have occurred over time so that they have left behind differences between the organisms studied (Felsenstein 1982, Farris 1983, Riggins and Farris 1983, Hawkins *et al.* 1997, Mishler 2000). The characters used in the analysis must also be independent of each other.

With the development of PCR and automated DNA sequencing methods, sequence-level data have become increasingly important as a source of information in phylogenetic analyses. This development has also drawn attention to a novel problem: the alignment of DNA bases. If the functional constraints for a particular

genomic region are not strong enough, the region is subject to insertion and deletion events as well as to rapid changes in base composition. DNA sequences of non-transcribed regions, and even protein-coding genes or genes coding the conservative parts of ribosomal or transfer RNAs, are often of different lengths in different organisms. This can make it difficult to determine homology between bases of sequences from different, even closely related, organisms.

The very first alignment method at the beginning of the molecular taxonomy era was to align the sequences manually, judging by eye the homology of the bases. Very soon, however, alignment programs, such as Clustal (Higgins and Sharp 1988), Malign (Wheeler and Gladstein 1994), and DiAlign (Morgenstern *et al.* 1998) were developed. The alignments that these programs produce are objective and repeatable. However, it is not unusual to improve the alignments obtained from programs manually when the user disagrees with the program. This may violate the requirement of objectivity and repeatability, but on the other hand, using prior knowledge to make alignments more biologically plausible seems justified. For example in protein coding regions one would avoid single or two-base indels that result in frame-reading shifts or stop codons. Similarly, for many Type II introns, non-transcribed spacers etc, there are secondary structural considerations that can aid alignment.

Whether one uses manual alignment or different algorithms of special programs to generate alignment, it is customary to use one alignment as a basis for ensuing phylogenetic analysis, despite this being only one of many possible alignments, the

number of different alignments being enormous especially in variable regions. Exploration of all of these alignments manually to a larger degree is impossible. To overcome this problem, Wheeler (1996) introduced a novel method, direct optimization, and developed the POY program, which incorporates algorithms to evaluate different alignments. In an average POY analysis, several million different character optimizations are tested to determine the most parsimonious one, or the one with the highest likelihood score. In this procedure, searches for the shortest tree and the alignment of sequences producing this tree are done simultaneously. Assumptions of base homologies are dynamically determined and uniquely tailored for each phylogeny (Wheeler 2001).

Although POY analyzes the alignments strictly according to a change matrix using the costs set by user, POY has been suggested not be suitable for alignment of sequences showing length variation (Belshaw and Quicke 2002, Caterino and Vogler 2002, Petersen *et al.* 2004). This would be a serious drawback since the capacity of POY to explore different ways of aligning regions showing even considerable length variation greatly increases the prospects of using such regions in phylogenetic analysis. So far, these highly variable regions have often been omitted from the analyses (e.g. Swofford *et al.* 1996, Naylor and Brown 1997), and this naturally leads to a reduction in the amount of potentially important phylogenetic data available. More discussion about the effect of indels on POY is provided in Study VI.

## Research Aim

The internal relationships of liverworts as well as their origin remains inconclusive, despite many attempts to use both morphological and molecular data to solve these relationships. The aim of this thesis was to provide new insight into the position of liverworts among land plants, the evolution of the main liverwort groups, and especially the position and internal relationships of what has been considered the leafy liverwort crown group, the order Porellales. The largest liverwort family, Lejeuneaceae (Porellales), which alone comprises over 10% of liverwort species (there are ca. 6000 - 8000 species of liverworts according to Crandall-Stotler and Stotler 2000 and ca. 1000 species of Lejeuneaceae according to Gradstein *et al.* 2003), is given special attention.

The studies were conducted in the order III, II, I, IV, V and VI, although in this thesis they are presented from the most general to the most focused for the sake of logical flow. In Study III, the monophyly of the Lejeuneaceae and the relationships of the main subfamilies were tested for the first time with analyses based on sequence-level data. ITS2, *rbcL*, and *trnL-trnF* region were utilized as molecular markers. According to the results obtained, the monophyly of Lejeuneaceae was equivocal due to the position of *Nipponolejeunea pilifera* as a sister to genus *Jubula* from Jubulaceae. The monophyly of the Lejeuneaceae was thus further tested in Study II including more species of Lejeuneaceae as well as representatives from three of the four families of the order Porellales. The monophyly of the order Porellales and the evolutionary relationships of its different

families were also investigated. ITS2, *rbcL*, and *trnL-trnF* were used in this study.

The monophyly of Porellales appeared ambiguous in Study II, e.g. *Ptilidium pulcherrimum* (Web.) Hampe, a taxonomically very distant species according to Crandall-Stotler and Stotler (2000), seemed to be part of Porellales, suggesting that the whole leafy liverwort evolution may be erroneously understood. In Study I, the limits of Porellales were explored by including in the analysis representatives from almost all the orders and suborders of Schuster (1979) and Crandall-Stotler and Stotler (2000). The emphasis was on the leafy liverwort evolution, but the evolutionary relationships of the other major liverwort groups, the complex thalloids and simple thalloids, were also examined. Since there were outgroups from all land plant groups as well as from “green algae”, the position of liverworts among the land plants could be tested. Gene regions *rbcL*, *trnL-trnF*, and *rps4* from the chloroplast were used as molecular markers.

