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Phylogeny of Vetaformaceae, Lepicoleaceae and Herbertaceae (including Mastigophoraceae) inferred from chloroplast *trn*L-F, nuclear ITS2, and morphology

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Phylogenetic relationships of Vetaformaceae, Lepicoleaceae, and Herbertaceae (including Mastigophoraceae) were reconstructed using chloroplast region *trn*L-F, nuclear ITS2, and 27 morphological characters. Forty-five species were included in the analysis, of which 37 belong to the ingroup. The data sets were analyzed simultaneously with direct optimization, as implemented in the program POY. The results confirm the sister relationships of Vetaformaceae and Lepicoleaceae as well as of Herbertaceae and former Mastigophoraceae. Within *Lepicolea* the species are divided into two sister clades. *Herbertus runcinatus* is sister to the rest of the genus. A clade of *H. oldfieldianus*, South American species and a species from the Azores form a separate lineage. The rest of the *Herbertus* species are grouped together but many unresolved nodes remain.

Key words: Herbertaceae, ITS2, Lepicoleaceae, Mastigophoraceae, phylogeny, systematics, *trn*L-F, Vetaformaceae

Introduction

The recent comprehensive molecular phylogenies of liverworts by He-Nygrén *et al*. (2004, 2006) and Heinrichs *et al*. (2005) have resolved the families Vetaformaceae, Lepicoleaceae, Mastigophoraceae, and Herbertaceae as a monophyletic group. The group shares the possession of antheridia enclosed by male bracteoles. Furthermore, the liverworts in the group are mostly isophyllous. As several studies using different datasets have produced the same topology and there is support from morphology, it is justified to assume the families as the ingroup. Contrary to earlier classifications based on morphology (Grolle 1983, Schuster 1984, 2000, Crandall-Stotler & Stotler 2000), the recent analyses that included sequence level data have resolved the four families as the most derived lineage of leafy liverworts (He-Nygrén *et al*. 2004, 2006, Heinrichs *et al*. 2005). According to these studies, Herbertaceae and Mastigophoraceae are most closely related to some lepicoleoid species, with the Vetaformaceae as sister group to Lepicoleaceae, and Mastigophoraceae sister to Herbertaceae. However, the sampling of the families

Vetaformaceae, Lepicoleaceae, Mastigophoraceae, and Herbertaceae in both studies referred to above is restricted. This study aimed to test the presented hypothesis with more extensive sampling of these families.

Vetaforma dusenii, the only species of Vetaformaceae, has been found in Argentina and Chile. The monogeneric Lepicoleaceae occur mostly in the southern hemisphere. Many species have been recorded for South America and Australasia, but only one occurs in Africa (Schuster 2000). *Lepicolea* was revised by Scott (1960), who reported seven species. Currently at least 10 species are recognized (Schuster 2000).

In Mastigophoraceae two genera have been recognized. *Dendromastigophora* is a New Zealand endemic. The genus *Mastigophora* has a wide, disjunct range and is found in British Columbia, mid-Atlantic islands, and also from Oceanic Europe to the Himalayas, China and to Australasia (Schuster 1987). Herbertaceae have been divided into two genera, *Triandrophyllum* and *Herbertus*. *Triandrophyllum* is a small southern temperate genus (Gradstein & da Costa 2003), while *Herbertus* has a circumpacific range with extensions to the Appalachian mountains, tropical Africa and Europe (Schuster 2000). The taxonomy of the genera remains mostly unresolved.

Mastigophora consists of three species. Monotypic *Dendromastigophora* was separated from *Mastigophora* by Schuster (1987). *Herbertus* is the largest genus of the group under consideration. The number of species in *Herbertus* remains uncertain; Gradstein (2001) gave an estimate of ca. 25 species worldwide. *Triandrophyllum* consists of 4–5 species of uncertain delimitation (Schuster 2000). Gradstein (2001) reduced *Olgantha*, a third genus recognized in Herbertaceae by Schuster (1996, 2000), to synonymy under *Triandrophyllum*.

The taxonomic position of the four families studied vary in recent classifications. Grolle (1983) placed all four in the order Jungermanniales. In Schuster's (1984, 2000) classification scheme Vetaformaceae, Lepicoleaceae, and Herbertaceae follow *Haplomitrium*, which he considered to be the most primitive extant liverwort. They are all isophyllous, which Schuster (1984) believed to be a primitive feature. Schuster (1984) placed Mastigophoraceae in suborder Ptilidiineae, which he considered to be derived. Crandall-Stotler and Stotler (2000) classified Mastigophoraceae in the primitive Ptilidiineae (Lepicoleales), and Vetaformaceae and Lepicoleaceae in the closely related Lepicoleiineae (Lepicoleales), but Herbertaceae were in a more derived sub-order Herbertineae (Jungermanniales). He-Nygrén *et al*. (2006) placed all the families in their Lophocoleineae in Jungermanniales.

The relationships of Mastigophoraceae have been especially controversial. Inoue (1978) supported Grolle's (1972) view of *Mastigophora* belonging to Lepicoleaceae subfamily Mastigophoroidea based on his observations of the sporophyte characters, which were formerly unknown. However, Schuster (1987) rejected that placement and reiterated his earlier idea (Schuster 1972, 1979, 1984) of classifying Mastigophoraceae in Ptilidiineae, justifying this by virtue of the similarities of Mastigophoraceae and Ptilidiaceae in their leaf division, incubous orientation of leaves, and anisophylly. He also presented a detailed discussion on the morphological differences of Lepicoleaceae and Mastigophoraceae (Schuster 1987). In the classification by Crandall-Stotler and Stotler (2000) Mastigophoraceae remained in the suborder Ptilidiineae, which was, however, considered as a primitive suborder among leafy liverworts. Recently, Heinrichs *et al*. (2005) proposed to include Mastigophoraceae in the family Herbertaceae, based on chloroplast *rbc*L data. He-Nygrén *et al*. (2006) confirmed this result in their classification, which was based on phylogenetic analysis of numerous genes and morphology.

