Ann. Bot. Fennici 40: 317–329 ISSN 0003-3847
Helsinki 29 October 2003

Phylogenetic relationships of the generic complex *Chiloscyphus–Lophocolea–Heteroscyphus* (Geocalycaceae, Hepaticae): Insights from three chloroplast genes and morphology

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Received 5 Dec. 2002, revised version received 13 Feb. 2003, accepted 18 Feb. 2003

He-Nygrén, X. & Piippo, S. 2003: Phylogenetic relationships of the generic complex *Chiloscyphus–Lophocolea–Heteroscyphus* (Geocalycaceae, Hepaticae): Insights from three chloroplast genes and morphology. — *Ann. Bot. Fennici* 40: 317–329.

We attempted to reconstruct the phylogeny of the generic complex *Chiloscyphus–Lophocolea–Heteroscyphus* (Geocalycaceae, Hepaticae) by using sequence data from three regions of the chloroplast genome, *rbc*L, *trn*L-*trn*F and *psb*T-*psb*H, and 17 morphological characters, and to explore character evolution. Twenty-one taxa exemplars were selected and 2141 characters from both sequence and morphology were applied for parsimony-based analyses. The combined molecular data set, the morphological data set and the combined molecular and morphological data set were analysed. Our results identify the monophyly of the *Chiloscyphus–Lophocolea–Heteroscyphus* complex and support combining *Chiloscyphus* and *Lophocolea* into a single genus, *Chiloscyphus*. Our results also reveal that *Chiloscyphus s. lato* forms a sister group to the genus *Heteroscyphus*. *Chiloscyphus s. stricto* is closely allied to both subgenus *Lophocolea* section *Heterophyllae* and section *Lophocolea*, although morphologically section *Lophocolea* has a very different leaf form, leaf insertion and male and female inflorescences. The analyses compiled from the combined morphological and sequence data suggest that *Heteroscyphus* is the most derived group among the complex. The generic complex here includes the genus *Chiloscyphus*, consisting of subgenera *Chiloscyphus* and *Lophocolea*, and the genus *Heteroscyphus*. *Heteroscyphus* section *Connatus* is synonymized as section *Heteroscyphus* based on both morphological and molecular data. The analysis of morphological variation in combination with molecular data reveals that the individual morphological characters of the *Chiloscyphus–Lophocolea–Heteroscyphus* complex vary in their utility for classification.

Key words: *Chiloscyphus*, chloroplast genome, DNA sequence, Geocalycaceae, Hepaticae, *Heteroscyphus*, *Lophocolea*, Lophocoleoideae, morphology, parsimony, phylogeny, *psb*T-*psb*H, *rbc*L, *trn*L-*trn*F

Introduction

Chiloscyphus, *Lophocolea* and *Heteroscyphus* are closely allied hepatic genera in the family Geocalycaceae, subfamily Lophocoleoideae. Approximately 500 species have been described for the three groups, with the majority of species occurring in the tropics, especially in antipodal regions. The classification of three generic entities has stirred up controversy for a long time due to the complex morphology and inadequate taxonomic study of these genera. Historically, *Chiloscyphus* and *Lophocolea* were already recognized when the bulk of "modern" segregate genera were still placed in the single genus *Jungermannia*. *Chiloscyphus* was described by Corda (1829) and included *C. polyanthos* and *C. helferi* (*nom. nud.*). *Lophocolea* was treated as an independent genus by Dumortier (1835) and elevated from section *Lophocolea* of *Jungermannia*. The species *Jungermannia bidentata* and *J. heterophylla* placed in section *Lophocolea* were then assigned to genus *Lophocolea*. Both genera were established based on European taxa, and the generic concepts were mainly developed by Nees (1833–1838), Gottsche *et al.* (1844–1847), Montagne (1845) and Spruce (1885). At the time, the two genera were distinguished mostly on the basis of position of female inflorescences and perianth shape: on very short lateral branches and short and inflated perianths in *Chiloscyphus*; on long leafy branches and elongate and frequently triangular-prismatic perianths in *Lophocolea*. The early workers' accurate observations and thorough diagnoses, especially on fertility structures for both genera, allowed these generic concepts to withstand the test of time. However, these generic distinctions became questionable and ambiguous when species of the group from the Southern Hemisphere were taken into account (Mitten 1854–1855, 1873) and when taxonomic works were conducted on a world-wide basis (Schuster 1980, Engel & Schuster 1984).

Heteroscyphus is a segregate of *Chiloscyphus*. Schiffner (1910) transferred those taxa previously described under *Chiloscyphus* with spicate androecia on short abbreviated normally lateral branches to *Heteroscyphus*. The genus is also characterized by ventral branches, small male bracts, often toothed ventral leaf margins, well-developed trigones in leaf cells, and underleaves often connate with both sides of lateral leaves. Only the *Chiloscyphus polyanthos*/*C. pallescens* complex with a strictly Laurasian distribution remains in *Chiloscyphus s. stricto*. While questioned or rejected by some authors (Stephani 1917–1924, Hodgson 1943, Kuwahara & Hattori 1953), *Heteroscyphus* has been generally accepted as an independent genus (Fulford 1976, Grolle 1978, Piippo 1985, Srivastava & Srivastava 1990, Schumacker & Váňa 2000, Gradstein *et al.* 2001). However, separation of sterile plants of *Heteroscyphus* from *Lophocolea* may be difficult in some taxa.

Engel and Schuster (1984) evaluated genera of the subfamily Lophocoleoideae, and concluded that no basis exists for a generic separation of *Chiloscyphus* and *Lophocolea* and redefined the *Chiloscyphus–Lophocolea–Heteroscyphus* complex. They merged *Lophocolea* with *Chiloscyphus*, and recognized the genus *Chiloscyphus* to include the subgenera *Fragilifolia*, *Lophocolea*, *Notholophocolea*, *Phaeochiloscyphus* and *Chiloscyphus*, with the majority of species occurring in *Lophocolea* and *Chiloscyphus*. They described the genus *Heteroscyphus* as including subgenera *Heteroscyphus* and *Tetracymbaliella*. However, the merging of *Chiloscyphus* and *Lophocolea* by Engel and Schuster (1984) has not been accepted by a number of authors, particularly those working on the European and local floras (Grolle 1995, Paton 1999, Grolle & Long 2000, Gradstein *et al.* 2001, Srivastava & Srivastava 2002). Grolle (1995) stated that the treatment by Engel and Schuster (1984) hardly made progress in the taxonomy of the Lophocoleoideae, but rather obscured the isolated morphological and ecological position of the *Chiloscyphus polyanthos*/*C. pallensces* complex in this subfamily. He drew support from the lack of evidence of chemical affinity between *Lophocolea heterophylla*, *Chiloscyphus* and *Heteroscyphus* noted by Asakawa *et al.* (1990).