In Study IV, the novel taxonomical position of the genus *Nipponolejeunea*, which emerged in Study III, was further tested with more representatives from the genera *Nipponolejeunea* and *Jubula*. The ITS2, *rbcL*, and *trnL-trnF* regions as well as 26 morphological characters were used to obtain the phylogenetic hypothesis. Study V explored the possibility of cryptic speciation in *Ptychanthus striatus* from Lejeuneaceae. While analysis was based on ITS2 data, morphological features were also examined. Study VI explored the performance and suitability of POY for the kind of molecular data used in the above-mentioned studies, as POY was used to create the phylogenetic hypothesis in all of them. The performance

of POY when it encounters sequences with insertions or deletions was explored, and this was compared with the performance of NONA version 1.8 (A.P. Goloboff) and MrBayes (Huelsenbeck and Ronquist 2001). Manually manipulated *rbcL* sequences from 20 land plants were used as experimental data.

## Material and methods

### DNA sequence data and alignment

Molecular data were used to resolve the taxonomical problems in Studies I-VI. Four gene regions were utilized: nuclear region coding for the internal transcribed spacer II and the partial sequences of 26S and 5,8S rRNA genes surrounding it, here referred to as ITS2, and from the chloroplast Leucine transfer-RNA-Phenylalanine transfer-RNA region (*trnL-trnF*), partial sequence of the large subunit of the ribulose biphosphate carboxylase gene (*rbcL*), and *rps4* coding for the small ribosomal protein 4. These genomic regions are widely used in phylogenetic analysis of plants, and they have also been used in studies dealing with phylogeny of different groups of bryophytes (Lewis *et al.* 1997, Hyvönen *et al.* 1998, 2004, Shaw 2000b, Stech and Frey 2001, Vanderpoorten *et al.* 2001, La Farge *et al.* 2002, Pedersen and Hedenäs 2002, Magombo 2003, McDaniel and Shaw 2003, Pedersen *et al.* 2003, Virtanen 2003, Heinrichs *et al.* 2004, Davis 2004, Crandall-Stotler *et al.* 2005, Forrest and Crandall-Stotler 2005). ITS2 has mostly been used to resolve relationships between closely related taxa or even at the population level (e.g. Mindell and Honeycutt 1989, Zambino and

Szabo 1993, Kim and Jansen 1994, Shaw 2000b, Werner *et al.* 2003, Heinrichs *et al.* 2004), while *rbcL* is conservative and often used in deeper level phylogenies (Nishiyama and Kato. 1999, Newton *et al.* 2000, Nickrent *et al.* 2000, Magombo 2003, Heinrichs *et al.* 2005). The *trnL-trnF* region is the most controversial of the four regions; although it has been found to be informative within limited groups, e.g. angiosperms (Borsch *et al.* 2003) or liverworts (Stech and Frey 2001), a few elements may have had independent origins, as well as independent gains and losses in different groups of land plants (Quandt *et al.* 2004). Even *rps4* is today widely used in plant phylogenetic studies at many levels, for example Lueth and Goffinet (2005) used it to determine the identity of a particular *Splachnum* specimen and Forrest and Crandall-Stotler (2005) to investigate relationships in phylum Marchantiophyta. For more detailed descriptions of these gene regions, see Materials and Methods in Study III and Results in Study II.

*RbcL* and *rps4* are protein-coding genes and are thus fairly easily aligned. ITS2 and *trnL* and *trnF* contain besides conserved parts also highly variable regions. This is a problem when assumptions about base-to-base homologies are made prior to the phylogenetic analysis. In all studies here, direct optimization (Wheeler 1996) as implemented in the program POY (Wheeler *et al.* 2002, 2003) was used to solve this problem, in addition to the programs Clustal (Studies I-V), and Dialign (Study V). Clustal was mostly used to obtain a preliminary alignment before cutting the sequences to shorter fragments for POY analysis. This was done to save CPU time during the analyses.

## Morphology

Morphological characters were used in Studies IV and V to solve taxonomic relationships. In Study IV, morphological data were also included in the phylogenetic analysis. The aim was to use only independent characters that could be coded to discrete states, and to avoid overlapping continuous characters. Whether continuous characters should be used at all in phylogenetic analysis has been a topic much debated (see Cranston and Humpries 1988, Stevens 1991, Thiele 1993, Rae 1998, Wiens 2000, Grant and Kluge 2004). However, some concessions had to be made for continuous characters to also enable utilization of characters that have traditionally been used to separate taxa under investigation. One example of this kind of character is trigone volume. According to Stevens (1991), continuous characters can be used if they can be coded to clearly separate discrete states.

All of the morphological characters were treated as unordered in the analysis to avoid any *ad hoc* hypotheses about their evolution. For a more detailed description of the morphological characters, and the rationale underlying their inclusion or exclusion, see Appendix 1 of Study IV.

## Phylogenetic analyses

To be able to also utilize the variable sequence regions, which are difficult or impossible to align with other methods, phylogenetic analyses were in all studies performed with direct optimization as implemented in the program POY. However, static Clustal and Dialign alignments in

combination with the parsimony program NONA (I, IV - VI) the Bayesian inference program MrBayes (IV, V), and Paup\* in both parsimony and ML mode (VI) were also used for the sake of comparison and to assess POY's actual ability to handle length differences in the data.