In this study I address the following questions: (1) Are the families Vetaformaceae, Lepicoleaceae, and Herbertaceae, including Mastigophoraceae, monophyletic, and what are their phylogenetic relationships? (2) Are the genera included in this study monophyletic? (3) Can any infrageneric relationships within the two larger genera, *Herbertus* and *Lepicolea* be proposed? (4) Are there any evolutionary trends in morphological characters within the group? In order to obtain answers to these questions the *trn*L-F region of the chloroplast and ITS2 region of the nuclear genome were sequenced, and 27

morphological characters examined and included in a combined cladistic analysis (Kluge 1989, Nixon & Carpenter 1996).

Material and methods

Selection of terminals

Altogether 45 exemplars were studied, 37 belonging to the ingroup and eight to the outgroup (Table 1). The authors for the included species are given in Table 1. Representatives of all species of the ingroup genera for which I had recently collected specimens available were included. For *Herbertus aduncus* and *H. dicranus*, two species with wide ranges and large morphological variation, different subspecies or samples from different geographic regions were included.

According to the results of He-Nygrén *et al.* (2004, 2006) the Trichocoleaceae, Plagiochilaceae, and Geocalycaceae form a sister group to the ingroup families. I included representatives (*Trichocolea tomentella*, *Plagiochila asplenioides*, *Chiloscyphus profundus*) of each of the abovementioned families in the outgroup. Additional outgroup species *Blepharostoma trichophyllum*, *Jungermannia leiantha*, *Temnoma pilosum*, *Hygrolembidium acrocladum*, and *Ptilidium pulcherrimum* represent lineages of phylogenetically more distant leafy liverwort groups. In a recent liverwort classification by Crandall-Stotler and Stotler (2000), the monogeneric Chaetophyllopsidaceae was considered closely related to Mastigophoraceae, Vetaformaceae, and Lepicoleaceae. However, *Chaetophyllopsis* was not included here as the results of He-Nygrén *et al.* (2004, 2006) and Heinrichs *et al.* (2005) show *Chaetophyllopsis* as being distantly related to the species in the present study.

Morphological data

The morphological data set included 27 characters (Appendix 1). For the gametophytic characters the morphological matrix was compiled using the specimens used for DNA extraction. Sexual organs were observed from additional specimens available in the Botanical Museum (H) of the Finnish Museum of Natural History, and also loaned material from various herbaria. In many cases, however, no specimen contained sexual organs, and the coding was done according to literature (Schuster 1959, 1966, 1987, 2000, 2002, Scott 1960). The characters of the matrix are often used in familial or generic delimitations of the ingroup. However, no *a priori* assumptions of informative characters were made and all the characters were coded as unordered. No sporophyte characters were included, because for most of the taxa the sporophytes are unknown. Quantitative characters were generally excluded, except for those showing clear discontinuities (characters 11, 14, and 18). The morphological matrix is given in Appendix 2.

Molecular data

Two mostly non-coding DNA sequence regions were used for the phylogeny reconstruction, chloroplast *trn*L-F and nuclear ITS2. The primers used for sequencing the *trn*L-F region were C and F (Taberlet *et al.* 1991). For ITS2 region primers 5.SR http://www.biology.duke.edu/fungi/mycolab/primers.htm and LC4-R (Shaw 2000) or in some cases universal primers ITS3 and ITS4 (White *et al.* 1990) were used. Chloroplast *trn*L-F region includes the partial sequence of Leucine transfer RNA (UAA), an intergenic spacer, and a partial sequence of Phenylalanine transfer RNA (GAA). Most of the length variation is concentrated in the *trn*L intron, and some in the non-coding *trn*L-F spacer. One out of 45 sequenced taxa were lacking the *trn*L-F sequence (2%). Nuclear Internal Transcribed Spacer 2 (ITS2) is situated between the ribosome coding 5,8S and 26S genes. ITS2 sequence was lacking for 17 out of 45 taxa (38%).

DNA extraction, PCR and sequencing

The sequences were produced in two different laboratories, *trn*L-F sequences mainly in the Laboratory of Molecular Plant Systematics, NHN-Utrecht branch, here referred to as U, and ITS2 sequences mainly in Molecular Ecology

Table 1. The GenBank accession numbers for the sequences used in the phylogenetic analysis. The accession numbers for sequences taken from GenBank are shown in italics. The accession numbers published in He-Nygrén *et al.* (2004, 2006) are marked with asterisk. The voucher specimens for novel sequences herein, as well as references for sequences taken from the GenBank are listed under vouchers. Liverworts are arranged to families according to He-Nygrén *et al.* (2006) classification.

