Phylogenetic relationships of the Thuidiaceae and the non-monophyly of the Thuidiaceae and the Leskeaceae based on *rbc*L, *rps*4 and the *rps*4-*trn*S intergenic spacer

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Abstract. We explored molecular data in order to establish the phylogenetic relationships of the Thuidiaceae. We sampled nine genera and 13 species of Thuidiaceae, and included representatives of 15 families that have been considered related to Thuidiaceae at some point. We used two chloroplast codifier genes (*rbc*L and *rps*4) and the *rps*4-*trn*S intergenic spacer. Our combined parsimony analyses retrieved a clade containing 12 exemplars of Thuidiaceae representing eight genera (*Thuidium*, *Thuidiopsis*, *Pelekium*, *Aequatoriella*, *Abietinella*, *Rauiella*, *Haplocladium* and *Actinothuidium*) but with the inclusion of *Leskea polycarpa* and exclusion of *Hylocomiopsis* making the Thuidiaceae non-monophyletic as currently defined, and the Leskeaceae polyphyletic. The name Thuidiaceae is retained for the informal ''thuidioid'' group of taxa. The Rhytidiaceae (*Rhytidium rugosum*) was found sister to the clade of Thuidiaceae s.lat and *Leskea polycarpa*. The *rps*4-*trn*S spacer added characters that improved resolution and may be of value for similar studies at family level in other pleurocarpous mosses.

Keywords. Bryopsida, Hypnales, Thuidiaceae, molecular phylogeny, Rhytidiaceae, gap codes, Leskeaceae.

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The Thuidiaceae are a widely distributed family of pleurocarpous mosses. They colonize diverse substrates and some representatives of the family have an important role forming carpets on forest floors contributing to water retention, serving as germination beds and as microhabitat for many arthropods. The Thuidiaceae have been characterized by their pinnate gametophyte covered by abundant paraphyllia (linear or ramified), foliar dimorphism between stem and branches, papillae on leaf and paraphyllia cells, sometimes on setae and perfect hypnaceous peristome (e.g., Buck & Crum 1990; Crum & Anderson 1981; Touw 2001). The Thuidiaceae belong to the order Hypnales. This order

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contains most of the diversity of pleurocarpous mosses with over 40 families and ca. 400 genera (Goffinet & Buck 2004). Recent phylogenetic analyses of pleurocarpous mosses have recognized the monophyly of the order Hypnales, including the formerly recognized Leucodontales and Hypnales (Buck et al. 2000; De Luna et al. 1999, 2000; Goffinet & Buck 2004; Goffinet et al. 2001; Tsubota et al. 2004). At least one exemplar species of Thuidiaceae was sampled in each of these studies. All analyses have indicated that the Thuidiaceae certainly belong to the Hypnales. However, historically there have been considerable differences in opinion about the relationships of the Thuidiaceae. Different authors have suggested relationships between Thuidiaceae and Leskeaceae. For example, Kindberg (1897) proposed the Thuidiaceae, with five genera, and related it to the Leskeaceae. Fleischer (1922) expanded the Thuidiaceae with 18 genera and placed it close to the Leskeaceae, Amblystegiaceae, Theliaceae, Fabroniaceae and Brachytheciaceae. Also, Brotherus (1924, 1925) linked the Thuidiaceae with the same families, recognizing 20 genera. Stepputat and Ziegenspeck (1929) also considered Thuidiaceae and Leskeaceae as closely related families.

In modern times, most classifications have related the Thuidiaceae to the Leskeaceae as well as to the Anomodontaceae, since the collection of genera placed under the Leskeaceae were segregated into these families. The morphological diversity and close relationships among the Thuidiaceae, Leskeaceae, Theliaceae and Fabroniaceae led Smith (1978) to propose a new order, the Thuidiales. Later, Crum and Anderson (1981) highlighted the relationship of the Thuidiaceae with the Leskeaceae and Amblystegiaceae indicating similarities in gametophytic characters. Indeed, Buck and Crum (1990) emphasized the close relationship of the Thuidiaceae to the Leskeaceae, and reässigned some genera to the Hylocomiaceae, Helodiaceae and Pterigynandraceae. Furthermore, using morphological characters, Hedenäs (1997) identified the Amblystegiaceae as the sister group of Thuidiaceae. Recently, Touw (2001) distinguished 16 genera of Thuidiaceae with no mention about relationships of the family. On the other hand, the phylogenetic study by Gardiner et al. (2005) revealed the heterogeneous nature of the Leskeaceae and recommended that the ''relationships of *Leskea* and *Haplocladium* with Thuidiaceae need additional studies.'' Their representation of the Thuidiaceae was sparse but appropriate in the context of the relationships of Leskeaceae. Despite previous revisions and phylogenetic studies of some exemplars of the Thuidiaceae, relationships of the family are still controversial. The establishment of a reliable phylogenetic hypothesis of the Thuidiaceae among pleurocarpous mosses will allow future evaluations of character evolution for traits traditionally related to water retention such as paraphyllia and papillae (e.g., Goebel 1969), a topic of interest to the lead author which will be discussed elsewhere.