## Results and Discussion

### Origin of liverworts

The results of Study I place liverworts as a sister group to all other land plants. This is in accordance with Lewis *et al.* (1997), Kelch *et al.* (2004), and Groth-Malonek *et al.* (2005), but not with e.g. Hedderston *et al.* (1996) Nishiyama and Kato (1999), Nickrent *et al.* (2000), or He-Nygren *et al.* (2005), who present hornworts grouping in a sister position to the rest of land plants, or with Nishiyama *et al.* (2004) and Goremykin and Hellwig (2005), who present the bryophytes as a monophyletic sister group to the tracheophytes. The sampling in Study I was biased towards liverworts, and this may have obscured the overall placement of the liverwort clade. However, the view that the liverworts diversified first, or even that all early land plants had liverwort features, gets support from the first land plant fossils. Spores from the layers over 500 million years old resemble spores of extant liverworts (Gray and Boucott 1971, Gray 1985, 1991, Kendrick and Crane 1997, Wellman *et al.* 2003). This means that liverworts have retained these very old features, at least in their spores. However, surprisingly, these early spores seem to lack elaters, which are today the hallmark of both

liverworts and hornworts (Wellman *et al.* 2003).

It is remarkable though that, despite all evidence pointing to liverworts being one of the oldest land plants groups, the first unequivocal liverwort macrofossils appear only in the Devonian Period (Schuster 1966, Krassilov and Schuster 1984, Oostendorp 1987, Kenrick and Crane 1997, Edwards 2000). The fragile structure of liverworts could be one reason. However, one would think that during the 100 million years that elapsed between the appearance of liverwort-like spores and the first recognizable liverwort macrofossils at least a few exemplars of early liverworts should have been preserved.

### Relationships of the major groups of liverworts

The phylogenetic analysis in Study I places *Blasia pusilla* L. as sister to all other liverworts. *Blasia* L. has been considered a simple thalloid (Renzaglia 1982, Schuster 1992, Wheeler 2000) and has even been placed in its own class, Blasiopsida, between Marchantiopsida and Jungermanniopsida (Stech and Frey 2001). Garbary *et al.* (1993), Pass and Renzaglia (1995), Davis (2004), Forrest and Crandall -Stotler (2004), (2005), Crandall-Stotler *et al.* (2005), Heinrichs *et al.* (2005), and He-Nygren *et al.* (2005) support the position of *Blasia* as a sister to complex thalloids. Indeed, family Blasiaceae H. Klinggr. differs from all other liverworts in having ventral scales composed of numerous cells and ventral auricles filled with colonies of *Nostoc* cyanobacteria (however *Cavicularia densa* Steph., the only other species assigned to the Blasiaceae

besides *Blasia pusilla*, was not included in this thesis). *Blasia* shares, for example, the ventral scales on the thallus, the microanatomy of the spermatozoid, and blepharoplast structure with the complex thalloids (Duckett *et al.* 1982, Pass and Renzaglia 1995). Even if *Blasia* is shown to be sister to complex thalloids (possibly a link between these and the early simple thalloids) it may still resemble the common ancestor of the liverworts, or alternatively may represent the peak of a long evolutionary lineage in which all other members are extinct. It should also be noted that one of the oldest liverwort fossils, *Blasiites*, R. M. Schust. (Schuster 1966) at least superficially resembles the extant *Blasia*.

The ancestral liverwort may indeed have been thalloid, as the leafy liverworts appear derived in Study I. Moreover, the oldest liverwort fossils are thalloid (*Pallaviciniites devonicus* (Hueber) R. M. Schust., *Blasiites*). However, the latest results place the genera *Treubia* K. I. Goebel and *Haplomitrium* Nees in a sister position to the rest of liverworts (Forrest and Crandall-Stotler 2004, 2005, Crandall-Stotler *et al.* 2005, Heinrichs *et al.* 2005, He-Nygrén *et al.* 2005). *Treubia* and *Haplomitrium* both have a tetrahedral apical cell, so if they do indeed form a sister clade to the other liverworts, this may support the tetrahedral apical cell as plesiomorphic in liverworts. Interestingly, however, *Haplomitrium* is in this position only when *Treubia* is included in the analysis, otherwise it is found among or close to the simple thalloids (Davis 2004, Study I). This warrants a careful examination to ensure that the placement of *Haplomitrium* in these studies is not caused by long branch attraction. If so, the situation

might resemble those described by Siddall and Whiting (1999) and Pol and Siddall (2001), in which the removal of one of two long branches attracted to each other allowed the second to find its correct place (see also Bergsten 2005).

In Study I Sphaerocarpaceae Cavers and Monocleales R. M. Schust. are clustered within Marchantiales Limpr. This is in accord with the results of Boisselier-Dubayle *et al.* (2002), and Forrest and Crandall-Stotler (2004). In Davis (2004), Forrest and Crandall-Stotler (2005), Crandall-Stotler *et al.* (2005), Heinrichs *et al.* (2005), and He-Nygrén *et al.* (2005) Sphaerocarpaceae are in a sister position to the Marchantiales.

The complex thalloids do not seem to be the most advanced group of liverworts, as claimed by Schuster (1972), instead forming a clade that is as old as the clade containing simple thalloids and leafy liverworts. This scenario appears also in the results of Davis (2004), Forrest and Crandall-Stotler (2004, 2005), Crandall-Stotler *et al.* (2005), Heinrichs *et al.* (2005) and He-Nygrén *et al.* (2005). This topology and the sister position of *Treubia* and *Haplomitrium* to the rest of liverworts gain further support from the occurrence of fungal associations. It appears that *Treubia* and *Haplomitrium*, as well as the complex and simple thalloid liverworts, are usually associated with fungi from the Glomeromycota forming arbuscular mycorrhizal symbioses reminiscent of those of many vascular plants. Leafy liverworts, by contrast, are associated with fungi from Asco- and Basidiomycota when symbiotic association is present (Heinrichs *et al.* 2005, Selosse 2005).

