The family Herbertaceae and its novel systematic position within liverworts

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Academic dissertation
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The family Herbertaceae and its novel systematic position within liverworts

Aino Juslén

This thesis is based on the following articles:


These are referred to in the text by their Roman numerals.
Contributions

The following table shows the main contributions of authors to the original articles or manuscripts.

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Introduction

The original idea of this thesis was to study the taxonomy of the liverwort genus *Herbertus* Gray and its phylogenetic relationships. The taxonomic revision was delimited to Asia (I, II), an area with many species of *Herbertus*, but without modern comprehensive study of the genus. Another region with a rich *Herbertus* flora is South America, which has been studied for example by Van Reenen (1982), Feldberg *et al.* (2004), and Feldberg and Heinrichs (2005a, 2006).

For decades *Herbertus* has been considered to be one of the most primitive genera of liverworts (Grolle 1983, Schuster 1979, 1984, 2000). Based on this hypothesis, my original plan for the phylogenetic study was to include Herbertaceae and other “basal” leafy liverworts. However, the first comprehensive phylogeny of leafy liverworts based on sequence level data (IV) showed that *Herbertus* actually is a derived genus. Based on the results of studies IV, V, and Heinrichs *et al.* (2005), the ingroup of study III was selected to include the Vetaformaceae, Lepicoleaceae, and Herbertaceae including Mastigophoraceae.

The aim of studies I and II was to examine and evaluate the morphological characters useful for identification of Asian *Herbertus* and solve the taxonomic relationships of the species. In III, my aim was to reconstruct the phylogenetic relationships of Herbertaceae and its close relatives. In IV and V we aimed to test the formerly presented hypotheses of liverwort relationships (Schuster 1979, 1984, 2000, 2002, Crandall-Stotler & Stotler 2000) based on molecular and morphological data. In study V, we aimed to establish a new classification for liverworts, which would reflect phylogenetic relationships.

Historical outline

Taxonomy of *Herbertus*

Species of *Herbertus* show great plasticity in their size and colour, and in some characters of their leaves such as the bifidness (Van Reenen 1982) and the width of the sinus (Inoue 1977). There are currently ca. 100 binomials of *Herbertus* (Geissler & Bischler 1990). Due to the variability of the plants, numerous species have been described, especially by Stephani (1895, 1898-1924). Evans (1917), Schuster (1957), and recently Gradstein (2001) predicted a large reduction in species number within *Herbertus*. In their recent revision of Neotropical *Herbertus*
Feldberg and Heinrichs (2006) recognized only two species instead of earlier number of 10–11 species.

The studies on Asian *Herbertus* are few. Miller (1965) worked on the Asian and Pacific taxa, but failed to clarify the taxonomy, as no logical characters for delimiting the species were presented. He listed 47 species (Miller 1965). Inoue (1977) studied Taiwanese species and recently So (2003) revised the South Pacific and Australasian *Herbertus*. For more detailed information on history and studies of *Herbertus*, see the introduction of study II.

The phylogenetic relationships of Herbertaceae

Classifications presented by various workers, based on morphology, have recognized the families Vetaformaceae, Lepicoleaceae, and Herbertaceae as close relatives, regarding them as the most primitive lineages of leafy liverworts (Grolle 1983, Schuster 1984). The relationships of Mastigophoraceae have been controversial. Grolle (1972, 1983) and Inoue (1978) placed the family within Lepicoleaceae, but Schuster (1984, 1987) regarded *Mastigophora* Nees to belong to Ptiliidinae, followed also by Crandall-Stotler and Stotler (2000). The recent molecular phylogenies supported the monophyly of the group formed by Vetaformaceae, Lepicoleaceae, Herbertaceae, and Mastigophoraceae as one of the most derived lineages of liverworts (IV, V, Heinrichs et al. 2005). Heinrichs et al. (2005) proposed the inclusion of Mastigophoraceae in Herbertaceae. Besides in III, the relationships within the largest genus of the group, *Herbertus*, have been studied using nuclear ITS sequences by Feldberg et al. (2004) and Feldberg and Heinrichs (2005b, 2006).

Liverwort classification

The liverworts were first recognized as an independent plant lineage by de Jussieu in 1789. Since then, the relationships of liverworts have remained controversial. Macrofossil records of liverworts are also fairly few (see Krassilov & Schuster 1984, Oostendorp 1987). The early classifications emphasized the division between the complex thalloid and other liverworts (Lindberg 1875, Goebel 1882, Schiffner 1893, Wettstein 1903–1908, Müller 1939–1940). Schiffner (1893) established two groups: the Jungermanniales akrogynae (the leafy liverworts) and the Jungermanniales anakrogynae (the simple thalloid liverworts).
The hypothesis that liverworts have a common ancestor with an erect, radial gametophyte and a tetrahedral apical cell was first introduced by Wettstein (1903–1908). This hypothesis was reflected in the classification schemes of Verdoorn (1932), Buch (1936), Evans (1939), Grolle (1983), and Schuster (1966, 1972, 1979, 1984, 2000). According to Schuster, the liverworts and mosses share a common ancestor, with *Haplomitrium* Nees representing the oldest lineage among the extant species. He arranged leafy liverworts assuming that the same kind of evolutionary trends had been repeated in many different leafy liverwort suborders, with evolution proceeding from erect and isophyllous to creeping and anisophyllous plants (Schuster 1984). This view was generally accepted until recently (Grolle 1983, Schuster 1984, 2000, 2002, Crandall-Stotler & Stotler 2000, see Table 1). For a more detailed history of liverwort classification see the introduction in IV and V.