Species	*trn*L-F	ITS2	Voucher specimen for sequences
Vetaformaceae			
Vetaforma dusenii (Steph.) Fulford & J.Taylor	AY463593*	DQ293965	Chile, *Engel 11423* (H)
Lepicoleaceae			
Lepicolea attenuata (Mitt.) Steph.	*AY007629*		Stech & Frey 2001
Lepicolea ochroleuca (L.f. *ex* Spreng.) Spruce	AY463566*	DQ293961	Peru, *Frahm et al. s.n.* (H)
Lepicolea pruinosa (Taylor) Spruce	AY463567*		
Lepicolea ramentifissa Herzog	DQ293988		Venezuela, *Griffin & López F. PV-1419* (H)
Lepicolea rara (Steph.) Grolle	DQ293989		Papua New Guinea, *Norris 62782* (H)
Lepicolea scolopendra (Hook.) Dumort. *ex* Trevis.	AY463568*		
Herbertaceae			
Dendromastigophora flagellifera (Hook.) R.M.Schust.	AY463555*		
Herbertus acanthelius Spruce	DQ293966		Ecuador, *Buck 10228* (H)
		AJ783339	Feldberg *et al.* 2004
Herbertus aduncus Gray subsp. *aduncus* China	DQ293967	DQ293945	China, *Koponen et al. 51204*
Herbertus aduncus Gray subsp. *aduncus* Usa	DQ293969		United States, *Norris 88922* (UC)
Herbertus aduncus Gray subsp. *hutchinsiae* R.M.Schust.	DQ293968	DQ293946	Wales, *Juslén 1685*
Herbertus armitanus (Steph.) H.A.Mill.	DQ293970		Papua New Guinea, *De Sloover 43158* (H)
Herbertus circinatus (Steph.) H.A.Mill.	DQ293972		Papua New Guinea, *De Sloover 42753* (H)
Herbertus dicranus Nepal (Taylor *ex* Gottsche) Trevis.	AY463559*		Nepal, *Long 17542* (H)
Herbertus dicranus China (Taylor *ex* Gottsche) Trevis.	DQ293973		China, *Koponen et al. 50760*
Herbertus divergens (Steph.) Herzog	DQ293974	DQ293948	Panama, *Salazar & Gradstein 9286* (H)
Herbertus gracilis (Mont.) Steph.	DQ293975	DQ293949	Hawaii, *Weber & Randolph B-89,875* (UC)
Herbertus grossispinus (Steph.) Fulford	DQ293976	DQ293950	Ecuador, *Gradstein et al. 6822* (H)
Herbertus juniperoideus (Sw.) Grolle	DQ293977		Honduras, *Allen 12097* (H)
Herbertus kurzii (Steph.) H.A.Mill.	DQ293978	DQ293951	Nepal, *Long 30447* (H)
Herbertus longifissus Steph.	DQ293979	DQ293952	Papua New Guinea, *Norris 63194* (H)
Herbertus longispinus Jack & Steph.	DQ293980	DQ293953	Vietnam, *Averyanov et al. BR 015* (MO)
Herbertus oldfieldianus (Steph.) Rodway	DQ293981	DQ293954	New Zealand, *Glenny 8857* (H)
Herbertus pilifer Schiffn.		DQ293955	Papua New Guinea, *Kluiving 1453* (H)
Herbertus ramosus (Steph.) H.A. Mill.	DQ293982	DQ293956	Vietnam, Averyanov *et al.* BR 020 (MO)
Herbertus runcinatus (Taylor) Herzog	DQ293983		Chile, *Hyvönen 2960* (H)
Herbertus sendtneri (Nees) Lindb.	DQ026620*		Bhutan, *Long 28864* (H)
Herbertus sp1.	DQ293984	DQ293957	Vietnam, *Harder et al. 5093* (MO)
Herbertus sp2.	DQ293971	DQ293947	Azores Islands, *Flores D5*
Herbertus stramineus (Dumort.) Lett.	DQ293985	DQ293958	Norway, *Hassel* (H)
Herbertus '*subdentatus' non* (Steph.) Fulford	DQ293986	DQ293959	Tanzania, *Pòcs et al. 88123/D* (H)
Herbertus '*subdentatus*' *non* (Steph.) Fulford	DQ293987		Venezuela, *Griffin & López F. PV-610* (H)
		AJ413177	Heinrichs *et al.* 2002

Mastigophora diclados (Brid. *ex* F. Weber) Nees	DQ293990			Seychelles Islands, *Pòcs 9320/D* (H)
Mastigophora woodsii (Hook.) Nees	AY463574*	DQ293962		Australia, *Frahm*, CANB639918
Triandrophyllum heterophyllum (Steph.) Grolle	DQ293991	DQ293963		Papua New Guinea, *Koponen 32208*, (CANB)
Triandrophyllum subtrifidum (Hook. & Taylor) Fulford & Hatcher	AY463589*	DQ293964		Chile, *Hyvönen 5334* (H)
Ptilidiaceae				
Ptilidium pulcherrimum (Weber) Hampe	*AY251186*	*AY257506*		Ahonen 2004
Jungermanniaceae				
Jungermannia leiantha Grolle	*AY149857*			He-Nygrén & Piippo 2003
		AY316351		Ahonen 2004
Trichocoleaceae				
Blepharostoma trichophyllum (L.) Dumort.	AY463548*	DQ293944		Finland, *He-Nygrén & Piippo 1471*
Temnoma pilosum (A. Evans) R.M. Schust.	AY463588*			
Trichocolea tomentella (Ehrh.) Dumort.	AY463590*			
Geocalycaceae				
Chiloscyphus profundus (Nees) J.J. Engel & R.M. Schust.	*AY149874*			He-Nygrén & Piippo 2003
		AJ422240		Heinrichs 2002
Plagiochilaceae				
Plagiochila asplenioides (L.) Dumort.	*AY149858*			He-Nygrén & Piippo 2003
		AJ414268		Renker *et al.* 2002
Lepidoziaceae				
Hygrolembidium acrocladum (Berggr.) R.M. Schust.	AY463560*	DQ293960		Australia, *Streimann 16733* (H)