In this paper we attempt to resolve the phylogenetic relationships of the Thuidiaceae by using three molecular markers, one of which, the *rps*4-*trn*S intergenic spacer, is used for the first time for pleurocarpous mosses. Our approach to the problem with relationships of the Thuidiaceae was to sample genera covering the morphological variation pointed out by Touw (2001) within the Thuidiaceae and also to include genera from several families that at some point have been considered closely related to the Thuidiaceae. We analyzed the chloroplast genes *rbc*L and *rps*4, which have been used previously by several authors for establishing moss relationships at different taxonomic levels within acrocarps (Cox et al. 2000; Goffinet et al. 1998; Hyvönen et al. 1998, 2004; La Farge et al. 2000; Magombo 2003; Virtanen 2003) and within pleurocarps (Bell & Newton 2005; Buck et al. 2000, 2005; De Luna et al. 2000; Goffinet et al. 2001; Pedersen & Hedenäs 2002; Tsubota et al. 1999).

Materials and Methods

Taxonomic sampling. We sampled 15 families putatively related to the Thuidiaceae among pleurocarpous mosses within the Hypnales. These were selected according to previous ideas of relationships within the suborders Leskeacanae, Brachytheciacanae and Hypnacanae (Buck & Vitt 1986), the morphological analysis of the Amblystegiaceae, Thuidiaceae and Hypnaceae by Hedenäs (1997), and the phylogenetic study by Gardiner et al. (2005) on the Leskeaceae. In total we

sampled 35 genera. Our sampling included the three families very closely related to the Thuidiaceae (number of genera per family in parentheses): Leskeaceae (3), Amblystegiaceae (4) and Anomodontaceae (3). We also sampled all families putatively related to the Thuidiaceae placed in the Leskeacanae (Buck & Vitt 1986): Rigodiaceae (1), Echinodiaceae (1) and Pterigynandraceae (1). In order to sample the phylogenetic diversity of the rest of the Hypnales, we sampled at least one representative of most families grouped in the Brachytheciacanae and Hypnacanae (Buck & Vitt 1986). From the former, we sampled the Rhytidiaceae (1), Lembophyllaceae (1), Stereophyllaceae (1) and Brachytheciaceae (2). From the latter, we included the Entodontaceae (1), Sematophyllaceae (3), Hylocomiaceae (2) and Plagiotheciaceae (1).

The representation of the Thuidiaceae in our analysis was based on the proposal of Touw (2001). He recognized the Thuidiaceae with 16 genera and 72 valid species. We sampled nine genera covering the exemplars of his three informal groups within Thuidiaceae: *Thuidium*, *Pelekium*, *Aequatoriella* and *Thuidiopsis* from the Thuidioid group; *Abietinella*, *Haplocladium* and *Rauiella* from the combined group; *Hylocomiopsis* and *Actinothuidium* from the Heloidioid group. Each genus was represented by one species, except the genera *Thuidium* and *Pelekium*. The former was represented by four species (including *T. tamariscinum*, the type species) and the latter by two species, *P. velatum*, the type species, and *P. siphotheca*, an American representative previously known as *Cyrto-hypnum mexicanum* (Touw 2001). In total, we sampled 13 species from this family.

The Leskeaceae includes 22 genera according to Buck and Goffinet (2000). Gardiner et al. (2005) found four clades of Leskeaceae. Among the six genera which were not transferred to the Pseudoleskeaceae and Pylaisiaceae, we sampled *Leskea* and *Pseudoleskeella*. Both genera remain in Leskeaceae sensu Buck and Goffinet (2000) and Gardiner et al. (2005). We also included *Claopodium* which is in the Thuidiaceae according to Fleischer (1922) and Brotherus (1924), Leskeaceae sensu Buck and Goffinet (2000), but Gardiner et al. (2005) found it related to *Anomodon*. The Anomodontaceae according to Buck and Goffinet (2000) contain seven genera. We represented it by *Anomodon*, *Herpetineuron* and *Haplohymenium*, genera that Fleischer (1922) and Brotherus (1924) included in Thuidiaceae. The Amblystegiaceae are a large family with 15 genera (Buck & Goffinet 2000). Analyses by Vanderpoorten et al. (2001) revealed core genera of Amblystegiaceae. We sampled one exemplar of *Amblystegium*, *Campylium*, *Hygroamblystegium* and *Calliergonella*. This family was suggested as the sister group of the Thuidiaceae (Hedenäs 1997).

Three genera were included to represent additional families putatively related to Thuidiaceae in the Leskeacanae (Buck & Vitt 1986). The Pterigynandraceae contain six genera (Buck & Goffinet 2000), among which we sampled *Heterocladium*. This genus was placed within Thuidiaceae by Fleischer (1922) and Brotherus (1924), until Buck and Crum (1990) transferred it to the Pterigynandraceae. The Rigodiaceae are monogeneric (Buck & Goffinet 2000; Zomlefer 1993). The Echinodiaceae (Buck & Goffinet 2000; Churchill 1986) has been considered monogeneric but recent evidence (Stech et al. 2006) suggests that the six species belong to three different families. The exemplar that we included (*E. umbrosum*) may be close to the Thamnobryaceae.

The rest of the Hypnales was sampled with five genera from families in the Brachytheciacanae (Buck & Vitt 1986). The Rhytidiaceae are monospecific (Buck & Goffinet 2000), consisting of *Rhytidium rugosum*, which we included. The Lembophyllaceae contain eight genera according to Buck and Goffinet (2000), but the phylogenetic analyses by Tangney (1997) and Quandt et al. (2000) circumscribed the family to only five genera. We sampled *Lembophyllum* as an exemplar of this family. The Stereophyllaceae are composed of eight genera (Buck & Goffinet 2000). We included one exemplar of *Stereophyllum*. The Brachytheciaceae are a large family with 40 genera (Buck & Goffinet 2000). Huttunen and Ignatov (2004) found it related to the Meteoriaceae, Hylocomiaceae and Amblystegiaceae. We sampled *Brachythecium* and *Helicodontium*. The latter was classified in Myriniaceae along with other five genera (Buck & Goffinet 2000), but it was found nested within Brachytheciaceae by Huttunen and Ignatov (2004).