In the present study, we undertook a phylogenetic study of the generic complex *Chiloscyphus–Lophocolea–Heteroscyphus* using both molecular and morphological characters. Our primary goal was to clarify phylogenetic relationships of the three "genera", to test previous

phylogenetic hypotheses inferred from morphological data and to provide a framework for further taxonomic and phylogenetic studies on the family Geocalycaceae, which are polymorphic at different taxonomic levels. We address the following questions: (1) Is the generic complex a monophyletic group? (2) Are *Chiloscyphus*, *Lophocolea* and *Heteroscyphus* monophyletic? (3) What is the evolutionary trend of morphological characters within the generic complex?

Three regions of the cpDNA genome were sequenced for molecular characters, together with 17 morphological characters to address the above questions. All three regions are located in the large single-copy region of the genome: (1) the protein-coding *rbc*L; (2) the *psb*T-*psb*H region, which consists of partial *psb*T gene, the non-coding intergenic spacer between *psb*T and *psb*N, *psb*N gene, the non-coding intergenic spacer between *psb*N and *psb*H, and partial *psb*H gene. The tandemly arranged *psb*T, *psb*N and *psb*H are protein-coding genes of the photosystem II subunit; (3) the *trn*L-*trn*F region, which comprises the non-coding tRNA L (leucine, UAA) intron, tRNA L 3´exon and non-coding intergenic spacer between tRNA L and partial tRNA F (phenylalanine, GAA). Morphological characters were selected from both gametophyte and sporophyte anatomy and structure.

Materials and methods

Taxon sampling

A total of 21 species were studied (Table 1). Sixteen ingroup taxa were selected based on available material to represent the morphological and geographical diversity of each "genus". Two species are from *Chiloscyphus*, including the generitype *C. polyanthos*, seven from *Lophocolea*, including the generitype *L. bidentata*, and seven from *Heteroscyphus*, including the generitype *H. aselliformis*. Five outgroup taxa, all from the *Jungermanniales*, are *Jungermannia leiantha* from Jungermanniaceae; *Pedinophyllopsis abditus* from the subfamily *Leptoscyphoideae* of Geocalycaceae; *Pedinophyllum truncatum* and *Plagiochila asplenioides* from Plagiochilaceae; and *Scapania undulata* from Scapaniaceae. *Pedinophyllum*, previously also assigned to Leptoscyphoideae, is now in Plagiochilaceae, a close relative of Geocalycaceae. All trees are rooted with *Jungermannia leiantha* because the node from which *Jungermannia* is derived, as a terminal taxon, is considered the most basal among the sampled taxa according to studies on morphological characters (*see* Crandall-Stotler & Stotler 2000). Within the ingroup, taxonomical treatment for lower-level *Lophocolea* follows Schuster (1980), all sampled taxa belonging to the subgenus *Lophocolea*, which is the group most taxonomically problematic in the genus. Species from three of the four sections of the subgenus *Lophocolea* were sampled (Table 1). Section *Microlophocolea* was not included in this study because we were unable to obtain sequence data. *Lophocolea japonica* is treated as belonging to section *Bicornutae* based on its underleaves often being connate with both sides of lateral leaves and with the broad sinus. All specimens studied and sequenced in the present study are deposited in H.

DNA extraction, PCR-amplification and sequencing

Total genomic DNA was extracted from apical portions of herbarium specimens. Voucher specimen information and GenBank accession numbers are provided in Table 1. DNA was extracted using the modified CTAB protocols (*see* Rogers & Bendich 1994) or NucleoSpin Plant Kits (Macherey-Nagel). Manufacturers' instructions were followed, except that water was used for final elution and long-term storage. Double-stranded DNA templates were amplified using polymerase chain reaction (PCR) with primers from *rbc*L, *trn*L-*trn*F and *psb*T-*psb*H regions. The primers were referred from Ahonen *et al.* (2003) for *rbc*L, Taberlet *et al.* (1991) for *trn*L-*trn*F and Hong *et al.* (1995) for *psb*T-*psb*H. Each PCR reaction mixture contained 10.7 μl of distilled sterile water, 2.5 μl of 10X Biotaq™ reaction buffer, 1 μl of 25 mM l^{-1} $MgCl_2$, 1.5 μl of a mix of each dNTP at 5 mM mix, 1 μl of each primer of 10 pmol, 0.3 μl of Biotaq™ DNA polymerase 5000 μ ml^{-1} and 7 μl of the template DNA. Amplification was conducted in a PTC-

100 thermocycler. PCR cycle parameters were set as follows: initial denaturation step 10 min at 95 °C, denaturation of template DNA 1 min at 95 °C, primer annealing 1 min at 49 °C, primer extension for 1 min at 72 °C. After 35 cycles, a final extension of 7 min at 72 °C was added to allow completion of unfinished strands. Amplification products were checked by electrophoresis of 5 μl of the product through an agarose gel. The remaining products were purified with the

Table 1. List of taxa sampled for molecular sequences with GenBank accession numbers.

Taxon	Collection	*rbc*L	*psb*T-H	*trn*L-F
Outgroup				
Jungermannia leiantha	Finland, Nuuksio National Park, 2000 *He-Nygrén & Piippo 1466*	AY149838	AY149816	AY149857
Pedinophyllopsis abditus	Chile, Osorno Prov., Parque Nacional Puyehue, 1992 *Hyvönen 5839*		AY149819	AY149860
Pedinophyllum truncatum	China, NW Sichuan, Minshan Range, 1991 *Koponen 46768*	AY149855	AY149836	AY149878
Plagiochila asplenioides	Finland, Nuuksio National Park, 2000 *He-Nygrén & Piippo 1467*	AY149839	AY149817	AY149858
Scapania undulata	Finland, Nuuksio National Park, 2000 *He-Nygrén & Piippo 1468*	AY149840	AY149818	AY149859
Ingroup				
Chiloscyphus pallescens	Poland, Silesian upland, 1993 *A. Stenel (W-4)*	AY149849	AY149832	AY149871
Chiloscyphus polyanthos	Finland, Nuuksio National Park, 2000 *He-Nygrén & Piippo 1469*	AY149851	AY149833	AY149873
Heteroscyphus argutus	Nepal, Sankhuwasabha district, *D. G. Long 30333*		AY149820	AY149861
Heteroscyphus aselliformis	Borneo, Sabah, 1986 *Menzel et al. 3109*	AY149841	AY149821	
Heteroscyphus coalitus	Nepal, Sankhuwasabha district, *D. G. Long 30316*	AY149844	AY149825	AY149865
Heteroscyphus inflatus	Nepal, Sankhuwasabha district, *D. G. Long 30457*	AY149853	AY149835	AY149875
Heteroscyphus planus	Japan, Honshu, Gifu-ken, 1992 *Mizutani 15828*	AY149850		AY149872
Heteroscyphus splendens	Papua New Guinea, E de Mom Prov., 1989 *Hoffmann 89-749*	AY149854		AY149876
Heteroscyphus zollingeri	China, Hunan Prov., Yan-Ling Co., 1998 *Koponen et al. 57927*	AY149856	AY149837	AY149879
Lophocolea sect. Lophocolea:				
Lophocolea bidentata (*Chiloscyphus latifolius*)	Poland, Silesian upland, 1994 *K. Jedrzejko & A. Stenel (W-58)*	AY149842	AY149823	AY149862
Lophocolea cuspidata (*Chiloscyphus cuspidatus*)	China, Hunan Prov., Sang-Zhi Co., 1998 *Koponen et al. 48430*	AY149845	AY149826	AY149866
Lophocolea sect. Heterophllyae:				
Lophocolea heterophylla (*Chiloscyphus profundus*)	Finland, Nuuksio National Park, 2000 *He-Nygrén & Piippo* 1470	AY149852	AY149834	AY149874
Lophocolea itoana (*Chiloscyphus itoanus)*	China, Hunan Prov., Jiangyong Co., 1999 *Piippo 60709*	AY149846	AY149828	AY149868
Lophocolea minor (*Chiloscyphus minor*)	China, Hunan Prov., Zhangjiajie, 1999 *Rao 58428*	AY149843	AY149824	AY149864
Lophocolea sect. Bicornutae:				
Lophocolea japonica (*Chiloscyphus japonicus*)	China, Hunan Prov., Sang-Zhi Co., 1998 *Koponen et al. 50238*	AY149847	AY149829	AY149869
Lophocolea martiana (*Chiloscyphus martianus*)	French Guiana, Kourou, Mt. des Singes, 1986 *Gradstein 6265*	AY149848		AY149870