The most surprising result in Study I is the paraphyly of simple thalloids, the



grouping of *Metzgeria*, *Noteroclada* Taylor ex Hook. & Wils., and *Pleurozia* together, and the sister position of this clade with leafy liverworts. However, a terminal elaterophore, a two-layered capsule wall, 2-4 valves of the capsule, and one-spiral elater are morphological features in all of these taxa that support this novel arrangement. This result also gains support from Davis (2004), Forrest and Crandall-Stotler (2004) and (2005), Crandall-Stotler *et al.* (2005), Heinrichs *et al.* (2005) as well as from He-Nygrén *et al.* (2005).

The leafy liverworts are divided into two clades, and their evolution seems to have proceeded quite differently from the scenarios presented in earlier hypotheses (Schuster 1972, 1979, 1980, 1996a). They do not form a bush-like phylogeny, the allegedly primitive isophyllous liverworts are derived, and the Porellales together with Radulales and Lepidolaenineae form one of the main clades. In essence the same clades also appear in He-Nygrén *et al.* (2005), as well as in Davis (2004) and Heinrichs *et al.* (2005), although the species compositions are different in the latter two studies. Both Heinrichs *et al.* (2005) and He-Nygrén *et al.* (2005) name these clades Porellales and Jungermanniales.

### **Porellales-Lepidolaenineae clade**

In Study I the order Porellales groups together with the suborder Lepidolaenineae, forming one of the two main clades within the plants traditionally treated as leafy liverworts. This result is confirmed by Davis (2004), Heinrichs *et al.* (2005), He-Nygrén *et al.* (2005), and *Lepidogyna hodgsoniae* from Lepidolaenineae groups within

Porellales even in Stech and Frey (2004). These two groups share a number of similar characters - *Frullania*-type galeate lobules, *Frullania*-type branching, *Frullania*-type sporeling development, rhizoids restricted to the underleaf bases, cortical cells differentiated into thickened outer cells and thin-walled medullary cells, the presence of a stylus, small homogenous oil-bodies, reddish and brownish pigments, broad ventral merophytes, endosporic germination, and asexual reproduction via discoid gemmae or caducous leaves - which makes it surprising that they have not been united earlier. Admittedly, Schuster (1984, 1992) considered Lepidolaenineae and Porellales to share a common, probably Ptilidiineae R. M. Schust. -like, ancestor, but even he considered Lepidolaenineae more primitive than Porellineae. Crandall-Stotler and Stotler (2000) placed Lepidolaenineae in the basal position in their classification in the order Lepadiales while Porellales was considered one of the orders in most apical position among the leafy liverworts. The more primitive status given to Lepidolaenineae is mostly due to the presence of a coleocaulis in this suborder, and the lack of a perianth. However, according to the results of paper I, the coleocaulis in Lepidolaenineae is not a primitive but a derived feature.

The Porellales-Lepidolaenineae clade overall has strong support, while the internal nodes are not as well supported (Study I, Figure 1). Also a few surprising elements within the clade need to be pointed out. The most surprising may be the presence of *Ptilidium pulcherrimum*. *Ptilidium* Nees does not have a *Frullania*-type sporeling, endosporic germination, a stylus, asexual reproduction, or even a lobule. Nevertheless,

in Study I *Ptilidium pulcherrimum* appears sister to Lepidolaenineae and *Goebeliella* Steph., together with *Neotrichocolea bisseti* (Mitt.) S. Hatt. However, new results (Heinrichs *et al.* 2005, He-Nygrén *et al.* (2005) place the *Ptilidium-Neotrichocolea* clade in a sister position to the whole Porellales-Lepidolaenineae clade, thus supporting Schuster's (1984) view of *Ptilidium* as resembling a common ancestor of Lepidolaenineae and Porellales. In Davis (2004), the *Ptilidium-Neotrichocolea* clade jumps in different analyses from a sister position to the Porellales-Lepidolaenineae clade to a sister position to the clade containing the rest of the liverworts. *Ptilidium ciliare* is placed in this latter position even in Stech and Frey (2004). Adding the other species of the genus *Ptilidium*, *P. ciliare* (L.) Hampe, as well as *Trichocoleopsis* (allegedly close to *Neotrichocolea*: Schuster 1972, Crandall-Stotler and Stotler 2000) might help to establish the true taxonomic position of *Ptilidium* and *Neotrichocolea*.

Radulaceae also seem to be part of the Porellales-Lepidolaenineae clade, grouping strongly together with *Frullania*. Since *Radula* exhibits some unique morphological features (rhizoids restricted to the leaf lobules, often only a single oil-body in the cell, presence of a special yellow pigment, *Radula*-type germination, a dorsoventrally flattened perianth, and lack of underleaves), it is difficult to understand the apparent close relationship of *Radula* to *Frullania*. *Radula* groups within Porellales even in the results of Davis (2004) and Heinrichs *et al.* (2005), although it does not form a clade with *Frullania*.

*Frullania* has traditionally been placed in the same family with *Jubula* (e.g. Guerke

1978, Schuster 1992, Crandall-Stotler and Stotler 2000), but the findings of Studies II and I do not confirm this grouping. The separation of *Frullania* and *Jubula* into different families also gains support from Wilson *et al.* (2004), Heinrichs *et al.* (2005), and He-Nygrén *et al.* (2005).