**Recent studies based on sequence level data**


The majority of the latest studies recognize liverworts as monophyletic (e.g. Beckert *et al.* 1999, Nickrent *et al.* 2000, Dombrovskva & Qiu 2004, Crandall-Stotler *et al.* 2005, Groth-Malonek *et al.* 2005). In addition, some morphological synapomorphies of the liverworts support the monophyly of the group: e.g. the presence of membrane-bound oil-bodies, the growth hormone lunularic acid, the unequal division of the archespore cell into a spore mother cell and an elater cell, and the sporophyte generation developing completely inside the gametophyte within the modified archegonium (see Crandall-Stotler & Stotler 2000, Ahonen 2005, Frey & Stech 2005). It has also been suggested that liverworts are the earliest embryophyte lineage, based on microfossil evidence (e.g. Wellman *et al.* 2003), and on molecular data (e.g. Kelch *et al.* 2004, Groth-Malonek *et al.* 2005). However, the relationships of the early embryophyte lineages are still controversial.

Traditionally the Metzgeriales or Metzgeriidae, including liverworts with simple structured thallus and anacrogynous leafy-like liverworts, have been classified together (Schuster 1984, Crandall-Stotler & Stotler 2000). However, several molecular phylogenetic analyses have shown that the group is paraphyletic. In these studies Metzgeriales *s.str.* are resolved as sister to the leafy liverworts, while the other group of “traditional” simple thalloids, including Fossombironiales and many genera of former Metzgeriales, form a clade of their own (*IV*, Forrest & Crandall-Stotler 2004, 2005, Davis 2004, Crandall-Stotler *et al.* 2005, Heinrichs *et al.* 2005, *V*, Forrest *et al.* 2006).

The understanding of evolutionary relationships of leafy liverworts, comprising 85% of liverwort species (Schuster 1984), has dramatically changed following the advent of molecular phylogenies. Schuster’s (1984) idea of repeated evolution from isophyllous to anisophyllous plants, and a bush-like phylogeny, has been rejected. The leafy liverworts are basically divided in to three groups; an independent lineage of *Pleurozia* Dumort., and two larger groups; Porellales and Jungermanniales (*IV*, Davis 2004, Heinrichs *et al.* 2005, *V*, Forrest *et al.* 2006). Conflicting with the earlier hypotheses, the isophyllous liverworts have been resolved as derived lineages within different groups of leafy liverworts.

Heinrichs *et al.* (2005) analysed phylogenetic relationships of a large number of taxa based on chloroplast *rbcL*, and derived a higher level classification from the results.
Table 1. Comparison of different classifications of liverworts.

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*In addition, Frey & Stech (2005) recently proposed a new classification of liverworts (Hepaticophytina) based on reviewed molecular evidence and re-evaluation of morphological and ultrastructural characters (Table 1). They emphasized the importance of the sporophyte-gametophyte junction based on the studies by Frey et al. (2001) and Camara et al. (2003).*
Materials and methods

Taxonomic studies of Asian *Herbertus*

The taxonomy of Asian *Herbertus* was studied by microscopic examination of 1300 specimens. The revised area covers As 1 (Asian Russia + the former central Asian Soviet republics), As 2 (China including Hong Kong and Taiwan, Mongolia, Japan, North Korea, and South Korea), As 3 (India, Nepal, Bhutan, Pakistan, Sri Lanka, Myanmar, Laos, Vietnam, Cambodia, and Thailand), and As 4 (Indonesia, East Timor, Malaysia, Brunei, Singapore, and The Philippines; excluding Papua New Guinea, that was treated by So (2003)) (see van der Wijk 1959 for areal division). The study of *Herbertus* from China, Hunan Province (I) was based on collections by Koponen *et al.* (2000, 2004). Study I initiated the revision of all the Asian taxa (II). Approximately 90 different characters were studied and evaluated, providing the basis for species delimitation based on morphology. Unfortunately, only a very few specimens of Asian *Herbertus* have sexual organs, sporophytes are rare and identification is mostly based on gametophyte characters. Representative specimens listed under each species are specimens that I consider to be “typical” for the particular species. In some cases also specimens that I consider to represent quite extreme variation induced by environmental factors are included as no other specimens from the country were available. These are discussed separately, as e.g. *H. millerianus* (=*H. dicranus*) and Vietnamese specimen of *H. ramosus*.