QIA™ Quick PCR Purification Kit (Qiagen). Purified DNA products were sequenced with both forward and reverse primers using the Big Dye Terminator Sequencing Kit (Perkin Elmer) on an ABI 377 automated sequencer (PE Biosystems).

Sequence manipulation and alignment

Electropherograms were edited and forward and reverse sequences were assembled for each DNA region using SeqMan II (LaserGene System Software, DNAStar Inc.) and aligned using ClustalX version 1.8 (Thompson *et al*. 1997). All three genomic regions, the coding *rbc*L, the intron, the exon, the intergenic spacer and partial exon of the *trn*L-*trn*F region, and the partial *psb*T gene, the intergenic spacer, the *psb*N gene, another intergenic spacer and the partial *psb*H gene of the *psb*T-*psb*H region were delimited by comparing the sequences with available GenBank accessions. All sequences obtained in this study were submitted to GenBank (*see* Table 1).

Sequence variation

Sequence data of *rbc*L, *trn*L-*trn*F and *psb*T-*psb*H were assembled for 21 taxa. *rbc*L sequences of *Pedinophyllopsis abditus* and *Heteroscyphus argutus*, *trn*L-*trn*F sequence of *Heteroscyphus aselliformis*, and *psb*T-*psb*H sequences of *Lophocolea martiana*, *Heteroscyphus planus* and *H. splendens* were not obtained and therefore were not included in the analyses. Fifty-eight sequences were used in this study. Following alignment, 2124 characters were included in the analyses, among which 1045 were from the *rbc*L gene, 565 from the *trn*L-*trn*F region and 524 from the *psb*T-*i*H region. Among included sites, there were 319 informative positions, of which 157 were from the *rbc*L gene, 55 from the *psb*T-*psb*H region and 107 from the *trn*L-*trn*F region.

Morphological data

Seventeen morphological characters were selected. Some of these characters have been used to delimit *Chiloscyphus*, *Lophocolea* and *Heteroscyphus* in previous studies (Schuster 1980, Schuster & Engel 1982, Engel & Schuster 1984, Piippo 1985, Srivastava & Srivastava 2002). The characters were coded into discrete states according to these studies and our own observations from herbarium specimens (Appendix). The character state was coded as a question mark when information for the taxon was unknown. All characters were treated as unordered and equally weighted. The characters and character states are listed below.

1. Secondary pigmentation: 0 = present, 1 = absent.
2. Rhizoid: 0 = scattered, 1 = restricted to underleaf base.
3. Lateral leaf arrangement: 0 = alternate, 1 = opposite or subopposite.
4. Dorsal connation of lateral leaves: 0 = free, 1 = connate dorsally.
5. Leaf apex: 0 = entire, 1 = lobed or dentate.
6. Trigones in leaf cells: 0 = indistinct, 1 = distinct, 2 = coarse.
7. Underleaf connation to lateral leaves: 0 = free from lateral leaves, 1 = connate at one side, 2 = connate at both sides.
8. Gynoecia position: 0 = on apex of main shoot, 1 = on apex of main shoot and also on short lateral-intercalary branches, 2 = terminal on abbreviate lateral branches.
9. Vegetative leaves of gynoecia branch: 0 = present, 1 = absent.
10. Size of female bracts: 0 = similar or larger than vegetative leaves, 1 = considerably smaller than vegetative leaves.
11. Female bracteoles: 0 = absent, 1 = present.
12. Number of the perianth keel: 0 = 0 keel, 1 = 2 keels, 2 = 3 keels.
13. Shape of the perianth: 0 = nontrigonous, 1 = trigonous.
14. Androecia position: 0 = on leading branches, 1 = on specialized short branches.
15. Size of male bracts: 0 = similar to leaves in size and shape, 1 = much smaller than leaves.
16. Thickness of the antheridial stalks: 0 = 2(4)-seriate, 1 = 1-seriate.
17. Thickness of the capsule wall: 0 = more than 4 layers, 1 = 2–4 layers.

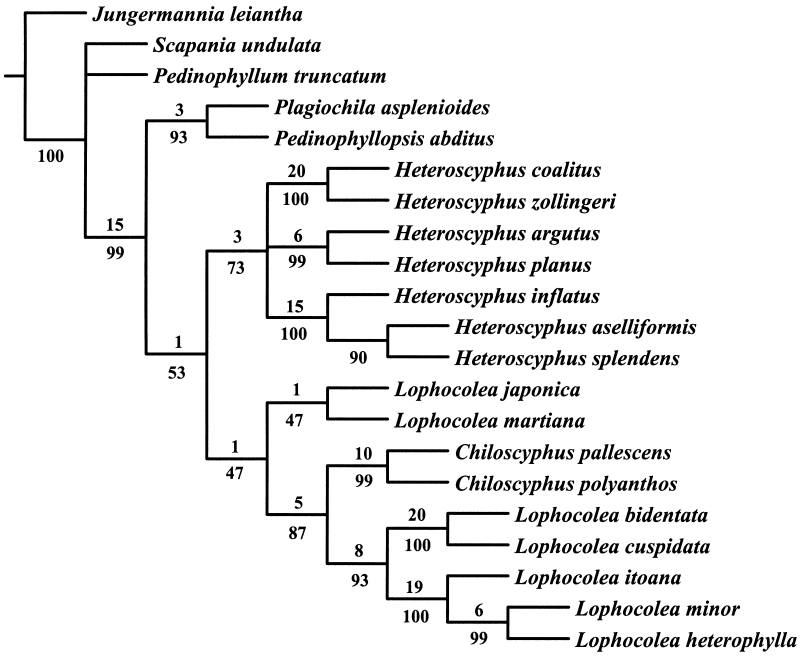

Fig. 1. Strict consensus topology of three equally most-parsimonious trees (*L* = 813, CI = 0.54, RI = 0.57) compiled from the combined *rbc*L, *psb*T-*psb*H and *trn*L-*trn*F sequences. Jackknife values are indicated below the branch and Bremer-support values above.

