

The systematic position of *Gradsteinia andicola* Ochyra (Donrichardsiaceae, Bryopsida) : evidence from nrDNA internal transcribed spacer sequences

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Abstract. Nuclear ribosomal DNA internal transcribed spacer (ITS) 1/2 sequences of the Colombian endemic *Gradsteinia andicola* were determined and compared with those of 16 other species of the Hypnales (Amblystegiaceae, Brachytheciaceae, Hypnaceae, Plagiotheciaceae and Rhytidiaceae). In a maximum parsimony tree *Gradsteinia andicola* belongs to a well supported clade consisting of *Amblystegium*, *Cratoneuron*, *Cratoneuroopsis*, *Hypnobartlettia* and *Palustriella*, and seems to be closely related to *Cratoneuroopsis relaxa* from New Zealand. *Gradsteinia andicola* is therefore transferred to Amblystegiaceae, but the genus *Gradsteinia* is maintained. The systematic relationship of Amblystegiaceae and Donrichardsiaceae is discussed.

Introduction

The genus *Gradsteinia* was established by Ochyra (1990) for *Gradsteinia andicola* from Colombia. *Gradsteinia andicola* is a remarkable aquatic moss distinguished from all other pleurocarpous species by the concave boat-shaped and cucullate leaves with variously forked costae and polystratose longitudinal laminal streaks. A second species, *Gradsteinia torrenticola* Ochyra, Schmidt & Bültmann was described from Tenerife, Canary Islands (Ochyra et al. 1998).

According to its gametophytic appearance, *Gradsteinia* fits into the Donrichardsiaceae, a small family of mostly monotypic genera, introduced by Ochyra (1985) for *Donrichardsia macroneuron* (Grout) H. Crum & L. E. Anderson. Species included in the Donrichardsiaceae after the investigations of Ochyra (1985, 1986a,b, 1990) were *Donrichardsia macroneuron*, *Gradsteinia andicola* and *Richardsiopsis lacustris* (Herz. & Rich.) Ochyra in subfam. Donrichardsioideae

and *Sciaromiella bartlettii* (Crum & Steere) Ochyra and *Sciaromiopsis sinensis* (Broth.) Broth. in subfam. Sciaromiopsioideae (Tab. 1). All these species are aquatic mosses easily recognizable by a unique combination of gametophytic characters, including linear-flexuose, prorate lamina cells, very strong costae, polystratose leaf borders or laminae with variously polystratose streaks, and pseudoparaphyllia.

Similarly, the Hypnobartlettiaceae, also established by Ochyra (1985), comprised three species (*Hypnobartlettia fontana* Ochyra, *Koponenia holoneuron* (Herz.) Ochyra, *Ochyraea tatrensis* Vana) with the same areolation, strong or ill-defined costae, completely bi- or polystratose laminae and pseudoparaphyllia, but also filamentous paraphyllia not present in the Donrichardsiaceae.

However, the systematic position of the genera included in Donrichardsiaceae and Hypnobartlettiaceae and the systematic value of both families remain doubtful because all species are only known in sterile condition and are restricted to very narrow geographical ranges, resulting in an unusual distribution pattern (Fig. 1).

Recent analyses indicated that the Donrichardsiaceae are artificial and that the species belong to Amblystegiaceae or Brachytheciaceae, respectively. *Richardsiopsis lacustris* was synonymized with *Drepanocladus perplicatus* (Dus.) Roth (Amblystegiaceae, Hedenäs 1997). The first sporophytic and molecular evidence came from the new species *Platyhypnidium mutatum* Ochyra & Vanderpoorten (Brachytheciaceae), found in 1997 in the Black Forest, Germany. Originally, Ochyra & Vanderpoorten intended to describe this moss as a new species of *Donrichardia*, but finally it was placed in *Platyhypnidium* because sporophytes and DNA sequences (except for one position) were identical with those of *Platyhypnidium riparioides* (Hedw.) Dix., growing abundantly at the same waterfall (Ochyra & Vanderpoorten 1999, Stech & Frahm 1999). It was supposed that *Platyhypnidium mutatum* was derived from *P. riparioides* by

mutations of genes responsible for the formation of its different gametophytic characters, a broader costa and irregularly bi- or polystratose lamina. The type species of Hypnobartlettiaceae, *Hypnobartlettia fontana* Ochyra from Waikoropupu Springs, New Zealand, was shown to belong to Amblystegiaceae based on non-coding sequences of cpDNA (*trnL* intron) and nrDNA (ITS1/2) (Stech et al. 1999^{LIA}).