DNA sequence data

Molecular data were used in phylogeny reconstruction in studies III, IV, and V. Five DNA sequence regions were utilized. Chloroplast regions used were the Leucine transfer-RNA-Phenylalanine transfer RNA region (rrnL-F), partial sequences of the large subunit of the ribulose bisphosphate carboxylase gene (rbcL), and the gene region coding for the small ribosomal protein 4 (rps4). The nuclear regions were the Internal Transcribed Spacer 2 (ITS2) and partial sequences of 26S rRNA gene (26S). The chloroplast regions have been widely used in phylogenetic analyses of plants, including numerous studies of bryophyte phylogeny (e.g. Lewis *et al.* 1997, Hyvönen *et al.* 1998, 2004, Stech & Frey 2001, La Farge *et al.* 2002, Pedersen & Hedenäs 2002, Magombo 2003, Pedersen *et al.* 2003, Virtanen 2003, Shaw *et al.* 2003, Ahonen 2004, 2006). *RbcL* seems to be the quite conservative, and often used to resolve deeper level phylogenies throughout the plant kingdom (Nishiyama & Kato 1999, Newton
et al. 2000, Nickrent et al. 2000, Heinrichs et al. 2005). Rps4 has proved to be informative at various taxonomical levels, including within species and genera (Lüth & Goffinet 2005) and between phylogenetically more distant groups (Davis 2004, Forrest & Crandall-Stotler 2005). \textit{TrnL-trnF} has been extensively used and found to be informative at many levels (e.g. Stech & Frey 2001, Vandeerporten \textit{et al.} 2003a, Huttunen \textit{et al.} 2004). However, certain parts of the \textit{trnL-trnF} region have been assumed to have independent origins, or independent gains and losses in different land plant groups (Quandt \textit{et al.} 2004). In study \textbf{IV} the intergenic spacer was excluded from the analyses and in study \textbf{V} the gene areas P6 and P8 of \textit{trnL-trnF} were excluded in addition. In study \textbf{III}, for reconstruction of phylogeny of closely related genera, the whole length of \textit{trnL-trnF} was used.


Because both \textit{rbcL} and \textit{rps4} are protein coding genes they show essentially no, or, very little length variation. On the other hand, in \textit{trnL-trnF} there is considerable length variation, mostly in the intron, and to a lesser degree in the intergenic spacer (included in the analysis only in the study \textbf{III}). ITS2, used in study \textbf{III}, showed length variation from 273 base pairs to 320 bp. 26S, used in study \textbf{V}, contained little length variation within the ingroup, liverworts, but much greater variation across the whole dataset; from 1037 bp in the liverwort of the genus \textit{Nardia} Gray to 1243 bp in the charophyte alga of the genus \textit{Chara} L..

Morphological characters

Morphological characters were used in the studies of phylogeny of Vetaformaceae, Lepicoleaceae and Herbertaceae (\textbf{III}), and all the liverworts (\textbf{V}). The first analysis included 27 morphological characters, and the larger one 90. The morphological characters were primarily coded based on the voucher specimens. However, the liverwort specimens rarely contain sexual organs, and sporophytes may be unknown. Therefore many of these characters were coded based on literature (as listed in detail in each study).
Oil bodies have been extensively used in studies of liverwort systematics as diagnostic characters (e.g. Gradstein et al. 1977, 1981, Kis & Pócs 1982, 1997). However, their use in phylogenetic analyses is so far limited as reliable coding should be based on fresh material. Crandall-Stotler and Stotler (2000) used the number and occurrence of oil bodies in different cells as character states.

Phylogenetic analyses

The primary optimality criterion for all the studies in this thesis was parsimony (III, IV, V). Traditionally, alignment of DNA sequences and phylogenetic analyses are performed separately. The purpose of the alignment is to identify homologous nucleotides in sequences from different terminals in the analysis. The alignment, performed before the analyses, is static and the resulting alignment gaps are mostly treated as missing data. The novel approach of direct optimization evaluates the nucleotide homology directly in reference to topology (Wheeler 1996, Wheeler et al. 2004). The indels are treated as evidentially equivalent to any other kind of inferred transformation (Frost et al. 2006). In all the phylogenetic studies (III, IV, V) of the present thesis, the approach of direct optimization, as implemented in the program POY (Wheeler et al. 1996–2003), was utilized. In direct optimization, the search for the shortest tree, and the most parsimonious hypothesis of character homology are performed simultaneously. Only the results provide an “alignment” of the sequence data, which presents a hypothesis for the most parsimonious, optimal solution, of the character transformations. In order to save limited computation time, some of the data sets were cut into shorter segments based on preliminary alignments performed with the programs Clustal (Thompson et al. 1994) (IV) or Dialign (Morgestern 1999) (III, V). The number of replicates was restricted by the available CPU time. The analyses in study IV included five replicates and ten builds per replicate, while in study V we were able to run five replicates and 50 builds per replicate. The smaller dataset in the analyses of study III allowed running as many as 250 replicates and 50 builds per replicate. In study IV we compared the stability of the different clades based on different gap:transversion:transition costs (see study IV, table 2). However, Frost et al. (2001), Grant and Kluge (2003), and Huttunen (2004) concluded that the parsimonious results (in terms of minimizing the number of assumed transformations) are obtained only when all the characters are weighted equally. In the studies III and V only gap costs 1:1:1 were used. The analyses were run with the parallel version of POY in computer clusters of the CSC, IT Centre for Science, Espoo, Finland, or in the Beowulf cluster of the Finnish Museum of Natural History.
We also conducted analyses using other approaches; parsimony analysis based on static alignment using the program Nona (Goloboff 1993) (IV, V), and Bayesian inference with the program MrBayes (Huelsenbeck & Ronquist 2001) (V). The results were compared to the results obtained by POY. For these approaches the data was aligned using either Clustal (IV) or Dialign (V).