Table 1. Species list by alphabetic order of families represented in these analyses. Herbarium at which voucher is located is indicated as British Museum (BM) and Leiden Herbarium (L). The abbreviations indicate the source of sequences as follow: DGA and AEN—sequences obtained at BM; XAL—sequences obtained at the Instituto de Ecología, AC. For new sequences voucher information is shown after the slash line. Sequences downloaded from GenBank are indicated with their respective accession number.

Family	Sequences lab origin/voucher		Herbarium or database
Species name	*rps*4 and *rps*4-*trn*S	*rbc*L	*rps*4/*rbc*L
Amblystegiaceae			
Amblystegium varium	DGA/*Newton 4385*	DGA/*Newton 4385*	BM
Campylium chrysophyllum	AF143048	XAL	GenBank/XAL
Hygroamblystegium tenax	AF143047	AF233565	GenBank
Calliergonella lindbergii	AF143035	AB029390	GenBank
Anomodontaceae			
Anomodon rugelii	AF143023	DGA/*Long 22155*	GenBank/BM
Haplohymenium triste	AF143022	DGA/*Newton 4289*	GenBank/BM
Herpetineuron toccoae	DGA/*Newton 4580*	AB019474	BM/GenBank
Brachytheciaceae			
Brachythecium plumosum	AF143078	AF233566	GenBank
Brachythecium rutabulum	AF023818	AEN/*Bell 912*	GenBank/BM
Helicodontium capillare	AF143043	AF233571	GenBank
Cyrtopodaceae (outgroup)			
Bescherellia brevifolia	AJ251313	AJ275184	GenBank
Echinodiaceae			
Echinodium umbrosum	AF143044	AF233568	GenBank
Entodontaceae			
Entodon brevisetus	AF143057	XAL/*De Luna 2261*	GenBank/XAL
Hookeriaceae (outgroup)			
Hookeria acutifolia	AF143071	AF158170	GenBank
Hookeria lucens	AJ251316	AY631185	GenBank
Hylocomiaceae			
Loeskeobryum brevirostre	AF143079	AB024658	GenBank
Rhytidiadelphus squarrosus	AF143033	AB024667	GenBank
Hypnodendraceae (outgroup)			
Hypnodendron camptotheca	AF023821	AY524448	GenBank
Hypopterygiaceae (outgroup)			
Hypopterygium tamarisci	AF143077	AF232695	GenBank
Lembophyllaceae			
Lembophyllum divulsum	AF143045	AF233570	GenBank
Leskeaceae			
Leskea polycarpa	DGA/*Newton 5131*	DGA/*Newton 5131*	BM
Claopodium prionophyllum	DGA/*Long 16576*	DGA/*Long 16576*	BM
Pseudoleskeella tectorum	DGA/*Newton 5163*	DGA/*Newton 5163*	BM
Plagiotheciaceae			
Plagiothecium denticulatum	AF469828	AB024623	GenBank
Pterigynandraceae			
Heterocladium wulfsbergii	DGA/*Newton 5148*	DGA/*Newton 5148*	BM
Rhytidiaceae			
Rhytidium rugosum	DGA/*Newton 4261*	DGA/*Newton 4261*	BM
Rigodiaceae			
Rigodium toxarion	DGA/*Newton 4489*	DGA/*Newton 4489*	BM
Sematophyllaceae			
Acroporium pungens	AF143028	AF233572	GenBank

Table 1. Continued.

Family *Species name*	Sequences lab origin/voucher *rps*4 and *rps*4-*trn*S	 *rbc*L	Herbarium or database *rps*4/*rbc*L
Pylaisiadelpha tenuirostris	AF143053	AB039789	GenBank
Taxithelium planum	AF143054	AF233573	GenBank
Stereophyllaceae			
Stereophyllum radiculosum	AF469846	AB024637	GenBank
Thuidiaceae			
Abietinella abietina	AEN/*Newton 5928*	AF005519	BM/GenBank
Actinothuidium hookeri	DGA/*Long 24165*	DGA/*Long 24165*	BM
Aequatoriella bifaria	DGA/*Touw & Snoek 25290*	DGA/*Touw & Snoek 25290*	L
Pelekium siphotheca	DGA/*Newton 5835*	DGA/*Newton 5835*	BM
Haplocladium microphyllum	DGA/*Long 8507*	DGA/*Long 8507*	BM
Hylocomiopsis ovicarpa	DGA/*Koponen 36832*	DGA/*Koponen 36832*	L
Pelekium velatum	DGA/*Coode 5964*	DGA/Coode 5964	L
Rauiella lagoensis	AEN/*Sidwell et al. 831*	AEN/*Sidwell et al. 831*	BM
Thuidiopsis sparsa	DGA/*Streimann 44467*	DGA/*Streimann 44467*	L
Thuidium cymbifolium	DGA/*Long 17449*	DGA/*Long 17449*	BM
Thuidium delicatulum	AF143039	AF158177	GenBank
Thuidium plumulosum	DGA/*Touw & Snoek 25288*	DGA/*Touw & Snoek 25288*	L
Thuidium tamariscinum	DGA/*Newton 5140*	DGA/*Newton 5140*	BM

From the remaining Hypnales, we sampled eight genera from representative families of the Hypnacanae (Buck & Vitt 1986). The Entodontaceae contain four genera (Buck & Goffinet 2000). We included *Entodon*. The Sematophyllaceae are a large family with 46 genera (Buck & Goffinet 2000), among which we selected three: *Acroporium*, *Taxithelium* and *Pylaisiadelpha*. The Hylocomiaceae include 13 genera, of which we sampled *Loeskeobryum* and *Rhytidiadelphus*, genera not questioned as to their placement in the family (Chiang & Schaal 2000). Although *Hylocomiopsis* was transferred from Thuidiaceae to Hylocomiaceae by Buck and Crum (1990) we included this genus as an exemplar of the Thuidiaceae since Touw (2001) classified it there. The Plagiotheciaceae according to Buck and Goffinet (2000) are monogeneric, but phylogenetic analysis by Pedersen and Hedenäs (2002) found that it includes 12 genera. We included *Plagiothecium denticulatum* as an exemplar of this family.