and Systematics Laboratory of University of Helsinki (MES). The isolation of the DNA was mainly performed according to the CTAB technique described in Doyle and Doyle (1990), and the extracted DNA was purified with Promega Wizard Purification standard (Promega) (U). In some cases extraction was performed with Nucleospin Plant DNA Extraction Kit (Machery Nagel), following the manufacturer's instructions (MES). The polymerase chain reactions and cycle sequencing reactions were executed with GeneAmp PCR System 9700 of Applied Biosystems (U), or, Master cycler gradient of Eppendorf or PTC-200 of MJ Research (MES). The PCR settings for *trn*L-F were 5 min. 94 °C, 28 cycles (1 min. 94 °C, 1 min. 50 °C, 2 min. 72 °C) and a 10 min. 72 °C extension time, and for ITS2 10 min. 95 °C, 35 cycles (1 min. 95 °C, 1 min. 49 °C, 1 min. 72 °C), and a 7 min. 72 °C extension time. The PCR products were purified with QIAquick PCR Purification Kit (Qiagen), DNA Clean & Concentrator™-5 kit (Zymo research) (U), or GFX-PCR-DNA and gel Purification Kit (Amersham Biosciences) (MES). The cycle sequencing reactions were prepared using Big Dye 3.1 (Applied Biosystems) (U) and Big Dye 1.0 (Applied Biosystems) (MES) following the manufacturer's instructions. Cycle sequencing was performed using the following programs: in U 25 cycles (10 s 94 °C, 5 s 50 °C, 2 min. 72 °C) and 10 min. 72 °C extension and in MES 30 cycles (30 s 96 °C, 16 s 50 °C, 4 min. 60 °C) and 5 min. 15 °C. The sequencing products were purified with Millipore multiscreen sephadex filter for 96-well plates (U) and with Millipore Nucleospin sequencing reaction purification kit (MES). The sequencing products were resolved on the ABI 3700 (U) and MegaBACE (MES) sequencing machines. All the sequences were submitted to GenBank. A list of the accession numbers and voucher specimens is given in Table 1.

Phylogenetic analyses

Phylogenetic analyses were performed using direct optimization (Wheeler 1996), as implemented in POY (Wheeler *et al.* 2003). In direct optimization alignment and phylogenetic anal-

ysis are performed simultaneously. In POY, unlike in traditional methods, indels are treated as transformations and not as character states. Furthermore, in optimization alignment numerous alternative hypotheses of primary homology are tested during the analysis in order to find the most parsimonious tree(s) and alignment(s). Therefore, the resulting equally parsimonious trees are based on different character optimizations, unlike in conventional phylogenetic analyses that are based on static, and possibly non-optimal alignments. Character optimizations for each tree are given as a result of analysis as an implied alignment. The method is further explained for example in Wheeler (1996) and Schulmeister *et al*. (2002).

Phylogenetic analyses were performed with a parallel version of POY using 8 processors of 1.1 GHz each in CSC, the Finnish IT center for science (www.csc.fi). In order to save computation time and memory, *trn*L-F sequences were cut into three shorter fragments (*see* Wheeler *et al*. 2003). This was done in conservative regions within the *trn*L intron and between the *trn*L intron and exon 2 based on a preliminary Dialign 2 (Morgenstern 1999) alignment of sequences. The length variation observed at the beginning and end of sequences was filled with X's to avoid leading and trailing gaps, which would otherwise require a special cost to be assigned to them (*see* Schulmeister *et al*. 2002). As a kind of sensitivity analysis (*see* Wheeler 1995) the effects of different gap costs on the topology have often been used in phylogenetic analyses using direct optimization (*see* e.g. He-Nygrén *et al*. 2004, Huttunen & Ignatov 2004, Quandt *et al*. 2004). However, the necessity and value of such weighting has recently been criticized (Frost *et al*. 2001b, Grant & Kluge 2003, Huttunen 2004), and it has been claimed that only equal weighting is justified (Frost *et al*. 2001a). Thus, here the analyses were performed only using the equal weighting for all character changes. In the present study, the gaps and substitutions were weighted equally. The numerically coded morphological data is treated as if prealigned in the POY analysis (Wheeler *et al*. 2003). The command line applied was as follows: *poy -parallel -solospawn 7 -molecularmatrix Filename -norandomizeoutgroup -holdmaxtrees 20 -maxtrees 5 -nodiscrepancies -fitchtrees -random 250 -multibuild 50 -seed -1 -slop 3 -checkslop 30 -tbr -spr -sprmaxtrees 5 -drifttbr -numdrifttbr 5 -fuselimit 25 -fusingrounds 1 -impliedalignment -indices > Outfile*.

In order to evaluate the support for different clades, Bremer support values (Bremer 1994) were calculated for internal nodes, also with the program POY. The commands are the same as for the analysis but the commands *-bremer* and *-constrain* (with the necessary filename) were added. The constraint file of the output topologies of the original analysis is needed for calculating bremer supports. It was created with the program JACK2HEN, which is a supplementary program to POY and available from the same source. As compared with calculating Bremer support by consensing ever more inclusive sets of suboptimal trees, the approach used by POY is faster but it may overestimate group support (Wheeler *et al*. 2003).

Results

In the *trn*L-F data set the shortest sequence was that of *Dendromastigophora flagellifera* with 454 bp and the longest that of *Blepharostoma trichophylla* with 531 base pairs. In ITS2, there was length variation from 273 bp in *Plagiochila asplenioides* to 320 bp in *Hygrolembidium acrocladum*. The length variation of *trn*L-F region was in the *trn*L intron and in the intergenic spacer. One of the resulted POY implied alignments was studied as static alignment and indels treated as equal to base substitutions. It showed that in the *trn*L-F data set 36% of base positions were parsimony informative, and in ITS2 data set 45% (Table 2). Within the genus *Herbertus* the number of parsimony informative positions was relatively low: ca. 10% in *trn*L-F data, and 14% in ITS2 data (Table 2). With the combined molecular and morphological data set POY gave eight equally parsimonious trees of length 1922 steps. The analysis took ca. 80 hours and 183 692 990 alignments were evaluated.