**Results and discussion**

**Taxonomy of Herbertus**

In papers I and II, based on the study of morphological characters, altogether 12 species of *Herbertus* were accepted for Asia: *H. aduncus* (Dicks.) Gray subsp. *aduncus*, *H. armitanus* (Steph.) H.A. Mill., *H. buchii* Juslén sp. nova, *H. ceylanicus* (Steph.) Abeyw., *H. circinatus* (Steph.) H.A. Mill., *H. dicranus* (Taylor ex Gottsche et al.) Trevis, *H. guangdongii* P.J. Lin & Piippo, *H. kurzii* (Steph.) R.S. Chopra, *H. longispinus* Jack & Steph., *H. pilifer* (Steph.) H.A. Mill, *H. ramosus* (Steph.) H.A. Mill., and *H. sendtneri* (Nees) Lindb. Eleven binomials were proposed as synonyms, and *H. subrotundatus* X. Fu & Y.J. Yi was excluded from the genus. The generic name of *Herbertus* was used in the form *Herbera* for decades, until the approval of *Herbertus* by the IUCN (Grole & Florschütz 1975). According to the nomenclatural rules all the names in the old form should be changed to the correct form (Greuter *et al.* 2000). This was correctly followed in study II, but not in I.

*Herbertus* is an extremely variable genus morphologically (see e.g. Schuster 1966, Feldberg *et al.* 2004, Feldberg & Heinrichs 2006). However, careful examination of ample material convinced me that the species can be delimited also morphologically. In particular, leaf length-width ratio, shape and structure of the leaf apex, vitta bifurcation point in the basal lamina, number of slime papillae in the leaf margins, and length of the stalks of the slime papillae proved to be useful characters. Leaf length-width ratio was rather constant, e.g. in *H. ceylanicus*, *H. longispinus*, and *H. sendtneri*. For instance Schuster (1957) and Fulford (1963) used leaf length-width ratio in their keys to North and Latin American species. In addition, Meinunger and Köckinger (2002) mentioned the leaf length-width ratio as a rather constant character in *H. sendtneri*. Leaf lobes are usually either acute or acuminate. In Asian species, perhaps *H. pilifer* has the most distinctive apices, which are almost sharp with extensively elongated cells. The shape of the leaf apex has often been used in descriptions and identification keys (e.g. Evans 1917, Schuster 1957, Fulford 1963, Van Reenen 1982).
Furthermore, there are distinctive differences between species in the number of uniseriate cells in the leaf lobe apices. Of the Asian species, *H. armitanus, H. longispinus* and *H. pilifer* have numerous uniseriate cells in leaf apices. On the other hand, there are species, which may have only one uniseriate cell in the leaf apex, such as *H. ceylanicus, H. kurzii* and *H. sendtneri*, and additionally only a few rows two cells wide below the apex, so that the lobes are broad and almost blunt in appearance.

One of the most distinctive generic characters of *Herbertus*, the median band of elongated cells or vitta, is often used in taxonomy. Based on my studies, the bifurcation point of the vitta in the basal part of the lamina is a useful character in species discrimination. This character was also used by Fulford (1963) and Van Reenen (1982). In study I it was used as one of the characters separating *H. aduncus* and *H. dicranus*. Furthermore, it distinguishes e.g. *H. buchii* and *H. guangdongii* (II). Schuster (1957) used differences in the number of slime papillae as a diagnostic character differentiating *Herberta tenuis* (= *H. aduncus* subsp. *tenuis* (A.Evans) H.A.Mill. & E.B.Scott) from *H. butchinsiae* (= *H. aduncus* subsp. *butchinsiae* (Gottsche) R.M.Schust.). Meinunger and Köckinger (2002) stated that in *H. sendtneri* this character depends on the size of the leaves, so that small leaves often lack, or have only reduced slime papillae. However, they referred to slime papillae as one of the best characters to separate *H. sendtneri* (which often has distinctive slime papillae) from European *H. borealis* Crundw. and *H. stramineus* (Dumort.) Trevis. The number of slime papillae is markedly different in the two groups dealt with by Grolle (1978). The group affiliated with *H. dicranus* clearly has less conspicuous slime papillae than the group centred around *H. juniperoides* (Sw.) Grolle. The stalks of the papillae in the species of the *H. juniperoides*-group, occurring in South America, can be up to 10 cells long. The species related to *H. dicranus* often have no stalked papillae or else the stalks are only 1–2 cells long, which is the case in most of the Asian species. To some extent, this feature also characterises the two larger *Herbertus* lineages in the phylogenetic analyses in study III. Most of the Asian species have few slime papillae, which are mostly not stalked, or the stalks are only one to two cells long. *H. circinatus* and *H. pilifer* have distinctive slime papillae with stalks up to eight cells long. Coarse appendages on leaf margins with slime papillae are also characteristic for *H. sendtneri*.