Outgroups for the collection of 15 families in Hypnales were chosen based on previous cladistic analyses of pleurocarpous mosses (Bell et al. 2007; Buck et al. 2005; De Luna et al. 2000). In these analyses, the Hookeriales are the sister group of the Hypnales; the Ptychomniales are sister to both. These three orders together constitute the clade of ''homocostate pleurocarps'' (Bell et al. 2007); the Hypnodendrales are sister to this group. In our analyses, the Hookeriales were represented by the Hookeriaceae (*Hookeria acutifolia* and *H. lucens*) and Hypopterygiaceae (*Hypopterygium tamarisci*) and the Hypnodendrales were represented by Cyrtopodaceae (*Bescherellia brevifolia*) and Hypnodendraceae (*Hypnodendron camptotheca*). Our matrix was composed of 44 terminals (outgroups included).

Sources of data. A total of 47 new sequences were generated for this study (26 of *rbc*L and 21 of *rps*4 and *rps*4-*trn*S (**Table 1**). The sequences labeled XAL were obtained at the Instituto de Ecologia, AC using the protocol described in De Luna et al. (2000). Those labeled DGA and AEN were obtained at the Natural History Museum (BM) following standard protocols for DNA extraction, PCR amplifications and sequencing (described below). Other sequences used in the analyses were obtained from GenBank and used in the following studies: 20 sequences of *rbc*L (Arikawa & Higuchi 1999; Cox et al. 2000; De Luna et al. 2000; Goffinet et al. 1998; Tsubota et al. 1999) and 25 sequences of *rps*4 (Buck et al. 2000; Cox et al. 2000; Cox & Hedderson 1999; Pedersen & Hedenäs 2002).

Table 2. Primers used for *rbc*L and *rps*4 PCR amplification and sequencing reactions for the new sequences generated for this study. *NM34 is the name erroneously used for the primer M28 in several publications (e.g., Cox et al. 2000), for details see Hyvönen et al. (1998, 2004).

Gene	Primer	Sequence 5′-3′	Direction	Reference
*rbc*L	M28(NM34*)	GTT GTT GGA TTT AAA GCT GGT GTT	Forward	Cox et al. (2000)
	M745R	CTT CAC A(AT)G TAC CTG C(AG)G TAG C	Reverse	Lewis et al. (1997)
	M636	GCG TTG GAG AGA TCG TTT CT	Forward	Lewis et al. (1997)
	M1390	CTT TCC AAA TTT CAC AAG CAG CAG	Reverse	Lewis et al. (1997)
*rps*4	Rps5	ATG TCC CGT TAT CGA GGA CCT	Forward	Nadot et al. (1994)
	trnaS	TAC CGA GGG TTC GAA TC	Reverse	Cox et al. (2000)

Sequencing protocols. Details of protocols for sequences obtained from GenBank are given in the papers mentioned above. The protocol detailed below was used for the new sequences generated at BM.

A small quantity of gametophyte material from herbarium samples was ground with liquid N and sterile sand. The powder was put into a 1.5 ml Eppendorff tube for DNA extractions using the modified CTAB extraction procedure of Rogers and Bendich (1994). DNA was cleaned with the Wizard DNA Clean-up Kit (PROMEGA) according to the instructions of the manufacturer. A 100 µl PCR reaction was prepared for *rbc*L amplification using two pairs of primers: M28 + M745R and M636 + M1390R (**Table 2**). The cycles used for PCR reactions were as follows: an initial denaturing step of four min at 94°C, followed by 30 cycles of (30″94°C; 30″48°C; 60″72°C) and ending with a final extension step of 7 min at 72°C. The primers used for *rps*4 PCR amplification were forward rps5 and reverse trnaS (**Table 2**). The same cycle conditions were performed except for an annealing temperature of 50°C for *rps*4. The PCR products, for both genes, were cleaned with a QIA quick PCR purification spin column and were eluted in 30 µl of TE. A total of 10 µl sequencing reactions were prepared using the ABI prism Big Dye terminator Cycle Sequencing Ready reaction Kit. The primers were the same used for PCR amplification, and the DNA quantity varied according with the amount of material obtained in the PCR reactions. Sequences were run in an ABI 377 automated sequencer.

Alignment. The editing and assembly of contiguous sequences was done with the programs SeqMan II and SeqEdit. The alignment of *rbc*L and *rps*4 was performed manually by comparison with sequences of other pleurocarpous mosses previously aligned (Bell, unpublished) using the text editor of PAUP 4.0b10 (Swofford 2000). With the primers employed for PCR amplification of *rps*4, it is common to obtain a fragment of *rps*4-*trn*S intergenic spacer in addition to the *rsp*4. In order to identify the proper length of *rps*4 (initial and stop codons) we used the *Marchantia polymorpha* (609 bp) sequence as reference (NC_001319 GenBank accession number). The sequences of *rps*4 used in this analysis had a total length of 600 bp because the first three codons were absent.