The families Vetaformaceae, Lepicoleaceae, former Mastigophoraceae, and Herbertaceae are all resolved as monophyletic. Vetaformaceae appear as an independent lineage as a sister to

Lepicoleaceae, and the former Mastigophoraceae are placed as a sister to Herbertaceae. All of the six genera included in the ingroup appear as monophyletic. In the Lepicoleaceae, the sole genus *Lepicolea* appears as two distinct lineages as follows: *L. rara* is a sister to *L. pruinosa* and *L. ramentifissa* while *L. ochroleuca* is a sister to *L. attenuata* and *L. scolopendra*. The monotypic genus *Dendromastigophora* appears as the sister lineage to two species of the genus *Mastigophora*. *Triandrophyllum* is a sister to *Herbertus*. Within *Herbertus*, *H. runcinatus* is a sister to all other species sampled. Furthermore, the analyses resolved *H. oldfieldianus* as sister to a group of South American species and a specimen from the Azores. Within this clade *H. grossispinus* and *H. divergens* are sister species. The specimen from the Azores is a sister to *H. acanthelius* and *H. juniperoideus*. The relationships of the remaining species are not unambiguously resolved, as six nodes are collapsed in the strict consensus tree (Fig. 1). However, some pairs of sister species occur in all of the equally most parsimonious trees (*see* Fig. 1). The analysis without ITS2 data resulted in 20 equally parsimonious trees 1020 steps long. The strict consensus of those was similar to strict consensus of combined data except that the position of *H. oldfieldianus* was unresolved between the two larger clades of *Herbertus*. Furthermore, the largest clade of *Herbertus* was totally unresolved.

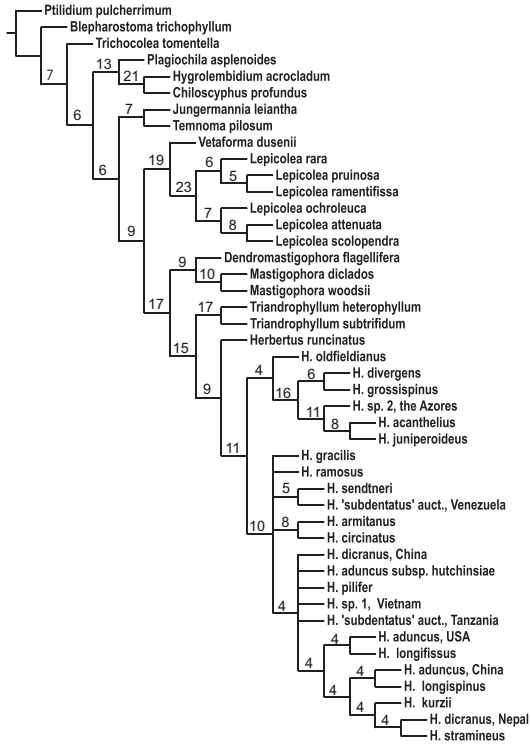

Fig. 1. Strict consensus tree of eight equally parsimonious trees obtained using POY with combined data. The numbers above the nodes are the Bremer support values.

The relationships of the families and genera in the present topologies from POY analysis were similar to topologies obtained from NONA, MrBayes, and POY analyses based on multigene and morphology data by He-Nygrén *et al.* (2004, 2006) as well as to those obtained from maximum likelihood analyses based on *rbc*L data (Heinrichs *et al.* 2005).

Discussion

Methodology

Direct optimization is a relatively novel method (Wheeler 1996), so far implemented only in one program, POY (Wheeler *et al.* 2003). From the beginning, it has been used largely in studies of arthropod phylogeny (e.g. Wahlberg & Zimmermann 2000, Wheeler *et al.* 2001, Giribet *et al.* 2002, 2003). The number of published studies in other fields of systematics is, however, increasing rapidly (e.g. Stenroos *et al.* 2002, Giannini & Simmons 2003, Huttunen & Ignatov 2004, He-Nygrén *et al.* 2004, 2006). Direct optimization and POY are discussed by several authors (e.g. Schulmeister *et al.* 2002, Huttunen 2004).

Table 2. Number of characters and parsimony informative (pi) positions in POY character optimization for genomic regions *trn*L-F and ITS2 in the whole data set and within the genus *Herbertus*. Indels were treated as equal to base substitutions.

	Number of terminals	Number of characters	Number of pi positions
*trn*L-F	44	686	247
*trn*L-F (*Herbertus*)	24	686	72
ITS2	28	524	237
ITS2 (*Herbertus*)	17	524	74

The results of our other phylogenetic studies (He-Nygrén *et al*. 2004, 2006) and my own preliminary analyses showed that other gene regions generally used in phylogenetic studies of bryophytes such as e.g. *rbc*L, *rps*4, and *psb*T-H do not show enough variation to be useful at this level. In the preliminary analyses nuclear ITS1 region always showed double bands when the PCR reactions were run in the agarose gel. Therefore it was considered too time-consuming and expensive to be used in the study. For studying phylogenetic relationships between families and genera the *trn*L-F and ITS2 regions seemed ideal. However, several equally parsimonious solutions and low support values (4) of some clades reveal that even these highly variable regions may not provide enough variation to unambiguously resolve the infrageneric relationships between all members of the genus *Herbertus*. Although missing ITS2 sequences have caused partly the low resolution within *Herbertus* (*see* also the beginning of the chapter Results).

On phylogenetic relationships of the families

The present results on the relationships between Vetaformaceae, Lepicoleaceae, and Herbertaceae including Mastigophoraceae support He-Nygrén *et al*. (2004, 2006) and Heinrichs *et al*. (2005). A notable morphological feature shared by the whole group is possession of antheridia enclosed by male bracteoles. However, bracteolar antheridia also occur in a few other genera of leafy liverworts, such as *Schistochila* and *Balantiopsis*. The phylogeny by He-Nygrén *et al*. (2006) resolved *Schistochila* as sister to the rest of the Jungermanniales, whereas the ingroup of the present study was derived. Therefore, the bracteolar antheridia could be a primitive character mostly lost in other Jungermanniales, or it could have developed repeatedly.