The study of numerous specimens confirmed that several characters, previously used in species delimitation, vary to great extent infraspecifically or overlap between species. These include, for example, leaf length, colour, bifidness, and width of the sinus. These characters have been criticized previously. Based on my study, leaf length varies greatly within Asian
Schuster (1957) illustrated leaf size variation using *Herbertus aduncus* subsp. *tenuis* (Evans) H.A. Mill. & E.B. Scott as an example. He concluded that the age of the colony strongly affects this character, more mature colonies often consisting of larger plants. Also Fulford (1963), Inoue (1977), and Van Reenen (1982) pointed out infraspecific variation in leaf size. Van Reenen (1982, fig. 15, p. 116) presented a clear illustration of variation in size of leaves. Meinunger and Köckinger (2002) stated that leaf size is dependent on habitat. Based on my study, Asian *Herbertus* species show a number of different colours, with all tones from bright or olive-green to dark green, and from orange-brown to almost black with a purple tint. However, colour can vary considerably according to habitat and is generally not useful in species identification. This has been mentioned previously by several authors (see e.g. Evans 1917, Schuster 1957, Miller 1962). An overemphasized character is the division of leaves – bifidness (Hodgetts 2001). The variation in this character between leaves of a single shoot can be remarkable, and it is very high between populations (for an illustration see Van Reenen 1982 fig. 16, p. 116). Another overemphasized character is the width of the leaf sinus, which is generally of no use in species delimitation. Inoue (1977) showed that the sinuses of Taiwanese *Herbertus dicranus* vary from 25° to 70° in a single population, and those of *H. sakuraii* (Warnst.) S.Hatt. growing in the Himalayas vary from ca. 20° to 65°. The thickness of the cell walls and the size and distinctiveness of the trigones vary considerably (Schuster 1957, Fulford 1963), but this variation is apparently more correlated with habitat conditions and altitudinal variation than with species (Schuster 1957). The red pigments and distinctive trigones that often develop in sites with ample sunlight can be judged – incorrectly – as specific characters if not enough specimens from various habitats have been studied (see I).

In studies I and II it was not possible to use scanning electron microscopy, but in other recent studies cuticle ornamentation has been used in species delimitation (So 2003, Feldberg et al. 2004, Felberg & Heinrichs 2005a,b). Previously, based on light microscopy only, the degree of roughness of the cuticle has not been found to be useful for separation of species (Evans 1917, Schuster 1957, Miller 1965). Inoue (1977) differentiated *Herbertus giraldianus* (Steph.) W.E.Nicholson primarily by its verrucose leaf cuticle. However, Long and Grolle (1990) compared specimens of *H. giraldianus* (synonymized with *H. dicranus* by Feldberg & Heinrichs 2005b) and found all degrees of verrucosity. According to Long and Grolle (1990), strong radiation at high altitudes might sometimes cause very distinctive verrucosity.

Since sporophytes occur in *Herbertus* very rarely, or are absent altogether, characters of sporophytes and reproductive structures are of little use in identification. The few
sporophytes and reproductive structures available for study showed very little variation in any of their characters. Schuster (1957) pointed out that there may be differences among subspecies, for example *H. aduncus* subsp. *hutchinsiae* produces antheridia and archegonia while *H. aduncus* subsp. *tenuis* is apparently unable to produce gametangia. The reason for this might be purely environmental rather than genetic (Schuster 1957). Descriptions of reproductive structures of *Herbertus* are rather few in the literature, e.g. by Evans (1917), Schuster (1957, 1966, 2000), Paton (1999), Hodgetts (2001), So (2003), and Feldberg and Heinrichs (2006) provide detailed studies.

Miller (1965) listed 47 species of *Herbertus* from Asia and the Pacific Islands. Combined with the results obtained by So (2003), it can now be stated that there are 16 species in the whole area of Asia, Australasia, and the Pacific Islands. This is in agreement with earlier predictions of a remarkable reduction in species number within *Herbertus* (Evans 1917, Schuster 1957, Gradstein 2001). There are still species that are morphologically distinct, but known only from type specimens, or from a few collections within a limited area (see So 2003, study II). More collections from poorly explored areas are needed to confirm or reject the specific status in these cases. Based on their nuclear ITS study, Feldberg et al. (2004) stated that morphological homoplasy and cryptic speciation are common in *Herbertus*. They further pointed out difficulties of delimiting species, especially in Asia and the Holarctic region (Feldberg & Heinrichs 2005b). Based on molecular phylogenies and morphological examination Felberg and Heinrichs (2006) came to a conclusion that only two species, *H. sendtneri* and *H. juniperoides*, can be recognized in the Neotropics, the latter one with four subspecies. The species get strong support from phylogenies, but the subspecies are for now circumscribed based mostly on morphology. The present morphological examination and the phylogenetic results of Asian *Herbertus* in the study III did not lead to as broad species concept as that used by Felberg and Heinrichs (2006). However, the morphological limits now given for the species should be further tested with more specimens from the poorly collected areas and by use of additional molecular markers.