*Goebeliella cornigera* (Mitt.) Steph. is situated in a position between morphologically very different taxa, the *Ptilidium-Neotrichocolea* clade and *Lepidogyna hodgsoniae* (Grolle) R.M. Schust. in the tree obtained in Study I (Figure 1). Support for this position is found in Davis (2004), who places *Goebeliella* as a sister to Lepidolaenineae. Heinrichs *et al.* (2005) place *Goebeliella* in sister position with *Radula*, and He-Nygrén *et al.* (2005) assign it together with the genus *Porella* in a sister position with Lepidolaenineae. This last result is consistent with the morphological characters. *Goebeliella* shares a 3- to 4-stratose capsule wall and a non-beaked perianth mouth with the genus *Porella* as well as free elaters with both *Porella* and Lepidolaenineae. *Goebeliella cornigera* forms one of the longest branches in the Porellales-Lepidolaenineae clade and has some unique morphological features, e.g. the horn-like lobules found in no other liverwort. It may well be a sole extant representative of a long evolutionary lineage, but the starting point of this lineage is obscure, warranting further studies.

Many different taxonomic positions both in Porellales and in Lepidolaenineae have been proposed for *Jubulopsis novae-zelandiae* (Hodgson 1965, Grolle 1966, Schuster 1970, Hattori 1972, Hamlin 1973, Schuster 1996b). In Study I, Porellales and Lepidolaenineae are merged, and *Jubulopsis* is situated among the species originally from

Lepidolaenineae, lending some degree of support to all of these authors. Interestingly, *Jubulopsis* groups inside the genus *Lepidolaena*. *Jubulopsis* does share a scaly coelocaulus and quadrifid underleaves with *Lepidolaena* but lacks water sacs on the underleaves, the special feature of the whole Lepidolaenaceae. However, this position was confirmed in Davis (2004), although her study only includes one species of *Lepidolaena*, and by He-Nygrén *et al.* (2005). This suggests that the genus *Lepidolaena* may need closer study.

### ***Jubula*-Lejeuneaceae clade**

The genus *Jubula* and the family Lejeuneaceae group together according to Studies I and II as well as to Wilson *et al.* (2004), Heinrichs *et al.* (2005), and He-Nygrén *et al.* (2005). This supports the suggestion by Mizutani (1961) that the genus *Jubula* should be placed within Lejeuneaceae, in a subfamily of its own. However, it is not possible to judge from the phylogenetic tree alone whether Jubuloideae should be treated as a subfamily of Lejeuneaceae, or whether these should be treated as two closely related families, Jubulaceae and Lejeuneaceae. *Jubula* and Lejeuneaceae share as synapomorphies 16 outer and 4 inner cells in the seta, a stylus reduced to a mere slime papilla, and elaters attached to the capsule wall. However, they also have the following differences: *Jubula* has ciliate leaves, two archegonia per perianth instead of one, no U-shaped central cells in the underleaf base, and no rosette-like protrusions on the spore surface. The taxonomic level of *Jubula* will likely become clear when the whole internal

taxonomy of the Porellales-Lepidolaenineae clade is resolved, including the positions of such intermediate genera as *Schusterella* S. Hatt., Sharp and Mizut., *Amphijubula* R. M. Schust., and *Neohattoria* Kamim., not present in this study. However, it already seems clear that at least the genera *Jubula* and *Frullania* should not be included in the same family.

### **Lejeuneaceae**

Ptychanthoideae and Lejeuneoideae appear as sister groups in Studies II and III, contrary to earlier hypotheses that the subfamily Ptychanthoideae would be more primitive than Lejeuneoideae (e.g. Mizutani 1961, Schuster 1980). This finding is supported by fossil data, with representatives of both subfamilies appearing at about the same time, 40 Ma BP (Grolle 1981, 1982, 1984a, 1984b, 1993). However, the initial division of these two lineages must have happened much earlier since the Lejeuneaceae flora of the Eocene was already rather developed, containing many present-day genera from both subfamilies. While in the *rbcL* phylogeny of Lejeuneaceae by Wilson *et al.* (2004) the relationships of Ptychanthoideae and Lejeuneoideae remain ambiguous, they are sister clades even in Heinrichs *et al.* 2005.

The tribe Brachiolejeuneae has Lejeuneoideae-type sporophytes and Ptychanthoideae-type gametophytes, and Krujt (1988) and Gradstein (1994) moved it from Ptychanthoideae to Lejeuneoideae. The sole representative of Brachiolejeuneae in Study II, *Odontolejeunea lunulata* (Weber) Schiffn., takes a sister position to both of these subfamilies. This surprising result was

confirmed by Groth-Malonek *et al.* (2004) and Davis (2004), although the latter study included very few Lejeuneaceae. Moreover, both Groth-Malonek *et al.* (2004) and Study **II** were partly based on ITS2, and therefore, this result could be due to some anomalous evolution in ITS2. In Heinrichs *et al.* (2005) a clade formed by *Odontolejeunea* and *Marchesinia* appears as a sister to the rest of Lejeuneoideae. However, as an intermediate taxon, Brachiolejeuneae is well suited to be an ancestral form of Lejeuneaceae.

There are probably too few representatives of Ptychanthoideae in Studies **I-IV** to resolve its internal phylogeny. *Ptychanthus* and *Spruceanthus* Verd. appear in an apical position in many of the most optimal trees of Studies **II, III, and IV**. Verdoorn (1934) separated *Spruceanthus* from *Ptychanthus* only because its perianth is three-keeled at the juvenile stage. Wilson *et al.* (2004) suggest no close relationship for *Spruceanthus* and *Ptychanthus*.

*Bryopteris* appears as a sister to the other Ptychanthoideae in both Studies **I and IV**. Also in Heinrichs *et al.* (2005), *Bryopteris* is found in a sister position to most of Ptychanthoideae. This is in accordance with Stotler and Crandall-Stotler (1974), who assigned *Bryopteris* into a family of its own. Gradstein (1975) also assigned it to a subfamily of its own within Lejeuneaceae.