Once the stop codon was identified at the end of *rps*4, we considered the rest of the sites as the *rps*4-*trn*S intergenic spacer (IGS). We aligned this region using an automatic alignment approach. The automatic alignment programs have different weight schemes for transitions and transversions, use similarity or distance matrix and apply different penalties for initial, opening and extension gap events. Most of the algorithms for computing an alignment are based on dynamic programming (see Sankoff 2000). The program ClustalW (Thompson et al. 1994) uses dynamic programming and works under the Feng and Doolittle (1987) algorithm executing progressive alignment. The weight scheme, gap penalties and the programming algorithm for comparing sequences defines the outcome alignment (Li 1997; Phillips et al. 2000; Vingron & Waterman 1994; Wheeler 2005) and it must be explicitly defined for any alignment and phylogenetic analysis performed.

The alignment of the *rps*4-*trn*S was executed on-line, with the program ClustalW (Thompson et al. 1994). This program has three essential steps: the first performs a pairwise comparison calculating a

Table 3. Character partitions, analyses performed, gap codes used and principal results obtained. Abbreviations: Ts = transitions, Tv = transversions, pres/abs = present/absent, CI = Consistency Index, RI = Retention Index, HI = Homoplasy Index, IGS = Intergenic Spacer, Thuid = Thuidiaceae, BS = Bremer Support, Jk = Jackknife.

Analyses performed Penalty values: Weight Ts and Tv = 1.0, Gap opening = 1.0, Gap extension = 1.0	Length	Trees	Informative characters	CI	RI	HI	Consensus Fork Index (CFI)	Number of nodes well supported by both (BS≥1) and (Jk≤≥50) values
Gaps=missing (IGS) + *rbc*L + *rps*4	1362	52	326	0.37	0.51	0.62	33	28
Gaps=pres/abs (IGS) + *rbc*L + *rps*4	1377	52	332	0.38	0.51	0.61	33	30
Gaps=new state (IGS) + *rbc*L + *rps*4	1668	18	372	0.36	0.50	0.63	34	27
*rbc*L + *rps*4	1223	439	292	0.49	0.48	0.62	22	21
*rps*4	338	1204	96	0.57	0.54	0.42	17	9
*rbc*L	861	54	196	0.35	0.48	0.64	24	19

distance between every pair of sequence using two methods (the slow-accurate option with full dynamic programming algorithms, and the fast-approximate option); the second step constructs a neighbor-joining dendrogram describing the similarities between sequences; and the final step performs a progressive alignment using a series of pairwise alignments following the branching order of the dendrogram as a guide. The parameters for pairwise comparison are as fundamental as those for multiple alignment options.

We used the following settings: pairwise comparison slow-accurate option: gap open penalty = 1.0, gap extension penalty = 1.0, ClustalW weight matrix for DNA; multiple alignment parameters: gap open penalty = 1.0, gap extension penalty = 1.0, Weight Transition = yes (Value = 1.0), ClustalW weight matrix for DNA, hydrophilic gaps = no. The outcome alignment was used without any posterior manual editing. We treated gaps in three different ways and produced a data matrix for each gap coding. Gaps generated during alignment were analyzed as missing data, as fifth state and as present/absent (1/0). The latter was applied to common indel events, independently of their length, identified across taxa, for example:

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Original data matrix     Gap coding data matrix
Taxa1 ACGTT-----TTAGT-   Taxa1 ACGTT[-----]1TTAGT[-]1
Taxa2 ACCTA-----TTTGGT   Taxa2 ACCTA[-----]1TTTGG[T]0
Taxa3 ACGTAAACCTTTAGA-   Taxa3 ACGTA[AACCT]0TTAGA[-]1
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Phylogenetic analyses and support. Separate analyses were performed for *rbc*L and *rps*4. We also implemented four combined analyses: *rbc*L + *rps*4 and three with *rbc*L + *rps*4 + IGS, which included each of the three different gap codings for IGS (**Table 3**). We used parsimony as optimality criterion for cladogram selection. A heuristic search was performed using PAUP 4.0b10 (Swofford 2000) with 3000 random addition sequence replicates using the Tree Bisection Reconnection (TBR) branch swapping algorithm. At each step, only 100 trees were held, saving three trees of equal or greater length of the shortest found in each replicate (steepest descent on). All trees obtained with this strategy were then used as initial trees for a new search but now saving all trees of minimal length found (steepest descent off). The same two-step search strategy was applied for all analyses of data partitions mentioned in the section above. When more than one tree was found a strict consensus was calculated.

Clade support was calculated with Bremer support (Bremer 1994) using reverse constraints as implemented in Autodecay 4.0 (Eriksson 1999) under the same conditions for tree search. The Jackknife values (Farris et al. 1996) were obtained deleting 30% of characters (Mort et al. 2000) with 100 replicates using the full heuristic search option of PAUP 4.0b10 holding 100 trees, 10 random addition replicates, TBR branch swapping, saving minimal trees. The consistency and retention indices (Farris 1989; Kluge & Farris 1969) are reported for six analyses as a measure of character fit.