**Phylogenetic relationships of Herbertaceae**

Vetaformaceae, Lepicoleaceae, and Herbertaceae were resolved as a monophyletic group in studies III, IV, and V which is in agreement with Heinrichs et al. (2005). The clade is among the most derived groups of the leafy liverworts; they are mostly isophyllous plants, and share bracteolar antheridia. In studies III, IV, and V, the Herbertaceae were resolved as sister to the
controversially defined Mastigophoraceae (see Grolle 1972, Inoue 1978, Schuster 1972, 1979, 1984, 1987, Crandall-Stotler & Stotler 2000). Our results confirm the view that the family Mastigophoraceae is a close relative of Herbertaceae, Lepicoleaceae, and Vetaformaceae (Grolle 1983) rather than belonging to Ptilidiineae (Schuster 1984, Crandall-Stotler & Stotler 2000). In our classification Mastigophoraceae was included in Herbertaceae as proposed by Heinrichs et al. (2005).

*Mastigophora* is a close relative of Herbertaceae, Lepicoleaceae, and Vetaformaceae. Mastigophoraceae was included in Herbertaceae as proposed by Heinrichs et al. (2005).

*Lepicolea* Dumort. is divided into two clades (see fig. 1 in III), and the results of study III indicate that stem paraphyllia might be a phylogenetically important character within the genus. Within Herbertaceae, *Mastigophora* and *Dendromastigophora* R.M.Schust. are sister to *Triandrophyllum* Fulford & Hatcher and *Herbertus*. The phylogenetic relationships within the genus *Herbertus* are not well resolved. However, *H. runcinatus* (Taylor) Kuhnem. is sister to the rest of the genus, and two other distinct lineages are recognized. One includes *H. oldfieldianus* (Steph.) Rodway, a taxon from the Azores, and South American species excluding *H. subdentatus* auct. non (Steph.) Fulford., while another clade includes all the other species of the genus. Feldberg et al. (2004) concluded that cryptic speciation causes problems in morphological definition of species within *Herbertus*, and they have recognized *H. sendtneri* (Nees) Lindb. as a species with wide distribution and much morphological variation based on their ITS analyses. However, according to study II, *H. sendtneri*, including *H. delavayi* Stephani synonymized by Inoue (1966), is morphologically a well-defined species.

The phylogeny of liverworts

*Treubia* and *Haplomitrium*

In study IV, *Haplomitrium* was resolved within the simple thalloid lineage. The same result was obtained in the studies by Stech and Frey (2001), Davis (2004), and in some of the analyses of Forrest and Crandall-Stotler (2004). It is noteworthy that those analyses did not include *Treubia*. When *Treubia* was added in study V, it appeared as sister to *Haplomitrium*, and these together were resolved as the earliest lineage of liverworts. This is in agreement with Forrest and Crandall-Stotler (2004, 2005), Crandall-Stotler et al. (2005), Heinrichs et al. (2005), Forrest et al (2006). Forrest and Crandall-Stotler (2004) specifically tested the influence of inclusion and exclusion of *Treubia* and *Haplomitrium*, and the results showed that *Haplomitrium* settled within the simple thalloids when *Treubia* was excluded. *Treubia* was always placed as the earliest diverging lineage. The Bayesian analysis in study V shows that branches leading
to both Treubia and Haplomitrium are exceptionally long, and long branch attraction cannot be ruled out as a possibility for this sister relation (see also Forrest & Crandall-Stotler 2004, Ahonen 2005). Although Treubia and Haplomitrium differ morphologically in many ways, they also share several features: tetrahedral apical cells, mucilage secretion from apically ventral slime papillae, leaf-wing origin from a single leaf initial, anacrogynous gametangial position, well-developed shoot calyptra, and the massive seta. Furthermore, they share massive blepharoplasts and spermatozoids, which have been considered to be features of old lineages of hepatics (Carothers & Rushing 1990). The phylogenetic analysis of male gametophytic characters also supported the sister relationship of these genera (Garbary et al. 1993). In study V, we have divided the liverwort phylum to three classes, Treubiopsida, Marchantiopsida, and Jungermanniopsida, reflecting phylogenetic relationships based on our multi-gene and morphological analyses. The class Treubiopsida includes the subclasses Treubiidae and Haplomitriidae. Heinrichs et al. (2005) included both Treubia and Haplomitrium within the class Haplomitriopsida; this concept was first introduced by Stotler and Crandall-Stotler (1977). Frey and Stech (2005) used different classes for both ancient lineages; Treubiopsida and Haplomitriopsida.