In this investigation, we present molecular data for *Gradsteinia andicola*, a species originally described in the Donrichardsiaceae, to define its position and to further evaluate the systematic value of the family. The nrDNA internal transcribed spacers 1/2 are used as molecular markers.

Material and methods

Plant material. Herbarium material of *Gradsteinia andicola* originated from the type locality in Colombia (Dept. Meta: Páramo de Sumapaz, Hoya Sitiales, 22.01.1973, A. M. Cleef 8236). Further species included in the analysis originated from field excursions in Central Europe and New Zealand (*Cratoneuroopsis relaxa*, *Hypnobartlettia fontana*). Herbarium specimens are deposited in the herbaria of the authors, duplicates of the New Zealand specimens in CHR.

Amblystegium serpens (Hedw.) B.S.G., Stech B980409.1, *Cratoneuroopsis relaxa* (Hook. f. & Wils.) Fleisch. in Broth., *Cratoneuron filicinum* (Hedw.) Spruce, Stech B900615, *Ctenidium molluscum* (Hedw.) Mitt., Stech B880103.2, *Eurhynchium striatum* (Hedw.) Schimp., Stech B971113.3, *Hookeria lucens* (Hedw.) Sm., Stech B880404.8, *Hygrohypnum smithii* (Sw.) Broth., *Hypnobartlettia fontana* Ochyra, *Palustriella commutata* (Hedw.) Ochyra, *Plagiothecium undulatum* (Hedw.) B.S.G., Stech B881003.2, *Platyhypnidium mutatum* Ochyra & Vanderpoorten, *Platyhypnidium riparioides* (Hedw.) Dix., *Rhynchostegiella jaquinii* (Garov.) Lindb., *Rhynchostegiella pumila* (Wils.) E. F. Warb., *Rhynchostegium murale* (Hedw.) B.S.G., *Rhynchostegium rotundifolium* B.S.G., *Rhytidium rugosum* (Hedw.) Kindb., Stech B930910.1.