*Trocholejeunea* Schiffn. is a sister clade to the other Ptychanthoideae, except *Bryopteris*, in Studies **I and IV**, and in a sister position to the rest of Ptychanthoideae in Studies **II and III**, where *Bryopteris* is not included. In Wilson *et al.* (2004), *Trocholejeunea* is also resolved between *Bryopteris* and the rest of the Ptychanthoideae. This position is consistent

with the structure of the seta in *Trocholejeunea* resembling that of *Bryopteris* (Gradstein 1994).

Concerning the subfamily Lejeuneoideae, the results are different in every study included in this thesis. A clade consisting of the species assigned to the subfamily Tuyamaelloideae and the tribe Calatholejeuneae form a monophyletic sister clade to the rest of the Lejeuneoideae in Study **III** but in Study **II** this clade is in an intermediate position in Lejeuneoideae, and in Study **I** it is apical. In Study **III**, tribal status could be assigned for this clade, yet in the other studies this would leave the rest of Lejeuneoideae paraphyletic. Likewise, the other species of Lejeuneoideae change places in every presented tree. The data are undoubtedly insufficient to resolve the internal taxonomy of both Lejeuneoideae and Ptychanthoideae. The results of Wilson *et al.* (2004) support most closely the findings of Study **I** with the Calatholejeuneae and *Lejeunea* species situated in sister clades, and the *Cheilolejeunea* species in a sister position to them.

### *Nipponolejeunea*

*Nipponolejeunea* groups very strongly with *Jubula* (Studies **I-IV**, Wilson *et al.* 2004, Heinrichs *et al.* 2005). Besides the molecular characters, this grouping is supported by the presence of Pycnolejeuneoid subfloral innovations (Mizutani 1970, Gradstein 1979, Gradstein *et al.* 2003), small trigones in leaf cells in both *Nipponolejeunea* and *Jubula*, and U-shaped central cells (Bischler 1969, Gradstein 2001) not being found in *Nipponolejeunea*. However, if

*Nipponolejeunea* is a sister genus to *Jubula*, it is difficult to explain why *Nipponolejeunea* has well-developed Lejeuneaceae-type lobules. One explanation could be that the Lejeuneaceae-type lobule is a juvenile feature and retaining it is a paedomorphy that has appeared at least twice in the Porellales-Lepidolaenineae clade, separately in Lejeuneaceae and *Nipponolejeunea* – or once in their common ancestor. Or possibly three times, as the genus *Trichocoleopsis*, which was not included in this thesis, but has been considered to be closely related to *Neotrichocolea* S. Hatt., also has Lejeuneaceae-type lobules. Actually, it is possible that most Porellales have an underlying capacity to develop Lejeuneaceae-type lobules. Indeed, juvenile *Frullania* has been described as having them (Schuster 1963b, Vanden Berghen 1977, Bisang 1987), as has *Porella platyphylloidea* (Schwein.) Lindb. in xeromorphic conditions (Schuster 1980). On the other hand, Frullaniaceae and Jubulaceae sometimes develop Porellaceae-type explanate lobules. Studies on the genetic control of lobule development are thus necessary to thoroughly understand the evolution and homology of the lobules.

### ***Ptychanthus striatus* and *P. africanus***

In Study V both parsimony and Bayesian analysis based on ITS2 data clearly distinguish two clades, one containing the Asian-Oceanic, and the other the African specimens of *Ptychanthus striatus* (Study V, Figures 6 and 7). These clades coincide with differences in oil-body structure. Grayish, botryoidal oil-bodies composed of numerous

globules characterize Asian-Oceanic specimens, and shiny, *Bazzania*-type oil-bodies composed of a few large segments African specimens (Kis and Pócs 1997). Nevertheless, the long-standing question of when two populations can be called different species arises here also. Morphological differences or reproductive isolation are the most traditional ways to define species (defined as typological and biological species concepts, respectively, Mayr 1942, 1963). Molecular differences, which we have been able to study only in the last few decades and which have given rise to the notion of cryptic speciation, can be regarded as equal to morphological differences in this respect. Several other species concepts have been proposed also, for example the phylogenetic species concept, which emphasizes phylogenetic history over factors such as possible interbreeding (e.g. Cracraft 1989, Nixon and Wheeler 1990), and the genealogical species concept, which defines a species as a set of populations for which gene trees at all loci examined are monophyletic (Baum and Shaw 1995).

No variation was found in *trnL-trnF*, *nad5* intron, or *rps4* sequences of the *Ptychanthus* specimens studied. This can be interpreted in several ways. It could be due to lineage sorting, these loci having become monophyletic by genetic drift while ITS2 has still retained several haplotypes. If this is the case, and the genealogical species concept is adopted, *Ptychanthus striatus* and *P. africanus* should not be recognised as separate species based on ITS2 data. However, it seems more likely that the ancestor of *P. striatus* and *P. africanus* had already reached monophyly in most gene lineages, and that the changes in ITS2 and in the oil-body structure are novelties acquired

during their geographic isolation from each other. In this case the two species can be recognised according to typological and phylogenetic species concepts. However, the sampling for ITS2 data is scarce in Study V, especially in Africa where all the specimens come from a limited geographical area. It is possible that corresponding ITS2 haplotypes would be found even in other areas. More DNA specimens should be collected from different geographical areas throughout the distribution range of both species.

Reproductive studies of *P. striatus* and *P. africanus* are also needed to conclude whether they are reproductively isolated by other barriers besides geography.