Blasia

Blasia was resolved as a sister to the rest of the liverworts in study IV, whereas in study V the position of Blasia was as sister to the complex thalloid liverworts. The latter position was found in various recent studies (Garbary et al. 1993, Pass & Renzaglia 1995, Davis 2004, Forrest & Crandall-Stotler 2004, 2005, Crandall-Stotler et al. 2005, Heinrichs et al. 2005, Forrest et al. 2006). The changes in the position of Blasia are simply due to significant increase in the material that we were able to include in our analyses. While there is clear conflict in terms of its position in these two studies it is not something that can be considered to be exceptional in phylogenetic analyses that always will challenge formerly proposed hypotheses. The blepharoblast ultrastructure (Pass & Renzaglia 1995), two rows of flattened multicellular scales (Schuster 1984), and Marchantialean type gametophyte-sporophyte junction with one sporophyte and two to three gametophyte placental cell layers with Marchantialean type transfer cells (Frey et al. 2001) connect Blasia to the complex thalloid liverworts. As an independent lineage having several unique morphological features, Blasiidae was recognized as a subclass in its own right within Marchantiopsida in our classification. Stech and Frey (2001) suggested the class Blasiopsida, which was included in their later classification scheme (Frey & Stech 2005), while Heinrichs et al. (2005) included Blasia in Marchantiopsida.
Complex thalloid liverworts

The sampling of complex thalloid liverworts in studies IV and V was relatively limited. In both studies, Monocleales is nested within Marchantiales, leaving Marchantiales as paraphyletic, which was also the result obtained by Wheeler (2000), Boisselier-Dubayle et al. (2002), Forrest and Crandall-Stotler (2005), Heinrichs et al. (2005), and Forrest et al. (2006). Traditionally, three orders have been recognized within complex thalloids, namely Sphaerocarpales, Monocleales, and Marchantiales (Schuster 1984), but studies IV and V do not support this view and Monoclea is included in the Marchantiales in our classification. The recognition of Sphaerocarpales is supported by distinct morphology and its position as the earliest diverging independent and strongly supported lineage within complex thalloid liverworts (V). This is in agreement with Boisselier-Dubayle et al. (2002), and Forrest and Crandall-Stotler (2005). Frey and Stech (2005) even separated an independent subclass Sphaerocarpiidae. By far the largest sampling of complex thalloids were analysed by Forrest et al. (2006). Based on equivocal results within the earliest lineages of complex thalloid liverworts, they proposed further work to resolve the relationships. However, it is totally clear, based on their analyses, that the traditional subdivision of the complex thalloids into orders Monocleales, Marchantiales, and Sphaerocarpales is not natural (Forrest et al. 2006).

Simple thalloid liverworts and Pleurozia

The traditional group of simple thalloid liverworts was resolved as paraphyletic in both studies IV and V. This surprising result has been confirmed by several other studies (Forrest & Crandall-Stotler 2004, 2005, Davis 2004, Crandall-Stotler et al. 2005, Heinrichs et al. 2005, Forrest et al. 2006).

Among anacrogynous simple thalloid liverworts, study IV separated mainly Pellia Raddi and the rest of the species as sister clades. In study V with larger sampling Pellia was resolved as the earliest diverging lineage within Jungermanniopsida, the latter consisting of a simple thalloid liverwort clade sister to the remainder of the group. In this study, the clades corresponding to Makinoa Miyake, Calycularia Mitt., Fossombroniineae, and Pallaviciniineae were also resolved. Our results showed a similar pattern to one obtained by Forrest and Crandall-Stotler (2004, 2005) and Crandall-Stotler et al. (2005), who specifically investigated relationships of simple thalloid liverworts, although in their studies Pellia appeared in the same monophyletic group as the other simple thalloids.
In study IV, *Metzgeria* Raddi and *Noteroclada* Taylor ex Hook. f. & Wilson were resolved as a sister group to the leafy liverwort *Pleurozia*. In study V *Noteroclada* was not included in the analyses. In V, the Bayesian approach resolved *Metzgeria*, *Verdoornia* R.M.Schust., *Aneura* Dumort., and *Lobatiriccardia* (Mizut. & S.Hatt.) Furuki as a sister clade to *Pleurozia*, but the results obtained with parsimony analysis using the program Nona and most of the POY analyses with direct optimization, resolved the *Metzgeria-Lobatiriccardia*–clade as sister to the leafy liverworts. Other recent phylogenetic analyses (Forrest & Crandall-Stotler 2004, 2005, Davis 2004, Crandall-Stotler et al. 2005, Heinrichs et al. 2005) have obtained the same result as our study IV and the Bayesian analysis of study V. The simple thalloid taxa in the group *Metzgeria-Lobatiriccardia* are actually acrogynous, with the archegonia restricted to highly abbreviated branches which do not grow apically after their formation (Schuster 1964). Besides this, a series of morphological characters supports the close relationships between the *Metzgeria-Lobatiriccardia*–clade and the leafy liverworts (see Forrest & Crandall-Stotler 2005, Ahonen 2005). The simple thalloid liverworts in the clade *Metzgeria–Lobatiriccardia*, and also *Pleurozia*, share a fundamental feature, the lenticular apical cell, which seems to be an old rather than a novel feature among the leafy liverworts. Furthermore, the first and second divisions of merophytes are anticlinal in *Pleurozia* and the simple thalloid hepatics, unlike in other leafy liverworts, which have periclinal second division. On the other hand, the leaf development of *Pleurozia*, involving three superficial leaf initials, resembles the ontogeny found in leafy liverworts (Crandall-Stotler 1976).