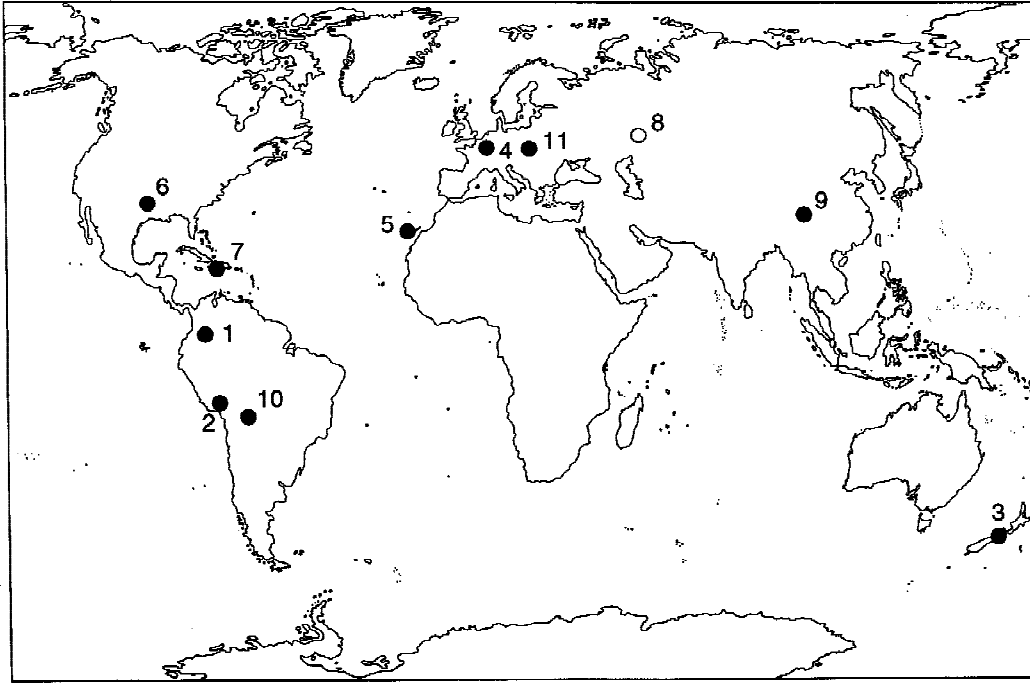


Fig. 1: World distribution of Donrichardsiaceae and species of Amblystegiaceae/Brachytheciaceae (systematic position according to recent analyses, Tab. 1) with variously polystratose laminae. 1-3 Amblystegiaceae: 1 *Gradsteinia andicola*, 2 *Drepanocladus perplicatus*, 3 *Hypnobartlettia fontana*; 4-5 Brachytheciaceae: 4 *Platyhypnidium mutatum*, 5 *Platyhypnidium torrenticola*; 6-9 Donrichardsiaceae: 6 *Donrichardsia macroneuron*, 7 *Sciaromiella bartlettii*, 8 *Sciaromiella longifolia* (fossil), 9 *Sciaromiopsis sinensis*; 10-11 species of former Hypnobartlettiaceae: 10 *Kopenenia holoneuron*, 11 *Ochyraea tatrensis*.

Plant material was prepared for DNA extraction through cleaning with water and ultrasonic treatment.

DNA extraction, PCR and sequencing reactions. DNA preparations were carried out following the method described by Doyle & Doyle (1990) but using only 70% (v/v) ethanol to wash the pellets after precipitation with cold isopropanol. PCR reactions were performed in a Biometra thermocycler using 1-3 U Taq DNA polymerase (Eurogentec or Qiagen), 1x buffer, magnesium chloride, 0.2 mM dNTPs (Roth), and 10 pmol of primers 18F/25R (after Baldwin 1992, primer sequences are available on request). The PCR protocol was 5 min 94°C, 35 cycles (45 s 94°C, 45 s 48°C, 1 min 72°C), 4 min 72°C

PCR products were purified using the QIAquick PCR purification kit (Qiagen). Cycle sequencing reactions (2 min 94°C, 35 cycles (30 s 94°C, 30 s 60°C, 30 s 72°C), 1 min 72°C) were carried out in a Perkin Elmer thermocycler using the SequiTherm Excel II DNA sequencing kit (Epicentre) and primers 18F/5.8R for ITS1 and 5.8F/25R for ITS2 (each 5' biotin-modified, Roth). Sequencing reactions were separated in the GATC-1500-system, transferred to Nylon membranes (Qiagen, Pall) and visualized applying a standard protocol with Streptavidin-Alkaline Phosphatase (Promega) and BCIP/NBT (Roth) treatment. Sequences were determined manually from the membranes. To verify the results, sequencing reactions were performed on two independent PCR products from each specimen.

Alignment and tree construction. An alignment of the sequences was created with the Alignment Editor Align32 (Hepperle 1997). Phylogenetic trees were evaluated according to the maximum parsimony principle with PAUP 4.0b3a (Swofford 2000), using *Hookeria lucens* as outgroup. A branch-and-bound search was performed with the following options: all characters unweighted and unordered, multistate characters interpreted as uncertainties, gaps coded as missing data, performing TBR branch swapping, collapse zero length branches, MulTrees option in effect. Character optimization was set as ACCTRAN. In addition, a heuristic bootstrap search with 1000 replicates, 100 random addition replicates per bootstrap replicate and the same options in effect was performed.

The sequences are deposited in the GenBank data base under the following accession numbers (ITS1/2):

Amblystegium serpens AF152387 / AF152390, *Cratoneurosis relaxa* AF152388 / AF152391, *Cratoneuron filicinum* AF230994 / AF231009, *Ctenidium molluscum* AF230989 / AF231004, *Eurhynchium striatum* AF230985 / AF231000, *Gradsteinia andicola* AF230992 / AF231007, *Hookeria lucens* AF230980 / AF230995, *Hygrohypnum smithii* AF230991 / AF231006, *Hypnobartlettia fontana* AF152386 / AF152389, *Palustriella commutata* AF230993 / AF231008, *Plagiothecium undulatum* AF230990 / AF231005, *Platyhypnidium mutatum* AF230982 / AF230997, *Platyhypnidium riparioides* AF230981 / AF230996, *Rhynchostegiella jaquinii* AF230987 / AF231002, *Rhynchostegiella pumila* AF230986 / AF231001, *Rhynchostegium murale* AF230983 / AF230998, *Rhynchostegium rotundifolium* AF230984 / AF230999, *Rhytidium rugosum* AF230988 / AF231003.