The topologies obtained support a Gondwanalandic origin of the Vetaformaceae–Lepicoleaceae–Herbertaceae clade. All terminals from the northern hemisphere, possibly of Laurasian origin, are in the group ranging from *Herbertus gracilis* to *H. stramineus*. This clade is in apical position in the trees, which indicates a more recent radiation among Laurasian species in *Herbertus*. Fossils do not offer any insights into the age of the ingroup, as there are no fossil records that resemble any of the taxa. The apical position of Vetaformaceae–Lepicoleaceae–Herbertaceae in the phylogenies by He-Nygrén *et al*. (2004, 2006) and Heinrichs *et al*. (2005) suggest that the clade has evolved rather recently. Furthermore, Feldberg *et al*. (2004) claimed that low ITS variation indicates a recent radiation of *Herbertus*. Similarly, the results of the present study show relatively low variation in *trn*L-F and ITS2 (*see* Table 2).

The genus *Grollea* was initially assumed to be closely related to the Herbertaceae or *Anthelia* (Schuster 1964), but was later placed in Blepharostomataceae (Schuster 1972). Hässel de Menendez (1980) moved *Grollea* to Antheliaceae. Recently it has been treated as a family of its own, Grolleaceae, within suborder Herbertineae (Crandall-Stotler & Stotler 2000, Schuster 2000) or within suborder Lophocoleineae (He-Nygrén *et al*. 2006). Unfortunately material of the rare *Grollea* has not been available for sampling in any phylogenetic studies, that have included sequence level data, and its relationships thus remain to be tested.

On Vetaformaceae and Lepicoleaceae

Vetaformaceae have been widely accepted as the group most closely related to Lepicoleaceae (Grolle 1983, Schuster 1984, 2000, Crandall-Stotler & Stotler 2000, He-Nygrén *et al*. 2006), which is also supported by results of this analysis. The Vetaformaceae–Lepicoleaceae clade shares the coelocaule, a structure derived from both stem and archegonium tissue protecting the developing sporophyte. The family Vetaformaceae, consisting only of *Vetaforma dusenii* was described by Fulford and Taylor (1959) and has been treated as a separate family in all the recent liverwort classifications. However, the sporophyte of *Vetaforma* has not been found and the final phylogenetic conclusions should be drawn only after its discovery (Schuster 2000). Morphologically *Vetaforma* is quite distinct from the rest of the ingroup in that it is the only species that has retained lateral intercalary branching. Furthermore, it lacks the

distinctly differentiated stem cortex cells found in other species of the ingroup.

Lepicoleaceae clade is one of the best supported clades in my analyses (Bremer support value 23). The family shares the character of having female bracts distinctly smaller than vegetative leaves. Within *Lepicolea* the clade of *Lepicolea rara*, *L. pruinosa*, and *L. ramentifissa* is distinguished by the presence of stem paraphyllia. In her revision of *Lepicolea*, Scott (1960) defined *L. rara* (as *L. loriana* = *L. rara*, synonymized by Piippo 1984) broadly including plants with and without stem paraphyllia, stating "paraphyllia usually present" (Schuster 2000). The specimen used in the present study has paraphyllia. Schuster (2000) anticipated a critical study of *Lepicolea* and especially *L. rara*. The present phylogeny suggests that the paraphyllia of the stem might be a structure of phylogenetic importance within *Lepicolea*. The presence of laciniae or cilia in primary divisions of leaves appeared as a homoplastic character. Strongly ciliate *L. ramentifissa* and *L. pruinosa* were resolved as sister species, while ciliate *L. ochroleuca* was placed separately as a sister to *L. atteanuata* and *L. scolopendra*.

Infrageneric relationships within *Lepicolea* seem to partially reflect geographical ranges, although the South American species are not all grouped together. Sister species *L. ramentifissa* and *L. pruinosa* are found exclusively in South America while their sister species *L. rara* is distributed in the Philippines, Indonesia, New Guinea, and the Pacific Islands, as well as in southern Chile. *L. ochroleuca* has its main distribution area in South America, but it extends to Africa as well as the only African species of the genus. Sister species *L. scolopendra* and *L. attenuata* occur in Australasia, but the range of *L. scolopendra* extends also to South America.

On Herbertaceae (including Mastigophoraceae)

The phylogenetic relationships of the former Mastigophoraceae have remained controversial (Inoue 1978, Grolle 1983, Schuster 1987, Crandall-Stotler & Stotler 2000), but recent studies by He-Nygrén *et al*. (2004, 2006) and Heinrichs *et al*. (2005) presented a novel hypothesis in which Mastigophoraceae are sister to Herbertaceae. The results of the present study confirm this sister relationship with relatively high Bremer support value (17).

Mastigophoraceae and Herbertaceae have many morphological similarities. The epidermal cells of the capsule wall bear "nodular" (vertical) thickenings in all but the shortest radial walls of Mastigophoraceae, and in Herbertaceae the cells of the outer layer also have nodular thickenings (*see* illustrations e.g. in Schuster 1987: 740, fig. 2: 10 Mastigophoraceae, and Schuster 2000: 98, fig. 18: 7 Herbertaceae). Conspicuous leaf cell trigones are characteristic for Mastigophoraceae (Schuster 1987), as well as for *Herbertus*, even though the cell walls of *Triandrophyllum* leaves lack trigones (Schuster 2000). Besides the development of asymmetrically trifid leaves (which also exist in *Triandrophyllum* of Herbertaceae) the incubous orientation of leaves and isophylly in Mastigophoraceae and Ptilidiaceae have been emphasized as evidence of affinity (Schuster 1987). The incubous orientation of leaves is also a character of Herbertaceae (Schuster 2000, Heinrichs *et al*. 2005). Heinrichs *et al*. (2005) also pointed out the shared occurrence of herbertane-type sesquiterpenes by the Mastigophoraceae and Herbertaceae (Harinantenaina & Asakawa 2004). Thus, inclusion of Mastigophoraceae in Herbertaceae seems to be very well justified.