Phylogenetic analyses

Parsimony-based phylogenetic analyses were conducted with NONA (Goloboff 1994) spawned from WinClada (Nixon 1999). The following search strategy was applied: 1000 random addition sequences using tree bisection and reconnection (TBR) holding 100 trees per replication, followed by swapping to completion (h/100; mult*1000; max*). One hundred strict consensus jackknife replications for each matrix were spawned from WinClada into NONA with each replicate, including the following parameters: ten random addition sequences holding 1000 trees (h/1000; mult*10). The Bremer-support values were also calculated with the following commands: suboptimal 50; hold 25 000; hold/20; mult*300; find; bsupport.

Three data sets were analysed using the same search strategy. The first set included the combined molecular data. The three molecular data matrices were fused into a single matrix for a combined data set. Each species was represented as a single terminal by fusing multiple species accessions to represent all the known variation. Therefore, all the data can be evaluated simultaneously to produce the most corroborated phylogenetic hypothesis (Nixon & Carpenter 1996). The second set included only morphological data. The purpose of these analyses was to investigate whether the result obtained from molecular data corresponds with the result compiled from morphological data and to test the hypotheses raised by Engel and Schuster (1984) based on morphological studies. The third data set combined both molecular and morphological data to obtain the most corroborated phylogenetic hypothesis. In addition, an analysis based on combined molecular and morphological data was conducted using more weight (weight 2, instead of 1) on the morphological data set.

Results

Combined molecular data set

Parsimony analysis of 319 potentially informative characters identifies three equally most-parsimonious trees (*L* = 813, CI = 0.54, RI = 0.57). Within the ingroup, the tree topologies of the first two trees are the same, and the third tree differs only slightly within the *Heteroscyphus* clade. In the strict consensus tree (*L* = 828, CI = 0.53, RI = 0.55; Fig. 1), two nodes collapse, but the topology does not change fundamentally. In all

four trees, the *Chiloscyphus–Lophocolea* topology remains unchanged. All four trees support the monophyly of the *Chiloscyphus–Lophocolea–Heteroscyphus* complex. All species of *Heteroscyphus* form a monophyletic group with relatively high jackknife support and a sister relationship to the *Chiloscyphus–Lophocolea* clade. The *Chiloscyphus–Lophocolea* clade is also a monophyletic group and is divided into two major clades. The *Chiloscyphus* clade is positioned among the Lophocoleas and forms a sister group to sections *Lophocolea* and *Heterophyllae*. The *Chiloscyphus* clade is not clustered with *Heteroscyphus* although they share female inflorescence characters. This may indicate that the short gynoecial branches have evolved twice independently. *Lophocolea japonica* and *L. martiana* of section *Bicornutae* are grouped together with weak support, and they become a sister group of the *Chiloscyphus* clade and the rest of the *Lophocolea* species. Therefore, our results based on molecular characters show that *Chiloscyphus s. stricto* is closely related to both *Lophocolea* section *Lophocolea* and section *Heterophyllae*, and the generic delimitation between *Chiloscyphus* and *Lophocolea* is dissolvable. Our results also suggest that *Heteroscyphus* should retain its independent generic status. These suggestions are in agreement with Engel and Schuster (1984), who united two genera to form the single genus *Chiloscyphus* and treated *Heteroscyphus* at the generic level. However, the conclusions by Engel and Schuster (1984) that *Chiloscyphus* is evolved from a common ancestral type with the section *Heterophyllae* and that *Heteroscyphus* is the most derived group among the generic complex are not fully supported by the molecular data alone in our study.

Morphological data set

Analysis of the 17 morphological characters identifies six equally most-parsimonious trees (L = 35, CI = 0.60, RI = 0.87). In the strict consensus (L = 43, CI = 0.48, RI = 0.79; Fig. 2), the *Chiloscyphus–Lophocolea–Heteroscyphus* complex is supported strongly as a monophyletic group by the female bracteole, number of the perianth keel and shape of the perianth. Within the ingroup, the *Heteroscyphus* clade is resolved as a monophyletic group, and it is the most derived among the generic complex, supported by non-homoplasious characters, such as lateral leaf arrangement, androecia position and the size of male bracts. Within the *Heteroscyphus* clade, *H. aselliformis*, *H. inflatus* and *H. splendens* form a further evolved clade supported by the well-developed trigones in leaf cells and the lateral leaves being dorsally connate. This group of taxa also differs from other members of *Heteroscyphus* by the entire or slightly lobed lateral leaves and the enlarged underleaves. The *Chiloscyphus* clade is weakly supported by gynoecia position, vegetative leaves of gynoecia branches and size of the female bracts. The species relationships of *Lophocolea* are poorly resolved. Based on the morphological data, the resolutions of the generic complex and the *Heteroscyphus* clade are worse than those obtained from the sequence data, although the jackknife and Bremer support values are higher. The unresolved relationship of the *Lophocolea* clade is, however, not surprising since various homoplasious characters, such as the trigones in leaf cells, the leaf apex, the dorsal connation of lateral leaves, the gynoecia position, the size of female bracts and the thickness of antheridial stalks, exist. Some of these characters occur repeatedly throughout the generic complex, making its phylogenetic relationships intricate. *Chiloscyphus* shares female inflorescence characters with *Heteroscyphus* but differs clearly from *Lophocolea*. However, its systematic position cannot be fully clarified based on the weak branch support, and its relations to both *Lophocolea* and *Heteroscyphus* remain uncertain on the basis of a badly resolved result. Consequently, combining *Chiloscyphus* and *Lophocolea* based on morphological data alone seems insufficiently grounded. On the other hand, in the light of evidence obtained from molecular data, the monophyletic entity, and the high jackknife and Bremer-support values, we would be prone to give *Heteroscyphus* a generic status. Furthermore, whether *Heteroscyphus* is evolved from section *Bicornutae* of *Lophocolea*, as speculated by Engel and Schuster (1984), is unknown because of the poor resolution in the *Lophocolea* clade. *Heteroscyphus* shares more characters with section *Lophocolea* and also with section