Results

Phylogenetic analyses. Results from separate and combined analyses of *rbc*L, *rps*4 and IGS are

presented in **Table 3**. Analyses of *rbc*L or *rps*4 alone did not resolve relationships of the Thuidiaceae with other families. The former retrieved 54 shortest trees (L=861, CI=0.35, RI=0.48) and the strict consensus was unresolved (results not shown). In contrast, the *rps*4 analysis retrieved 1204 shortest trees (L=338, CI=0.57, RI=0.54) and the strict consensus showed a clade (Bremer Support, BS=4, no Jackknife values above 50%), containing 16 exemplars, although with few internal branches resolved, from which 12 belong to the Thuidiaceae and the remaining four are from species of three different families: Amblystegiaceae (*Hygroamblystegium tenax* and *Calliergonella lindbergii*), Sematophyllaceae (*Acroporium pungens*) and Leskeaceae (*Leskea polycarpa*). The *rps*4 analysis indicates the Thuidiaceae are not monophyletic and the inclusion of *L. polycarpa* makes Leskeaceae also non-monophyletic. As in other analyses, *Hylocomiopsis* was placed distally among other families (results not shown). Analyses of *rbc*L in combination with *rps*4 produced a topology identical to *rbc*L alone (results not shown). This combined analysis retrieved 439 shortest trees of 1223 steps (CI=0.49, RI=0.48) and both genes contributed with 292 informative characters (**Table 3**), nevertheless the strict consensus was largely unresolved and it was not possible to determine the sister groups of the Thuidiaceae.

The data combination of *rbc*L, *rps*4 and IGS detected the following relationships irrespective of which gap code was applied: the Thuidiaceae and the Leskeaceae were not monophyletic. A clade containing 13 exemplars (see bold lines in **Fig. 1**) was recovered in which 12 species belong to the Thuidiaceae and one to the Leskeaceae. In contrast, differences were found depending on which gap code were applied. For example, when gaps were analyzed as missing data and as present/absent, the ''thuidioid'' group (sensu Touw 2001) was recovered as monophyletic (**Fig. 1**) but this did not happen under gaps as fifth state (**Fig. 2**). The species *Hylocomiopsis ovicarpa* (Thuidiaceae sensu Touw 2001) was placed as sister to a clade formed by *Heterocladium wulfsbergii* (currently in Pterigynandraceae) and *Echinodium umbrosum*. The other two members of Leskeaceae (*Pseudoleskeella tectorum* and *Claopodim prionophyllum*) were distantly located from each other; the former was sister to a clade formed by *Rhytidium rugosum* +''Thuidiaceae-Leskeaceae'' and there was not resolve position for the latter (**Fig. 1**). When gaps were treated as fifth state *H. ovicarpa* was sister to *Rigodium toxarion* + *Lembophyllum divulsum* clade; *P. tectorum* was sister group of *R. rugosum* and the position of *C. prionophyllum* was, again, not resolved (**Fig. 2**). The sister taxon to the ''Thuidiaceae-Leskeaceae'' clade was also different depending on gap code: *Rhytidium rugosum* was retrieved under gaps as missing and present/absent but *Hygroamblystegium tenax* when gaps were treated as a fifth state (**Figs. 1–2**).

Gap coding also had an effect on numbers of trees found, support values and number of informative characters. The analysis including gaps as missing data recovered 52 shortest trees of 1362 steps (CI=0.37, RI=0.51) based on 326 informative characters, gap coding as present/absent also retrieved 52 shortest trees (L=1377, CI=0.38, RI=0.51) but 332 informative characters. In contrast, gaps as fifth state identified 372 informative characters and 18 trees (L=1668, CI=0.36, RI=0.50) were found (**Table 3**). The Bremer support values for the ''Thuidiaceae-Leskeaaceae'' clade were high (BS=5, Jk=99; BS=7, Jk=52) under gaps as present/absent and fifth state, nevertheless jackknife values varied. The gap coding as present/absent retrieved the major number of nodes, 30 in total, well supported by both Bremer and Jackknife (**Table 3**).

Discussion

Phylogenetic relationships of the Thuidiaceae. Our results indicate that neither the Thuidiaceae nor the Leskeaceae are monophyletic groups. The sampling we included is a first approximation for the study of the Thuidiaceae relationships therefore these analyses are not yet conclusive and more Thuidiaceae and Leskeaceae exemplars are required for establishing nomenclatural decisions. At this point we can say that our taxonomic sampling allowed us to explore the phylogenetic position of taxa merged with the Thuidiaceae by Fleischer (1922), Brotherus (1924) and Hedenäs (1997) e.g., *Haplohymenium*, *Herpetineuron* and *Claopodium*. All these genera were consistently placed outside the Thuidiaceae in our