Triandrophyllum and *Herbertus* were resolved as a well-supported clade (Bremer support 15). They both lack terminal branching. However, the branching pattern in *Herbertus* is ambiguous. Fulford (1963) reported at least occasional *Frullania*-type terminal branching for *H. juniperoideus*, *H. grossispinus*, and *H. subdentatus* in her study of South American species. In his study of South American species, Gradstein (2001) also recorded *Frullania*-type branches for Herbertaceae and *Herbertus* in the family and generic level descriptions. On the other hand, Van Reenen (1982) described only *Bazzania*-type (= ventral intercalary) branching for species occurring in Colombia, including *H. juniperoideus*. I coded terminal branching as absent based on the specimens that I was able to study, including those from South America. Furthermore, lateral intercalary branching in

Herbertus remains questionable. Schuster (1966, 2000) has studied two species, the North American *H. aduncus* and the New Zealand *H. alpinus* (= *H. oldfieldianus* synonymized by So 2003), extensively. He reported rare lateral intercalary branching in generic descriptions in both studies, but again, I have coded it absent as not seen.

On *Herbertus*

Herbertus runcinatus alone was resolved as a sister to the rest of the genus. Its distribution is restricted to Patagonia, Chile. The other species sampled occur in two clades, one consisting of *H. oldfieldianus* from New Zealand, four South American species and one species from the Azores, and one comprising the rest of the genus. The latter clade includes species occurring in Europe, North America, Asia, New Guinea, and Hawaii, and one species, *H. subdentatus* auct. (= *H. sendtneri sensu* Feldberg *et al*. 2004), from South America. In the well-supported (Bremer support 16) clade consisting of the South American exemplars and the species from the Azores, most members have a distinctive vitta that covers a large part of the basal leaf lamina, and that bifurcates only just below the sinus. Slime papillae with conspicuously long stalks are also characteristic for most of the species. Feldberg and Heinrichs (2005a) synonymized *H. grossispinus* with *H. sendtneri* based on morphology. However, in my phylogeny *H. grossispinus* appears in a well-supported clade of South American species. *Herbertus sendtneri* and '*H. subdentatus*', which Feldberg *et al*. (2004) regard as synonymous, are in the largest clade, mostly unresolved in the strict consensus. It is not surprising that the specimen from the Azores appears in the same clade as the South American species, as it is very similar in its morphology, especially to *H. juniperoideus* of the same group. I have examined two kinds of *Herbertus* specimens from the Azores, one resembling *H. juniperoideus*, which was included in this study, and another that is closer to the *H. dicranus* complex in its appearance (specimens from the personal collection of F. Rumsey, BM). Most probably a plant of the latter type was included in the study of Feldberg *et al*. (2004), in which it was resolved in the same clade with samples earlier identified as *H. subdentatus*. On the basis of this result they synonymized *H. azoricus* with *H. sendtneri*, presumably regarding it as a uniform taxon and perhaps unaware of the taxonomic and nomenclatural confusion surrounding material from the Azores. Only very recently have authors, eg. Schumacker and Váňa (2005) recognised the presence of two taxa in the Azores. Contrasting views as to their identity and nomenclature remain to be clarified and resolved (F. Rumsey pers. comm.).

South American *H. subdentatus* plants were left without a name as Hodgetts (2003) argued that the type specimen of *H. subdentatus* (Steph.) Fulford from Guyana is actually another species, and that most of the South American and all African *H. subdentatus auct. non* (Steph.) Fulford records should be placed under synonymy of *H. dicranus*. As the South American *H. subdentatus auct*. is not closely related to *H. dicranus*, the proposed transfer (Hodgetts 2003) to *H. dicranus* is not supported by the present results. An African sample of *H. subdentatus* is in the same unresolved group with a specimen of *H. dicranus*, but a more thorough study at this level is needed in order to solve the relationships. In the study based on nuclear ITS and 5.8S sequences Feldberg *et al*. (2004) showed that South American *H. subdentatus* auct. belong to the same clade as the "*dicranus*" type *H. azoricus* and *H. sendtneri* from Austria. They identified all plants as *H. sendtneri*, a conclusion for which there is some support in the results presented here, in that *H. subdentatus auct. non* (Steph.) Fulford from South America is a sister to *H. sendtneri*. The *H. sendtneri* specimen of the present study was collected from Bhutan, in the Himalayas. In Feldberg and Heinrichs (2005b) the Bhutan plant is called *H. delavayi*. However, Hattori (1966) synonymized *H. delavayi* with *H. sendtneri*, which is well-justified according to study of the type specimens. Feldberg and Heinrichs (2005b) synonymized *H. borealis* with *H. delavayi* based on their phylogeny, which did not support Hodgett's (2003) synonymization of *H. borealis* with *H. dicranus*. My results, in which *H. sendtneri* from Bhutan is a sister to '*H. subdentatus*' from South America, are different from those of Feldberg and Heinrichs (2005b), in which the Bhutan

specimen is sister to British *H. borealis*, and that clade to British *H. stramineus*. The present phylogeny does not include *H. borealis* and *H. sendtneri* from Austria. However, based on morphology, the species definition of Feldberg *et al.* (2004) and Feldberg and Heinrichs (2005b) is broad, and the species concept should, I believe, be tested with more variable markers.

In the clade spanning *H. gracilis* and *H. stramineus* many nodes are unresolved in the strict consensus, but some sister species are grouped together. *Herbertus armitanus* and *H. circinatus* (Bremer support 8) resemble each other in that both are large, with falcate leaves terminated by 4–7 or 2–5 uniseriate cells. In addition, both have a strong vitta, and similar ranges in Indonesia, New Guinea, and the Philippines. *H. circinatus* is easily distinguished as it has unique, strongly circinate, leaves. However, for most of the sister species there are no obvious morphological characters connecting them. The Bremer support values for these clades are low, mostly four.