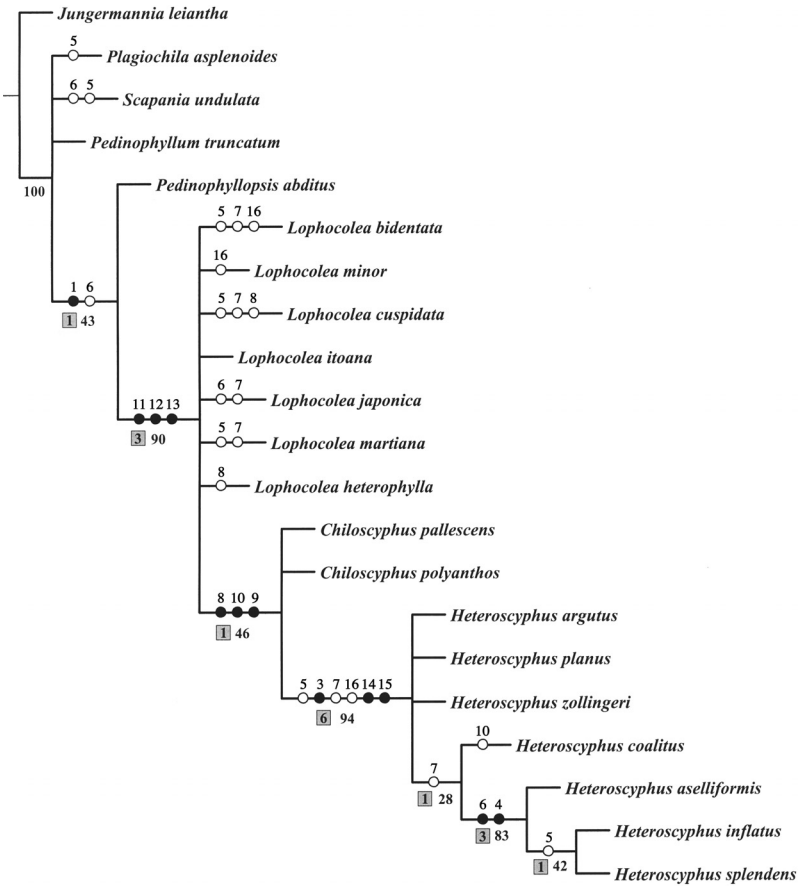

Fig. 2. Strict consensus topology of six equally most-parsimonious trees (*L* = 35, CI = 0.60, RI = 0.87) compiled from morphological characters. Character numbers are indicated above the branch, non-homoplasious characters are shown as solid black circles, and homoplasious characters as hollow circles. Jackknife values are indicated below the branch on the right and Bremer-support values are shown in grey on the left.

Bicornutae, including such characters as trigones in leaf cells, the leaf apex, underleaf connation and antheridial stalks. Morphologically, *Heteroscyphus* is indeed more allied with *Lophocolea* than with *Chiloscyphus*.

Combined sequence and morphological data set

Analysis of the combined sequence and morphological data set identifies one most-parsimonious tree (*L* = 857, CI = 0.54, RI = 0.59; Fig. 3). The topology strongly resembles that resulting from the sequence data set alone, but the jackknife and Bremer-support values are much higher at the *Chiloscyphus–Lophocolea–Heteroscyphus* clade and at the *Heteroscyphus* clade. The generic complex is resolved as a well-supported monophyletic group. The *Heteroscyphus* clade is supported fully as a monophyletic group, with only a slight clade alteration from the strict consensus tree obtained from the sequence data. The *Chiloscyphus–Lophocolea* clade remains basically the same as in the trees obtained from the sequence data. The *Heteroscyphus* clade and the *Chiloscyphus–Lophocolea* clade form a sister group. An analysis with a higher weight on morphological data (weight 2) concludes the same result. Our results based on the combined sequence and morphology data sets further support monophyletic origins of the *Chiloscyphus–Lophocolea–Heteroscyphus* complex, the *Heteroscyphus* clade and *Chiloscyphus–Lophocolea* clade. *Heteroscyphus* with high jackknife and Bremer supports can be treated as an independent genus. The *Chiloscyphus* clade forms a sister group with *Lophocolea* section *Lophocolea* and section *Heterophyllae*. However, the inference by Engel and Schuster (1984) that *Chiloscyphus s. stricto* is evolved from near section *Heterophyllae* is not supported. Our results also show

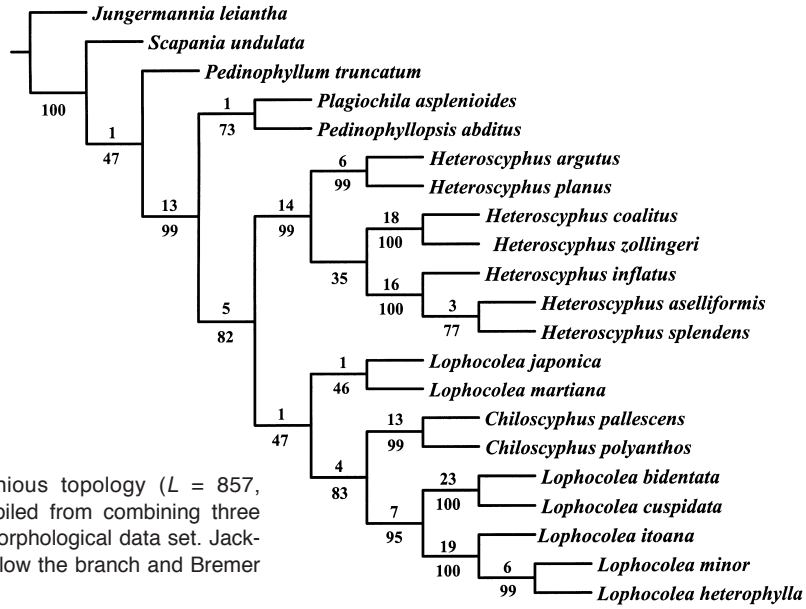

Fig. 3. The most-parsimonious topology (*L* = 857, CI = 0.54, RI = 0.59) compiled from combining three sequence data sets and a morphological data set. Jackknife values are indicated below the branch and Bremer support values above.

that *Chiloscyphus s. stricto* should be united with *Lophocolea* to form a single genus *Chiloscyphus*.

Discussion

On molecular and morphological characters

The results based on both morphological and molecular data sets clearly show that the sequence data not only provided many more characters but also played an important role in determining relationships within the *Chiloscyphus–Lophocolea–Heteroscyphus* complex. Furthermore, sequence data have provoked new discoveries and questions, which are addressed in the following sections.

Although morphology offered little resolution among terminals of *Lophocolea*, it had an overall stabilizing effect on the results. This can be seen from the higher branch support values on the clade of the generic complex and on the clade *Heteroscyphus* in the morphology-molecular tree. Together with the sequence data, our results suggest that *Heteroscyphus* is the most derived group among the generic complex and that it could have evolved from the lophocoleoid ancestors.