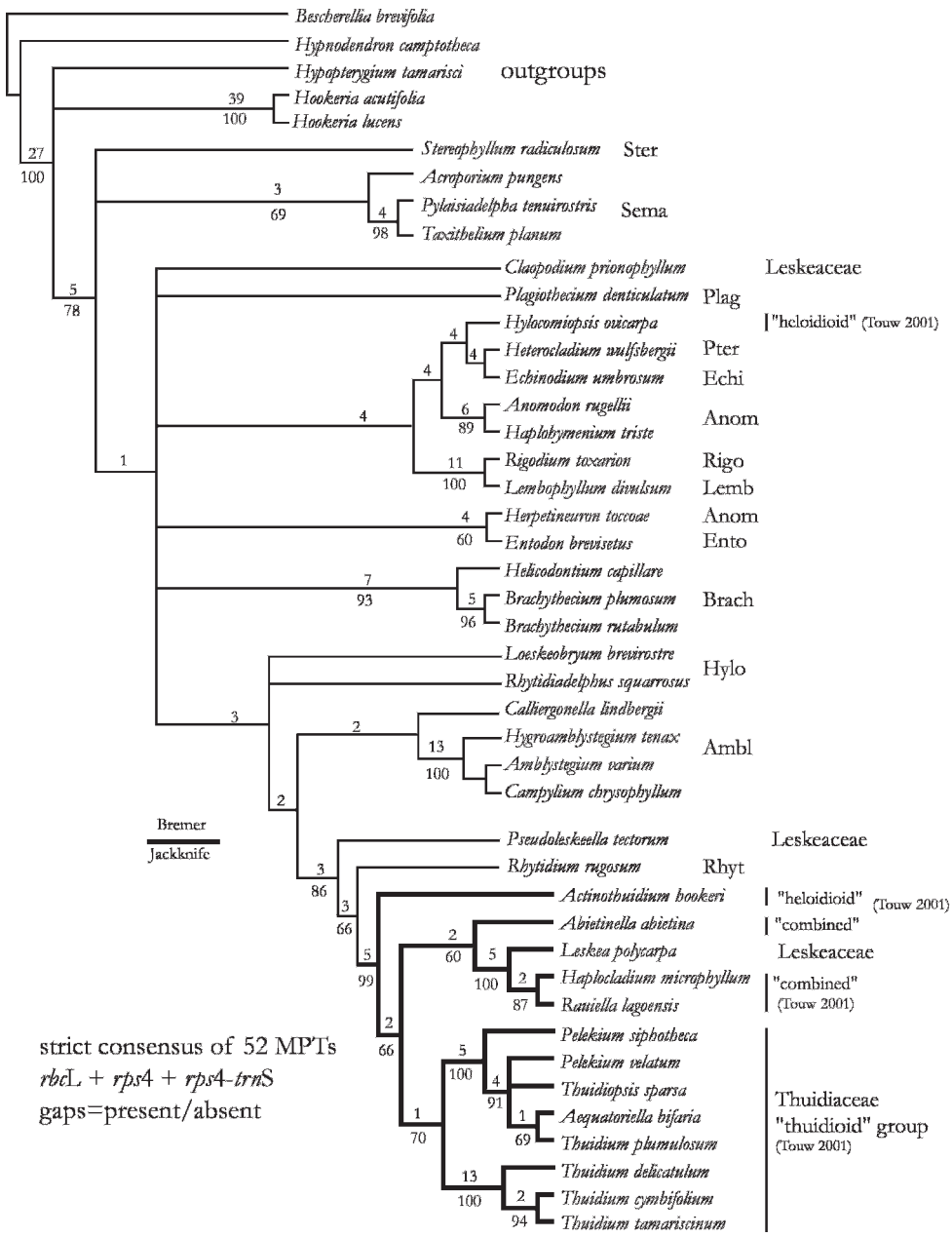

Figure 1. Strict consensus tree of 52 shortest trees (L=1377) retrieved by combined analysis of *rbc*L, *rps*4 and *rps*4-*trn*S with gaps of IGS coded as presence/absence. The same strict consensus topology was obtained when common gaps of IGS across taxa were treated as missing. Abbreviations are as follows: Rhyt = Rhytidiaceae, Ambl = Amblystegiaceae, Hylo = Hylocomiaceae, Brach = Brachytheciaceae, Ento = Entodontaceae, Anom = Anomodontaceae, Lemb = Lembophyllaceae, Rigo = Rigodiaceae, Echi = Echinodiaceae, Pter = Pterigynandraceae, Plag = Plagiotheciaceae, Sema = Sematophyllaceae, Ster= Stereophyllaceae. ''Thuidioid'', ''combined'' and ''heloidiod'' correspond to the informal groups mentioned by Touw (2001).

analyses. On the other hand, it was possible to determine the monophyly of the ''thuidioid'' group (sensu Touw, 2001) and non-monophyly of the ''combined'' and ''helodioid'' groups. Our analyses positioned *Hylocomiopsis ovicarpa* distantly related to the clade comprising 12 exemplars of the Thuidiaceae which also had *L. polycarpa* intermixed within the ''combined'' group of Touw (2001). The position of

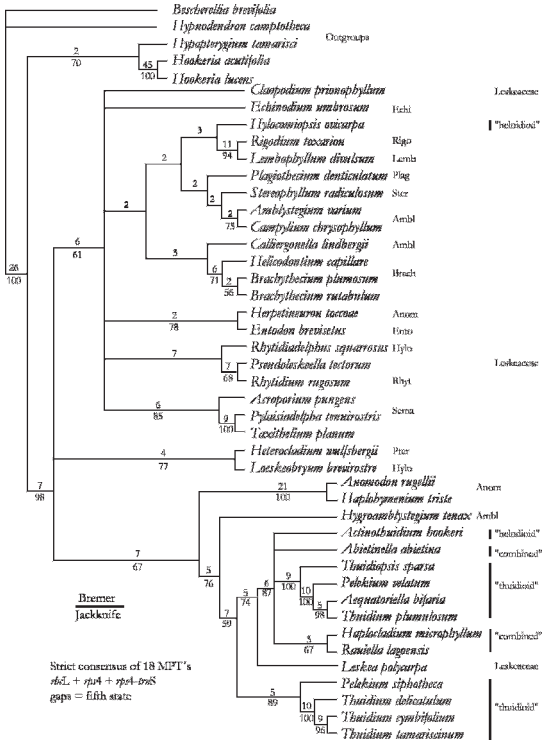

Figure 2. Strict consensus tree of 52 shortest trees (L=1668) obtained by combined analysis of *rbc*L, *rps*4 and *rps*4-*trn*S with gaps of IGS treated as fifth state. Abbreviations are as in Fig. 2.

this Leskeaceae species indicates the non-monophyly of this group, with the reservation that more taxa need to be included. The latter finding coincides with results by Gardiner et al. (2005) in a study addressing the limits of the Leskeaceae. They found two species of *Leskea* closely related to their *Haplocladium* species suggesting *Leskea* as the name for this clade and sister to them the clade formed by *Rauiella*, *Abietinella* and *Helodium* and sister to both clades the *Thuidium* clade (see their fig. 1, p. 653). They resolved part of the problem and resurrected the Pseudoleskeaceae and Pylaisiaceae.