The present results support division of *Herbertus* into three major lineages. The isolated lineage of *H. runcinatus*; a second lineage corresponding to the clade of mostly South American taxa; and a third lineage corresponding to the large apical clade in Fig. 1. Formerly only Miller (1965) has attempted to divide *Herbertus* into subgroups, with the five sections. However, his study included only species from the Pacific islands and Asia. In the present results those species are all placed in the mostly unresolved clade spanning *H. gracilis* to *H. stramineus*. The results of Feldberg *et al.* (2004) and the present study show that ITS and *trn*L-F variation in *Herbertus* is low. Testing species concepts and fully resolving the infrageneric relationships within *Herbertus* requires more variable molecular markers.

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Appendix 1. Morphological characters and character states used in the analyses.

0. Terminal branching: 0 = absent, 1 = present
1. Lateral intercalary branching: 0 = absent, 1 = present
2. Ventral intercalary branching: 0 = absent, 1 = present
3. Branching: 0 = irregular, 1 = regular
4. Geotropic flagelliform branches: 0 = absent, 1 = present
5. Trigones in leaf cell walls: 0 = absent, 1 = present
6. Leaf apex shape: 0 = rounded, 1 = ending to a uniseriate cell row
7. Leaf division: 0 = absent, 1 = bifid, 2 = trifid, 3 = quadrifid, 4 = bisbifid, 5 = regularly bifid and trifid
8. Primary divisions of leaves: 0 = entire, 1 = laciniate
9. Leaf insertion: 0 = incubous, 1 = transverse, 2 = succubous
10. Slime papillae present in mature lateral leaf margins: 0 = absent, 1 = present
11. Slime papillae stalks: 0 = not stalked or shortly (1–2 cells), 1 = long-stalked (3+ cells)
12. Leaf margins: 0 = entire, 1 = toothed, 2 = ciliate
13. Leaf vitta: 0 = absent, 1 = present
14. Vitta bifurcation at basal lamina: 0 = clearly in upper part of basal lamina, 1 = in half or lower part of basal lamina
15. Secondary pigmentation: 0 = absent, 1 = present
16. Gynoecia position: 0 = on apex of main shoot, 1 = on apex of main shoot and on short lateral-intercalary branches, 2 = terminal on abbreviated lateral branches, 3 = on leading leafy axes
17. Female bracteoles: 0 = absent, 1 = present
18. Size of female bracts: 0 = similar or larger than vegetative leaves, 1 = considerably smaller than vegetative leaves
19. Perianth: 0 = absent, 1 = present
20. Shoot calyptra: 0 = absent, 1 = present
21. Antheridia in male bracteoles: 0 = absent, 1 = present
22. Isophylly: 0 = absent, 1 = present
23. Stem cortex: 0 = absent, 1 = present
24. Stem cross section: 0 = oval, 1 = flattened oval, 2 = rounded or somewhat triangular
25. Underleaves division: 0 = absent, 1 = bifid, 2 = regularly both bifid and trifid, 3 = quadrifid, 4 = bisbifid
26. Paraphyllia on stem: 0 = absent, 1 = present

Appendix 2. Morphological character matrix.

Species	00000000001111111111222222201234567890123456789012345678
Blepharostoma trichophyllum	11100013011020-001?11010130
Chiloscyphus profundus	11100000020-00-011010000010
Hygrolembidium acrocladum	01101000-?0-00-041010000?00
Jungermannia leiantha	11000100-10-00-0?00100000-0
Plagiochila asplenioides	11000100-20-10-030010001000
Ptilidium pulcherrimum	10000115110-20-101010001010
Temnoma pilosum	10000113120-20-101011010030
Trichocolea tomentella	10010014120-20-031011000040
Dendromastigophora flagellifera	10010112000-10-121010101040
Herbertus acanthelius	00101111011101013101011101 0
Herbertus aduncus subsp. *aduncus* China	00101111011001113101011111 0
Herbertus aduncus subsp. *aduncus* USA	00101111011001113101011101 0
Herbertus aduncus subsp. *hutchinsiae*	00101111011001113101011101 0
Herbertus armitanus	00101111011001113101011101 0
Herbertus azoricus	00101111011101013101011101 0
Herbertus circinatus	00101111011101113101011101 0
Herbertus dicranus China	00101111011001113101011101 0
Herbertus dicranus Nepal	00101111011001113101011101 0
Herbertus divergens	00101111011101113101011101 0
Herbertus gracile	00101111011000113101011101 0
Herbertus grossispinus	00101111011101013101011101 0
Herbertus juniperoideus	00101111011001013101011101 0
Herbertus kurzii	00101111011001113101011101 0
Herbertus longifissus	00101111011001113101011101 0
Herbertus longispinus	00101111011001113101011101 0
Herbertus oldfieldianus	00101111011001013101011101 0
Herbertus pilifer	00101111011001113101011101 0
Herbertus ramosus	00101111011001113101011101 0
Herbertus runcinatus	00101111011101013101011101 0
Herbertus sendtneri	00101111011001113101011111 0
*Herbertus sp*1	00101111011001113101011101 0
Herbertus stramineus	00101111011001113101011101 0
Herbertus '*subdentatus*' Africa	00101111011101013101011101 0
Herbertus '*subdentatus*' South America	00101111011101113101011101 0
Lepicolea attenuata	10010114000-20-011101111040
Lepicolea ochroleuca	10010114100-20-011101111040
Lepicolea pruinosa	10010114100-20-011101111041
Lepicolea ramentifissa	10010114100-20-011101111041
Lepicolea rara	10010114000-20-011101111041
Lepicolea scolopendra	10010114000-21-011101111040
Mastigophora diclados	10010112000-20-121010111010
Mastigophora woodsii	10010112000-10-121010111010
Triandrophyllum heterophyllum	00101015000-00-131010111020
Triandrophyllum subtrifidum	00101015000-00-131010111020
Vetaforma dusenii	11100014010-00-031001110010