Results

Sequence lengths of the ITS1 range from 232 bp (*Hookeria lucens*) to 274 bp (*Rhynchostegiella pumila*). The ITS2 could not be determined completely in several species because of difficulties in reading larger parts or

at least a small GC-rich part of the 3' end. Approximately, sequence lengths of the ITS2 vary between 255 bp (*Rhynchostegiella pumila*) and 283 bp (*Hookeria lucens*).

The alignment of the nrDNA (Tab. 2) comprises 650 positions (ITS1: positions 1-365, ITS2 partial sequence: 366-650). The 5.8S rRNA gene and the 3' end of the ITS2 are not included. Two most variable parts of the alignment, one part of each ITS1 (positions 220-291) and ITS2 (452-506), were omitted from the phylogenetic analyses due to difficulties in aligning the sequences. Of the remaining 523 positions used for tree construction, 153 are variable and 73 parsimony-informative.

The maximum parsimony analysis (branch-and-bound search) yielded a single most parsimonious tree (length 231 steps, consistency index CI = 0.792, retention index RI = 0.787). This tree is shown in Fig. 2, with bootstrap values > 50% given above the branches.

Plagiothecium undulatum (Plagiotheciaceae) is separated from a cluster of all other species, which is supported by a high bootstrap value of 97%. Within this cluster, *Ctenidium molluscum* (Hypnaceae) branches off first. The cluster of the remaining species is divided into three well-supported clades. The first comprises all species of Brachytheciaceae (94%), while the second is formed by *Hygrohypnum smithii* (Amblystegiaceae) and *Rhytidium rugosum* (Rhytidiaceae) with a lower bootstrap support of 74%. The third clade consists of *Amblystegium serpens*, *Cratoneurosis relaxa*, *Cratoneuron filicinum*, *Hypnobartlettia fontana*, *Palustriella commutata* (Amblystegiaceae) and *Gradsteinia andicola* (Donrichardsiaceae), and is supported by a high bootstrap value of 96%. It is subdivided into three branches of two species, *Amblystegium/Hypnobartlettia* with only 67% bootstrap support, and *Cratoneurosis/Gradsteinia* and *Cratoneuron/Palustriella* with significant bootstrap values of each 94%.

Discussion

When Ochyra (1990) first described *Gradsteinia andicola*, he tried to define its systematic position