Distinguishing the species *Ptychanthus striatus* (Lehm. and Lindenb.) Nees and *Ptychanthus africanus* Steph. is thus mainly based on the difference in oil-body structure, although the present ITS2 data certainly supports the results. The two species were recognised mainly based on the typological species concept with consideration also of the phylogenetic and genealogical species concepts.

### **POY and the indels**

The direct optimization parsimony program POY, which was used as the main method to obtain phylogenetic hypotheses in this thesis, was found to be an efficient way to find the hidden signal in variable gene regions otherwise difficult to align reliably. POY optimizes the characters in all data sets simultaneously, aligning the data partitions according to the strongest signal in the entire data. It can thus find potential hidden support even in such data sets where the true signal is obscured by a random signal

(Gatesy *et al.* 1999, Cognato and Vogler 2001, Schulmeister *et al.* 2002).

At the same time, however, POY was found to be susceptible to long branch attraction caused by unrelated insertions and deletions in DNA sequences (Studies II and VI). Long branch attraction is defined here as in Andersson and Swofford (2004 p.441), as “any situation in which similarity due to convergent or parallel changes produces an artefactual phylogenetic grouping of taxa due to an inherent bias in the estimation procedure”. When gaps are treated as fifth character state in a phylogenetic analysis, they act as any nucleotide characters, and in that sense can be equated with the base-substitutions that cause classical branch attraction.

In POY gaps are treated as fifth character states, and as a consequence, they are regarded as synapomorphies when they occur in analogous positions in the alignment. Nevertheless, POY was slightly less susceptible to this type of branch attraction than e.g. NONA. The better performance of POY is due to its above-mentioned ability to find the hidden signal. When POY aligns the regions with deletions in some of the sequences (even insertions result in gaps to the other sequences) according to the strongest signal in the data, it readjusts the positions of gaps and even adds gaps so that enough synapomorphies are created for the alignment to support a particular topology. This, of course, can make POY alignment biologically unrealistic at times, but it does ensure the discovery of the correct topology until the deletions and insertions get too long for this mechanism to work. With higher gap costs, the gaps get more weight and gaps are pushed together, resulting in shorter

alignments. This leads to incorrect topologies already at shorter deletion and insertion lengths than with lower gap costs.

Deletion attraction may thus have affected the topologies obtained with higher gap costs here. The higher gap costs generally caused the groupings of taxa to become more and more spurious and different from all former taxonomic hypotheses (Studies I-III). In Study II, the complex thalloid liverwort *Marchantia polymorpha* and the leafy liverwort *Cheilolejeunea trifaria* were drawn together when gap costs higher than one were used. This effect was localized to a deletion of 100 base pairs in analogous positions in the *trnL* intron of both species. These deletions are probably independently attained because *M. polymorpha* and *C. trifaria* are, according to all classifications (e.g. Schuster 1979, Crandall-Stotler and Stotler 2000), taxonomically very remote.

Lloyd and Calder (1991) considered gaps of exactly equal length to be better than substitution characters. This may be so if the taxa being examined are taxonomically so close that the deletions are of homologous origin. However, with a taxonomically wide range of taxa, as was the case here, it is safest to use equal gap and change costs, or even treat gaps as missing data. The advantage of weighing gaps cautiously finds support also from Petersen *et al.* (2004) and Aagesen *et al.* (2005) who found that setting gap extension costs lower than the opening cost increases the congruence of the data sets.

The ability of POY to find the hidden signal in different data portions in combined analysis proved to be most valuable. This ability greatly enhanced the possibility of also utilizing highly variable gene regions,

like ITS2, which are otherwise difficult to align reliably. ITS2, aligned by POY according to the signal from other parts of the data, was also able to counteract the harmful effect of the above-mentioned *trnL* intron deletions in Study II. This is probably because ITS2 was easily aligned to contain enough “correct” synapomorphies that supported remote taxonomical positions for *Marchantia L.* and *Cheilolejeunea*.

Because the POY implied alignment may not be biologically correct and the alignment of all positions according to the strongest signal violates the requirement of independence of individual characters, Clustal and Dialign alignment as well as the phylogenetic programs NONA, Paup, and MrBayes were also used to compare the results. The results obtained with different methods were, however, usually very similar, indicating that if the signal in data is sufficiently strong the existing methods for phylogenetic inference work equally well. The quality of the data should thus be of primary concern in phylogenetic work.

## Conclusions

The results confirm monophyly of the liverworts and place them as a sister group to the other land plants. However, sampling among the outgroup taxa was scarce, and this may have biased the overall placement of liverworts. Studies with more balanced sampling from different land plant groups are needed to confirm the diversification order of the first land plants.

According to Study I, *Blasia pusilla* was sister to the other liverworts. The unique morphology of *B. pusilla* supports this position. However, in studies with both

*Haplomitrium* and *Treubia* included (Forrest and Crandall-Stotler 2004, 2005, Crandall-Stotler *et al.* 2005, Heinrichs *et al.* 2005, He-Nygrén *et al.* 2005), these two taxa together form a sister group to the rest of the liverworts and *Blasia* is sister to the complex thalloids. If not caused by long branch attraction, this indicates that *Treubia* and *Haplomitrium* could have diverged first from a common ancestor of the liverworts. This scenario, and the one in which complex thalloid liverworts form a sister group to simple thalloid and leafy liverworts rather than originating from leafy liverworts by reduction, gain support from fungal associations (Heinrichs *et al.* 2005, Selse 2005).