Based on our phylogenetic analyses, we have recognized in our classification (V) three subclasses within Jungermanniopsida; Pelliidae (the traditional simple thalloids), Metzgeriidae (the acrogynous simple thalloids), and Jungermanniidae (*Pleurozia* and other leafy liverworts). In our analyses (V), Pelliidae appeared as paraphyletic. However, in phylogenies based on larger sampling of simple thalloid liverworts, the group is resolved as monophyletic (Forrest & Crandall-Stotler 2004, 2005, Crandall-Stotler et al. 2005), and therefore it was considered as a subclass in our classification. Heinrichs et al. (2005) retained all the simple thalloids and *Pleurozia* within a paraphyletic Metzgeriidae. Frey and Stech (2005) elevated different lineages of simple thalloids to classes: Fossombroniopsida, Pallaviciniopsida, and Pelliopsida. Pleuroziidae and Metzgeriidae were recognized as subclasses within Jungermanniopsida.
Porellales and Jungermanniales

Studies IV and V included the largest sampling of leafy liverworts of any multi-gene phylogenetic studies by the time of their publication. The old idea of a bush-like phylogeny and homoplastic evolution in numerous suborders is rejected in the light of our results (IV, V) and other recent phylogenetic analyses (Davis 2004, Heinrichs et al. 2005, Forrest et al. 2006). The division into two clades, Porellales and Jungermanniales, was relatively well-supported; the same grouping has been found in essentially all recent analyses including leafy liverworts (Davis 2004, Forrest & Crandall-Stotler 2004, 2005, Crandall-Stotler et al. 2005, Heinrichs et al. 2005, Forrest et al. 2006). The plants in Porellales are epiphytic, anisophyllous, have incubic leaf orientation, and also share other morphological characters. Ahonen (2005) discussed Porellales in detail in her thesis.

The first diverging lineage of Jungermanniales was the well-supported Schistochilaceae, well distinguished also by its morphology (Schuster & Engel 1977, 1985). The relationships among the rest of the leafy liverworts were clarified in study V. Three larger monophyletic clades were resolved, principally the same groupings as were found in the analyses by Davis (2004), Heinrichs et al. (2005), and Forrest et al. 2006. There are no obvious morphological synapomorphies for any of the three major clades, namely Cephaloziineae, Jungermanniineae, and Lophocoleiineae. Our results suggest that the jungermannioid liverwort originally was anisophyllous, branched only from lateral merophytes, had bilobed leaves, scattered rhizoids, a massive seta, and an extensive haustorial foot. In contrast to the Porellales, most of the Jungermanniales grow on soil, peat, decaying wood, or rock substrate in forests or exposed habitats. The families of the traditionally defined suborders (Schuster 1972, 1984, Crandall-Stotler & Stotler 2000) are mixed in our topologies.

Conclusions

The main results of this thesis are the revision of the taxonomy of Asian Herbertus and the resolution of the phylogenetic position and relationships of Herbertaceae. According to my study, species of Herbertus can be morphologically delimited, despite of the great morphological variation they show. Surprisingly Herbertaceae was resolved as one of the most derived leafy liverwort families. Mastigophoraceae was confirmed as belonging to Herbertaceae. This study demonstrated that it is still necessary and valuable to patiently examine the morphology of plants in addition to making use of the massive amount
of information obtained from sequence level characters. Our multi-gene liverwort analyses were the largest by the time of their publication. The major lineages recognized are *Treubia-Haplomitrium* (Treubiopsida), *Blasia* together with the complex thalloid liverworts (Marchantiopsia), and the simple thalloid and leafy liverworts (Jungermanniopsida), including the orders Pelliales, Fossombroniales, Metzgeriales, Pleuroziales, Porellales, and Jungermanniales. The proposed new classification reflects the phylogenetic relationships of liverworts. Hypotheses of relationships between the larger groups of liverworts in all recent, numerous phylogenetic studies appear to be relatively similar, despite different methods used. Although major part of the liverwort genera are still lacking from the recent analyses, all the major lineages are represented. The new classification is needed, because our hypotheses of liverwort evolution have dramatically changed by the use of molecular characters. We are close to learning the secrets of the phylogeny of these early embryophytes and unravelling the signal hidden in their DNA. In the near future we can expect to obtain even larger amounts of information from whole genomes supplemented with wider sampling of the terminals, which undoubtedly will help us to understand the evolution of the earliest embryophytes even better. Resolving the evolutionary history of liverworts will involve a multidisciplinary, highly integrated approach that combines ultrastructural, anatomical, and developmental characters with those obtained from nucleotide sequences and genome structures.

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