We only sampled three Leskeaceae species (from over 20) and 13 species of the Thuidiaceae (from over 70) as defined today, and our expectation is that the addition of more exemplars from both families will give a more complete panorama and other changes may be necessary. The study by Gardiner et al. (2005) and our results confirm that hypotheses about the limits between the Thuidiaceae and the Leskeaceae are not yet conclusive and more intense sampling of both taxa and data is required.

According to our results and considering the study by Gardiner et al. (2005) it seems suitable to keep the name Thuidiaceae for the ''thuidioid'' group sensu Touw (2001) and take with caution the relationship of *Leskea* with the remaining taxa in the ''combined'' group of Thuidiaceae. Given that *Leskea polycarpa* is the type of the Leskeaceae, it may be necessary to recognize the ''combined'' group as members of the Leskeaceae. The addition of more data (morphological and other molecular markers) and more Thuidiaceae and Leskeaceae exemplars may alter their relationships or stabilize as currently found, providing enough support for a major nomeclatural change. It is also important to keep in mind that we sampled few examplars of other families putatively related to the Thuidiaceae such as Pterigynandraceae and we did not include others such as Fabroniaceae.

Our phylogenetic analyses retrieved Rhytidiaceae (*Rhytidium rugosum*) as the sister group of the Thuidiaceae-Leskeaceae clade; sister to them was another member of Leskeaceae (*Pseudoleskeella tectorum*) and sister to them was the Amblystegiaceae (**Fig. 1**). This finding is not surprising, since it was one possibility among previous hypothesis of relationships among Thuidiaceae, Leskeaceae, Amblystegiaceae and Rhytidiaceae. For example, according to Gardiner et al. (2005) the clade *Pseudoleskeella* + *Lindbergia* (Leskeaceae) is the sister group to their Thuidiaceae clade, which however includes two species of *Leskea*. On the other hand, the relationship of Amblystegiaceae-Thuidiaceae was suggested in previous phylogenetic analyses of pleurocarpous mosses based on morphological characters (Hedenäs 1997). He found a clade of Amblystegiaceae + Rhytidiaceae as sister to the Thuidiaceae. The same relationship between Amblystegiaceae-Thuidiaceae was detected with sequence level characters, even with reduced sampling (De Luna et al. 2000). Also, in studies of the Amblystegiaceae, Vanderporten et al. (2001) found Thuidiaceae sister to the core Amblystegiaceae. However, Vanderporten et al. (2002) included the Rhytidiaceae and found it was the sister group to *Leskea* + Thuidiaceae.

Phylogenetic information of* rbc*L and* rps*4. The evaluation of each partition separately gave us

information about its phylogenetic contribution. It has been argued that the phylogenetic content of molecular markers varies according to the taxonomic level explored but also depends on the group under study due to different rates of change (Mishler 2000; Soltis & Soltis 1998). The lack of resolution obtained here with *rbc*L could be an artifact of different factors such as our sampling among pleurocarps, and limits of resolution power of a single gene phylogeny. On the other hand, the phylogenetic content of *rps*4 seems to be quite variable. For the pleurocarpous mosses the utility of this gene has been recognized at the generic level and below (Buck et al. 2000; De Luna et al. 2000; Goffinet et al. 2001). By contrast, Nadot et al. (1994) demonstrated that the *rps*4 gene was useful at the subfamily and tribe level in angiosperms, such as the Poaceae, but it was useless between closely related genera; similar results were obtained by Souza-Chies et al. (1997) and Reeves et al. (2001) in their studies of the Iridaceae. The combination of both codifier genes did not resolve the relationships of the Thuidiaceae with other pleurocarps. Nevertheless, these relationships were solved when the *rps*4-*trn*S intergenic spacer was added to the analysis.

Contribution of* rps*4*-trn*S to phylogenetic relations of the Thuidiaceae. This spacer has not been used before for addressing higher-level phylogenetic relationships among pleurocarpous mosses. Previous authors working on phylogenetic studies of pleurocarps (e.g., Buck et al. 2000; De Luna et al. 2000; Goffinet et al. 2001) excluded the IGS from analysis due to its length variation and ambiguity in alignments. Werner et al. (2003) used the *rps*4-*trn*S intergenic spacer for addressing the systematic position of two species of *Tortula*, an acrocarpous moss, but they did not discuss its utility or variation within this group of mosses. In the tracheophytes, this spacer has been used for addressing phylogenetic relationships in the fern genera *Hymenophyllum* (Hennequin et al. 2003) and *Elaphoglossum* (Skog et al. 2004), in the horsetail *Equisetum* (Guillon 2004) and in the fern subfamily Taenitidoideae (Pteridaceae) by Sánchez-Baracaldo (2004). In our analyses, the use of the *rps*4-*trn*S in combination with *rbc*L and *rps*4 added phylogenetically important characters. The three analyses using different gap codes improved resolution of phylogenetic relationships of the Thuidiaceae among other pleurocarpous mosses. According to our results we recommend using the *rps*4-*trn*S intergenic spacer with gaps coded as present/absent in future phylogenetic studies of pleurocarpous mosses.

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