The simple thalloid liverworts appear paraphyletic, with *Metzgeria* and possibly *Noterochlada* grouping with *Pleurozia* as sister to the leafy liverwort species. This is supported by many recent studies (Davis 2004, Forrest and Crandall-Stotler 2004 and 2005, Crandall-Stotler *et al.* 2005, Heinrichs *et al.* 2005 He-Nygrén *et al.* 2005) and by the fact that *Metzgeria* forms an association with the Ascomycete *Xylaria* (Davis *et al.* 2003).

The traditional leafy liverworts except *Pleurozia* are divided into two clades, one of them containing the orders Porellales and Radulales, suborder Lepidolaenineae, and *Ptilidium pulcherrimum*. The other clade contains the rest of the leafy liverworts. Within leafy liverworts, contrary to earlier views, the isophyllous liverworts seem to be more derived than the anisophyllous liverworts.

The main results of this thesis are the taxonomic position and circumscription of the order Porellales, with the relationships of the subfamilies in one of its families,

Lejeuneaceae, being resolved. Contrary to earlier views (e.g. Schuster 1980), Porellales does not appear to be the crown group of the leafy liverworts. Furthermore, circumscription of the Porellales has been too narrow, and Porellales is now merged with Lepidolaenineae and Radulineae. Even the genus *Ptilidium* seems to be part of this clade. The clade is fairly uniform in its ecology and morphology, and appears to have retained many plesiomorphic characters also found in the *Pleurozia-Metzgeria-Noterochlada* clade, as well as in the Perssoniellineae R. M. Schust. clade which is the first diversified group among the rest of the leafy liverworts (Jungermanniales; Heinrichs *et al.* 2005, He-Nygrén *et al.* 2005). Such characters include epiphytism, presence of the lobule, and anisophylly. The affinities of the Mesozoic leafy fossil *Cheirorhiza* to the Porellales also lend support to the antiquity of this taxon (Krassilov and Schuster 1984, Schuster and Janssens 1989).

The internal nodes of the Porellales-Radulales-Lepidolaenineae-*Ptilidium* clade do not show particularly high Bremer support values (Figure 1 in Study I), and thus further studies are needed to confirm these relationships. However, two clades can be distinguished, one containing the genus *Jubula* and the family Lejeuneaceae, and the other the rest of the Porellales as well as Radulales, Lepidolaenineae, and *Ptilidium*, rendering Porellales sensu Schljakov (1972) and Crandall-Stotler and Stotler (2001) as paraphyletic. Another notable result of Studies I and II is the monophyly of genera *Radula* with the genus *Frullania*, although this needs to be confirmed with further studies. This is a novel result because thus



far *Frullania* and *Jubula* have been assigned to the same family, Jubulaceae.

The genus *Nipponolejeunea* is a sister group of the genus *Jubula* rather than a part of Lejeuneaceae. The Lejeuneaceae-type lobule in *Nipponolejeunea* may be a paedomorphy. A study on genetic control of lobule development is certainly warranted.

The results support the division of the family Lejeuneaceae into two subfamilies, Ptychanthoideae and Lejeuneoideae, which are in sister positions to each other. The tribe Brachiolejeuneae in Study II appears in a sister position to both of these subfamilies. This result is supported by Groth-Malonek *et al.* (2004) and Davis (2004), but more studies are necessary to confirm this placement. If the sister position of *Odontolejeunea* (Spruce) Schiffn. to the rest of the Lejeuneaceae proves to be correct, the ancestor of Lejeuneaceae may have possessed Lejeuneoideae-type sporophytes and Ptychanthoideae-type gametophytes.

Calatholejeuneae cannot be separated from Lejeuneoideae without leaving Lejeuneoideae paraphyletic, although species of Calatholejeuneae form a monophyletic entity with species of Tuyamaelloideae. However, this clade could only be given a rank below tribal level.

Based on the difference in oil-body structure, *Ptychanthus striatus* contains two cryptic species, *Ptychanthus striatus*, which is found in Asia and Oceania, and *Ptychanthus africanus*, which is found in Africa and Madagascar. The oil-body structure is the only morphological feature distinguishing these species. Molecular data from ITS2 supports the results, but wider range of specimens should be sequenced to fully understand the genetic variation within these taxa.

The choice of analysis method is crucial to the results of a phylogenetic analysis. The phylogenetic program POY proved to give good results as long as gaps were given equal weight with base substitutions. However, it might be more advisable to treat gaps as missing data, which can be done even with POY to some extent (see Study VI), unless confidence in their homologous origin prevails. Nonhomologous deletions in analogous positions become synapomorphies if gaps are treated as a fifth state, and they may cause long branch attraction when the data contain insufficient “correct” synapomorphies to counteract their effect. To prevent this type of long branch attraction, it is far better to add characters than taxa.

The results give many new insights into the evolution of liverworts, both challenging and confirming previous results in various taxonomic levels. The era of large-scale phylogenetic studies based on hundreds of taxa, numerous genes, and large morphological matrices investigating land plant and liverwort evolution has only just begun, and within a few years we will undoubtedly understand much more. Study I presents one of the most comprehensive published liverwort phylogeny to date. In addition to shedding light on land plant and liverwort origins, the results of this thesis indicate that some details of liverwort evolution have thus far been misunderstood, while simultaneously confirming many findings obtained at one time with merely a light microscope and accurate observations. Characters such as apical cells and slime papillae, warrant further research.

Currently, studies of liverwort phylogeny are like drawing a map of a recently found continent. We know some

details and features, but we still have a long way to go in figuring out how all big and small pieces of information fit together.

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