On Geocalycaceae and Plagiochilaceae

The result compiled from the sequence data set identifies a close relationship between *Pedinophyllopsis abditus* of Geocalycaceae subfamily Leptoscyphoideae and *Plagiochila asplenioides* of Plagiochilaceae. Morphologically, Leptoscyphoideae share with Plagiochilaceae the following characters: the bilabiate perianths, the vestigial female bracteoles and the rhizoids scattered over ventral merophytes. This morphological affinity had also been identified by Schuster and Engel (1982) in their study on the Leptoscyphoideae: “some of the genera with bilabiate perianths fall so close to the Plagiochilaceae that one is almost forced to draw arbitrary distinctions between Geocalycaceae and Plagiochilaceae”. However, Schuster and Engel (1982) maintained the distinctions between these two groups and assumed that they evolved independently from common ancestors, but they did not foot on retention of common criteria inherited from the ancestral types. The similarities between them were mainly due to identical tendencies towards alterations in bilateral symmetry. The present study, however, raises again the question: where do the Leptoscyphoideae end and the Plagiochilaceae begin? Taking both results obtained from the morphological and molecular data sets into

consideration, we assume that *Pedinophyllopsis* fits better into the Plagiochilaceae.

Surprisingly, *Pedinophyllum truncatum*, generally considered to belong in Plagiochilaceae, was not clustered together with *Plagiochila asplenioides*. Morphologically, it shares a laterally compressed perianth and thick capsule wall with members of Plagiochilaceae, but it stands out in various aspects: the autoecious inflorescence, the stem with a barely differentiated cortex, the leaf insertion not attaining stem midline, the rounded or shallowly bilobed leaf apex and the lack of a primary rhizogenous stem from which the leafy or aerial stem arises. The results obtained from the molecular data led us to the hypothesis that *Pedinophyllum* is related to *Plagiochila* remotely. Schuster and Engel (1982) considered *Pedinophyllum* better placed into the Leptoscyphoideae; however, that placement is not supported by our study.

On *Chiloscyphus* and *Lophocolea*

Engel and Schuster (1984) merged the two genera based mainly on similarities between *Chiloscyphus s. stricto* and *Lophocolea* section *Heterophyllae*. In the present study, both sequence data and combined sequence and morphological data reveal that *Chiloscyphus s. stricto* is closely allied to section *Heterophyllae* and, as well, section *Lophocolea*, although morphologically the latter has some different aspects in leaf form, leaf insertion and male and female inflorescences. Consequently, *Chiloscyphus s. stricto* could have evolved from lophocoleoid ancestors from either near section *Lophocolea* or near section *Heterophyllae*. Engel and Schuster (1984) postulated that the nearest extant taxon of the generic complex is Gondwanalandic *Chiloscyphus* subgenus *Notholophocolea* (type *Lophocolea boveana*), from which section *Bicornutae* is evolved. Unfortunately we could not include *Lophocolea boveana* in the present study to test this hypothesis.

Within *Chiloscyphus s. lato*, taxa are divided into a dichotomy, the *Lophocolea* section *Bicornutae* and a clade which consists of *Chiloscyphus s. stricto*, *Lophocolea* section *Lophocolea* and section *Heterophyllae*. Morphologically, section *Bicornutae* includes a series of taxa differing, in essence, from other members of *Lophocolea* in their nearly opposed leaves, widely united with the underleaves. The results compiled from molecular data also show that section *Bicornutae* may not be allied strongly to other members of *Lophocolea*. However, the taxonomic position of section *Bicornutae* cannot be clarified based on weak branch support and too few taxa. Here we tentatively treat this group of species as belonging in subgenus *Lophocolea*. The taxonomic position of the *Bicornutae* needs to be further tested by adding more taxa.

Morphologically closely related species of *Chiloscyphus s. lato*, such as *C. polyanthos* and *C. pallescens*, *C. latifolius* and *C. cuspidatus* and *C. minor* and *C. profundus*, are also shown to be closely allied molecularly. However, the taxonomic identity of each species based on molecular data can only be known after various population samples are examined. Taxonomically, *Chiloscyphus s. lato*, especially of those species from the Southern Hemisphere, are less studied, and generic revision on a world-wide basis is lacking.

On *Heteroscyphus*

Genus *Heteroscyphus* can be readily recognized by androecial structure: the androecia are spicate, slender, on abbreviated branches and of ventral-lateral or postical origin. The abbreviated gynoecial branches common to both *Chiloscyphus s. stricto* and *Heteroscyphus* are the major reason that these two groups have been assigned to the "genus" *Chiloscyphus*. However, results compiled from the sequence data suggest that the short gynoecial branches could have evolved independently. Therefore, *Heteroscyphus* would not be derived from chiloscyphoid ancestors but rather from lophocoleoid ancestors. Engel and Schuster (1984) assumed that ancestors of *Heteroscyphus* were not strongly differentiated from subgenus *Lophocolea* section *Bicornutae*. Their hypothesis, however, is rejected in this study since *Heteroscyphus* presents features that occur in both section *Bicornutae* and section *Lophocolea* and could therefore have evolved from ancestors near both sections.

Taxonomically, *Heteroscyphus* was treated as having two subgenera by Engel and Schuster

(1984), subgenus *Heteroscyphus* and subgenus *Tetracymbaliella*. The latter was previously treated as a genus by Grolle (1961) in the subfamily Lophocoleoideae, a close relative of *Chiloscyphus*, but was transferred to *Heteroscyphus* by Engel and Schuster (1984) based on its heteroscyphoid androecia. Subgenus *Tetracymbaliella* is distinct from subgenus *Heteroscyphus* by having pouches or concavities along lateral leaf and underleaf margins. In the present analyses, we were, however, unable to include molecular data on this group.

Srivastava and Srivastava (2002) treated Indian *Heteroscyphus* in two sections. Section *Connatus* (type *Heteroscyphus inflatus*) has entire or shortly bifid lateral leaves and large underleaves, lateral leaves that are dorsally connate, and coarse trigones in leaf cells, while section *Metaheteroscyphus* (type *Heteroscyphus argutus*) has dentate lateral leaves, lateral leaves that are never dorsally connate, small underleaves and small trigones in leaf cells. Our study shows that both the molecular and morphological characters of the generitype *Heteroscyphus aselliformis* fit in section *Connatus*. Therefore, section *Connatus* should be synonymized with section *Heteroscyphus* as follows:

Heteroscyphus section *Heteroscyphus*

Type: *Heteroscyphus aselliformis* (Nees) Schiffn., Oesterr. Bot. Zeitschr. 60: 172, 1910. — *Heteroscyphus* sect. *Connatus* A. Srivastava & S.C. Srivastava, Indian Geocalycaceae (Hepaticae): 77. 2002, *syn. nov.* — Type: *Heteroscyphus inflatus* (Steph.) S.C. Srivastava & A. Srivastava, Geophytology 16(1): 129–132. 1986.

We place *Heteroscyphus aselliformis*, *H. inflatus* and *H. splendens* in section *Heteroscyphus*, and the rest, *H. argutus*, *H. coalitus*, *H. planus* and *H. zollingeri*, in section *Metaheteroscyphus*.

Although *Heteroscyphus* is a large genus with major species diversity in the tropics and more than 100 valid names, a thorough taxonomical study on the genus is still lacking.

On character evolution

Table 2 summarizes the length and the indices of consistency and retention for each of the morphological characters on the equally most-parsimonious trees. Of the 17 potentially informative characters, nine show no homoplasy (secondary pigmentation, lateral leaf arrangement, dorsal connation of lateral leaves, androecia position, size of male bracts, vegetative leaves of female bracts, female bracteoles, number of perianth keel and shape of perianth), and four others provide at least some support for the grouping of taxa. The remaining four characters do not provide unambiguous support for any group of taxa (rhizoid, leaf apex, underleaf connation and thickness of antheridial stalks). Because the potential for characters to provide support is linked to taxon sampling, the four homoplasious characters should not be used in future studies on *Chiloscyphus* and *Heteroscyphus*.

Three of the morphological characters, the presence of female bracteoles, the 3-keeled and trigonous perianth, represent features of the *Chiloscyphus–Lophocolea–Heteroscyphus* complex, as well as of the subfamily Lophocoleoideae, except for the genus *Clasmatocolea*, which has reduced female bracteoles.

Within the *Chiloscyphus* subgenus *Lophocolea*, there appears to be no unambiguous character to address species relationships; consequently, taxonomic problems of this group

Table 2. Length, consistency and retention indices for morphological characters optimized onto the analysis of equally most-parsimonious trees.

Characters	*L*	CI	RI
1. Secondary pigmentation	1	1.0	1.0
2. Rhizoid	3	0.33	0.0
3. Lateral leaf arrangement	1	1.0	1.0
4. Dorsal connation of lateral leaves	1	1.0	1.0
5. Leaf apex	7	0.14	0.33
6. Trigones in leaf cells	5	0.40	0.62
7. Underleaf connation with lateral leaves	7	0.28	0.61
8. Gynoecia position	4	0.50	0.88
9. Vegetative leaf of gynoecia branch	1	1.0	1.0
10. Size of female bract	2	0.50	0.85
11. Female bracteole	1	1.0	1.0
12. Number of perianth keel	2	1.0	1.0
13. Shape of perianth	1	1.0	1.0
14. Androecia position	1	1.0	1.0
15. Size of male bract	1	1.0	1.0
16. Thickness of antheridial stalk	3	0.33	0.75
17. Thickness of capsule wall	2	0.50	0.50

cannot be resolved based on morphological characters alone. Subgenus *Chiloscyphus* differs from subgenus *Lophocolea* by three non-homoplasious characters, the gynoecia position, the size of female bract and the absence of vegetative leaves on female branches. These characters have been used intensively in the classification of *Chiloscyphus s. stricto*.

The genus *Heteroscyphus* is unambiguously distinct by the characters of lateral leaf arrangement, androecia position and abbreviated male branch. *Heteroscyphus* section *Heteroscyphus* can be recognized by unambiguous characters of dorsal connation of lateral leaves and coarse trigones in leaf cells. The ambiguous characters of *Heteroscyphus* are also present in subgenus *Lophocolea* sections *Lophocolea* and *Bicornutae*, which indicates that *Heteroscyphus* could have evolved from lophocoleoid ancestors.

The analysis of morphological variation in combination with molecular data reveals that the individual morphological characters of the *Chiloscyphus–Lophocolea–Heteroscyphus* complex vary in their utility for classification. However, the majority of the characters provided some level of grouping information within the generic complex. The lack of unique synapomorphic changes emphasizes the importance of using combinations of characters to delimit taxa of the generic complex.

Acknowledgements

We thank MSc Inkeri Ahonen, MSc Sanna Huttunen and Dr. Viivi Virtanen of the University of Helsinki for help with and comments on laboratory techniques and data analyses. We also thank Dr. David Long (Edinburgh) for providing the fresh material of *Heteroscyphus argutus*, *H. coalitus* and *H. inflatus* from Nepal.

References

Ahonen, I., Muona, J. & Piippo, S. 2003: Inferring the phylogeny of the Lejeuneaceae (Jungermanniopsida): a first appraisal of molecular data. — *Bryologist*. [In press].

Asakawa, Y., Toyota, M. & Ueda, A. 1990: Sacculatane and sclerodane-type diterpenoids from the liverworts *Porella perrottetiana* and *Heteroscyphus bescherellei*. — *Phytochemistry* 29: 2165–2167.

Corda, A. J. C. 1829: Genera Hepaticarum. — *Beitr. Naturgesch*. 12: 643–655.

Crandall-Stotler, B. & Stotler, R. E. 2000: Morphology and classification of the Marchantiophyta. — In: Shaw, A. J. & Goffinet, B. (eds.), *Bryophyte biology*: 21–70. Cambridge Univ. Press, Cambridge.

Dumortier, B. C. 1835: *Recueil d'observations sur les Jungermannicées*. Fasc. I: 3–27. — Imprimerie De J.-A. Blanquart, Tournay.

Engel, J. J. & Schuster, R. M. 1984: An overview and evaluation of the genera of Geocalycaceae subfamily Lophocoleoideae (Hepaticae). — *Nova Hedwigia* 39: 385–463.

Fulford, M. 1976: Manual of the leafy Hepaticae of Latin America. Part IV. — *Mem. New York Bot. Garden* 11: 393–535.

Goloboff, P. 1994: *NONA: a tree searching program*. — Programme and documentation available on the web at http://www.cladistics.com/about_nona.htm.

Gottsche, C. M., Lindenberg, J. B. G. & Nees von Esenbeck, C. G. 1844–1847: *Synopsis Hepaticarum*. — Sumtibus Meissnerianis, Hamburg.

Gradstein, S. R., Churchill, S. P. & Salazar-Allen, N. 2001: Guide to the bryophytes of tropical America. — *Mem. New York Bot. Garden* 86: i–viii, 1–577.

Grolle, R. 1961: *Tetracymbaliella*, eine neue Lebermoosgattung. — *Nova Hedwigia* 3: 47–53.

Grolle, R. 1978: *Heteroscyphus* Schiffn. nomen conservandum propositum. — *Taxon* 27: 127–128.

Grolle, R. 1995: The Hepaticae and Anthocerotae of the East African Islands. An annotated catalogue. — *Bryophyt. Biblioth*. 48: 1–178.

Grolle, R. & Long, D. 2000: An annotated check-list of the Hepaticae and Anthocerotae of Europe and Macaronesia. — *J. Bryol*. 22: 103–140.

Hodgson, E. A. 1943: A review of the New Zealand species of the genus *Chiloscyphus*, being the second of a series of papers on New Zealand Hepaticae. — *Trans. Proc. Roy. Soc. New Zealand* 73: 27–52.

Hong, L., Stevenson, J. F., Roth, W. B. & Hallick, R. B. 1995: *Euglena gracilis* chloroplast *psbB*, *psbT*, *psbH* and *psbN* gene cluster: regulation of *psbB-psbT* pre-mRNA processing. — *Molec. Gener. Gen*. 247: 180–188.

Kuwahara, Y. & Hattori, S. 1953: *Chiloscyphus javanicus* in Liukiu. — *J. Hattori Bot. Lab*. 9: 63–64.

Mitten, W. 1854–1855: Nat. Ord. Cl. Hepaticae, L. — In: Hooker, J. D. (ed.), *The botany of the Antarctic voyage*, Vol. II. Flora Novae-Zelandiae, Part II. Flowerless plants: 125–160 (1854); 161–172 (1855). Reeve Brothers, London.

Mitten, W. 1873: Jungermanniae and Marchantieae. — In: Seemann, B. (ed.), *Flora Vitiensis*: 404–419. L. Reeve, London.

Montagne, C. 1845: Plantes cellulaires. — In: Hombron & Jacquinot, Botanique. *Voyage au pole sud et dans l'Océanie, sur les corvettes l'Astrolabe et la Zélée* 1: 1–349. Gide, Paris.

Nees von Esenbeck, C. G. 1833: *Ordo tertius*. Hepaticae Hedw. — In: Martius, C. F. P. (ed.), *Flora brasiliensis seu enumeratio plantarum* 1(1): 295–390. J. G. Gotta, Stuttgart, Tübingen.

Nees von Esenbeck, C. G. 1833–1838: *Naturgeschichte der*

europäischen Lebermoose. Vols. 1–4. — Grass, Barth, und Comp., Breslau.

Nixon, K. C. 1999: *Winclada (beta) ver. 0.9*. — Published by the author, Ithaca, NY.

Nixon, K. C. & Carpenter, J. M. 1996: On simultaneous analysis. — *Cladistics* 12: 221–241.

Paton, J. A. 1999: *The liverwort flora of the British Isles*. — Harley Books, England.

Piippo, S. 1985: Bryophyte flora of the Huon Peninsula, Papua New Guinea. XII. Geocalycaceae (Hepaticae). — *Acta Bot. Fennica* 131: 129–167.

Rogers, S. O. & Bendich, A. J. 1994: Extraction of total cellular DNA from plants, algae and fungi. — In: Gelvin, S. B. & Schilperoot, R. A. (eds.), *Plant molecular biology manual* sect. D1: 1–8. Klewer Acad. Publ., London.

Schiffner, V. 1910: Über die Gattungen *Chiloscyphus* und *Heteroscyphus* n. gen. — *Oesterr. Bot. Zeitschr*. 60: 169–173.

Schumacker, R. & Váňa, J. 2000: Identification keys to the liverworts and hornworts of Europe and Macaronesia (Distribution and status). — *Documents de la Station Scientifique des Hautes-Fagnes* 31: 1–160.

Schuster, R. M. 1980: *The Hepaticae and Anthocerotae of North America*, 4. — Columbia Univ. Press, New York.

Schuster, R. M. & Engel, J. J. 1982: Austral Hepaticae XVI. Gondwanalandic Leptoscyphoideae (Geocalycaceae). — *Lindbergia* 8: 65–74.

Spruce, R. 1884–1885: Hepaticae Amazonicae et Andinae. — *Trans. Proc. Bot. Edinburgh* 15: 1–308, 1884 (part I); 309–589, 1885 (part II).

Srivastava, A. & Srivastava, S. C. 2002: *Indian Geocalycaceae (Hepaticae), a taxonomic study*. — Bishen Singh Mahendra Pal Singh, Dehra Dun, India.

Srivastava, S. C. & Srivastava, A. 1990: The genus *Heteroscyphus* Schiffn. in the Western Himalayas. — *Lindbergia* 15: 1–7.

Stephani, F. 1917–1924: *Species Hepaticarum* 6. — Imprimerie Jent, S. A., Geneve.

Taberlet, P., Gielly, L., Pautou, G. & Bouvet, J. 1991: Universal primers for amplification of three non-coding regions of chloroplast DNA. — *Plant Molec. Biol*. 17: 1105–1109.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. 1997: The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. — *Nucleic Acids Res*. 24: 4876–4882.

Appendix. Morphological character matrix. Character descriptions are provided in the Materials and methods section.

Taxon	Characters																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Jungermannia leiantha	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1
Pedinophyllopsis abditus	1	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	?
Pedinophyllum truncatum	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0
Plagiochila asplenioides	0	1	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0
Scapania undulata	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0
Chiloscyphus pallescens	1	1	0	0	0	0	0	2	1	0	1	2	1	0	0	0	1
Chiloscyphus polyanthos	1	1	0	0	0	0	0	2	1	0	1	2	1	0	0	0	1
Heteroscyphus argutus	1	1	1	0	1	0	1	2	1	1	1	2	1	1	1	1	1
Heteroscyphus aselliformis	1	1	1	1	1	2	2	2	1	1	1	2	1	1	1	1	1
Heteroscyphus coalitus	1	1	1	0	1	0	2	2	1	1	1	2	1	1	1	1	1
Heteroscyphus inflatus	1	1	1	1	0	2	2	2	1	1	1	2	1	1	1	1	1
Heteroscyphus planus	1	1	1	0	1	0	1	2	1	1	1	2	1	1	1	1	1
Heteroscyphus splendens	1	1	1	1	0	2	2	2	1	1	1	2	1	1	1	1	1
Heteroscyphus zollingeri	1	1	1	0	1	0	1	2	1	1	1	2	1	1	1	1	1
Lophocolea bidentata	1	1	0	0	1	0	1	0	0	0	1	2	1	0	0	1	1
Lophocolea cuspidata	1	1	0	0	1	0	1	1	0	0	1	2	1	0	0	0	1
Lophocolea heterophylla	1	1	0	0	0	0	0	1	0	0	1	2	1	0	0	0	1
Lophocolea itoana	1	1	0	0	0	0	0	0	0	0	1	2	1	0	0	0	1
Lophocolea japonica	1	1	0	0	0	1	1	0	0	0	1	2	1	0	0	0	1
Lophocolea martiana	1	1	0	0	1	0	2	0	0	0	1	2	1	0	0	0	1
Lophocolea minor	1	1	0	0	0	0	0	0	0	0	1	2	1	0	0	1	1