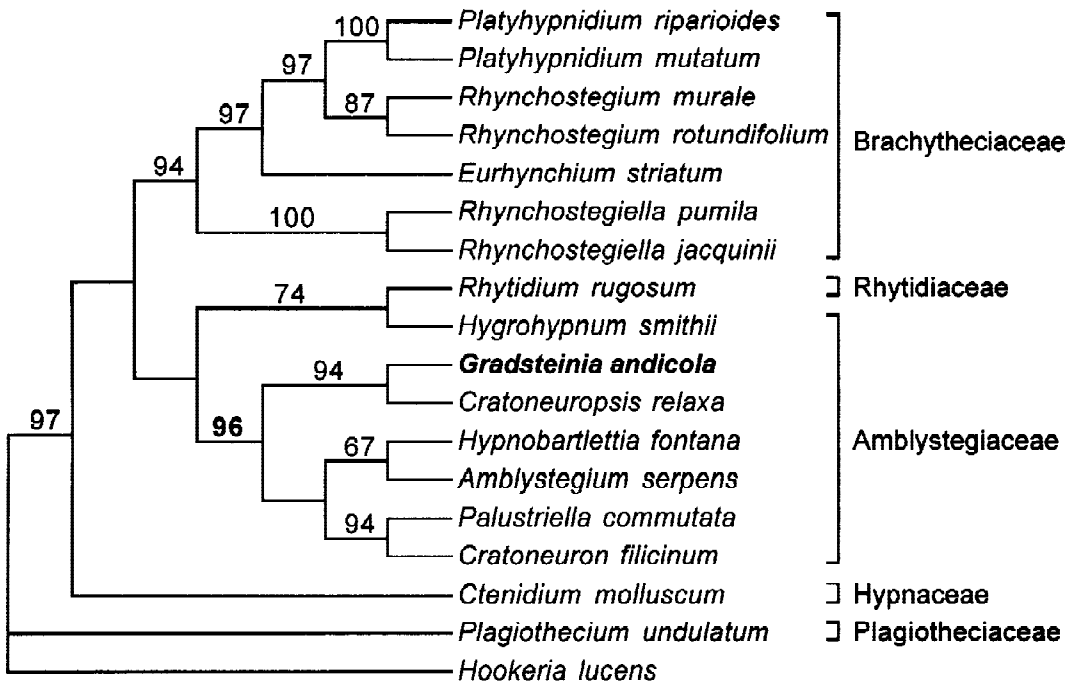


Fig. 2: Maximum parsimony cladogram of 17 species of the Hypnales and *Hookeria lucens* as outgroup based on nrDNA ITS1/2 sequences. Single most parsimonious tree (length 231, CI = 0.792, RI = 0.787) evaluated by a branch-and-bound search with PAUP 4.0b3a. Numbers above branches indicate bootstrap values from 1000 bootstrap replicates with 100 random addition replicates per bootstrap replicate.

among the diplolepidous pleurocarpous mosses based on the occurrence of gametophytic characters like bi- or polystratose laminae in the orders Leucodontales, Hookeriales and Hypnales. Although a tendency for the formation of polystratose laminae is rarely found also in the Leucodontales (e.g. *Muellerobryum* Fleisch., *Rhabdodontium* Broth., aquatic genera of Thamnobryaceae), Ochyra (1990) excluded any affinity because of the elongate lamina cells and a forked costa in *Gradsteinia* in contrast to short lamina cells and a single costa in the Leucodontales. Characters that might support a position in the Hookeriales include the bicellular axillary hairs and porose and thick-walled lamina cells. However, multistratose laminae do not occur in this order (except for partially bistratose laminae in *Diploneuron connivens* Bartr.), and double costae that are fused at the leaf base and thus can be interpreted as forked are also only rarely observed (e.g. in species of

Callicosta C. Müll.).

In contrast to the Leucodontales and Hookeriales, partially or totally bi- or polystratose laminae occur in several genera of the Hypnales, for which the families Donrichardsiaceae, Hypnobartlettiaceae and Vittiaceae had been established by Ochyra (1985, 1987). They were regarded as related to and perhaps segregated from the Amblystegiaceae. Compared with the genera of these families, *Gradsteinia andicola* seemed to be closest to *Donrichardsia macroneuron* due to a similar habit and leaf shape and the strong tendency for polystratose laminae and forked costae (Ochyra 1990), so the genus had to be placed in the subfamily Donrichardsioideae of Donrichardsiaceae (Tab. 1).

The systematic value of the Donrichardsiaceae has so far been difficult to evaluate, not only because all genera are only known in sterile

condition and show very restricted geographical ranges (Fig. 1), resulting in a distribution pattern that is difficult to explain, but also because strong costae and polystratose laminae might be convergences of not closely related aquatic mosses as a result of their environmental conditions. Already Ochyra (1990) stated that the Donrichardsiaceae seemed to be unnatural and polyphyletic. This was first supported by the exclusion of *Richardsiopsis lacustris* (Hedenäs 1997), which was synonymized with *Drepanocladus perplicatus* especially based on the presence of alar cells, but even the remaining species might not be a phylogenetic unit.

The first molecular analyses of species with polystratose laminae, *Platyhypnidium mutatum* (Stech & Frahm 1999) and *Hypnobartlettia fontana* (Stech et al. 1999) revealed their close relationship to Brachytheciaceae or Amblystegiaceae, respectively.

As shown in Fig. 2, the systematic position of *Gradsteinia andicola* can also be clearly determined based on molecular data. It belongs to the same well supported cluster as *Hypnobartlettia fontana*, together with *Amblystegium serpens*, *Cratoneuron filicinum*, *Cratoneuroopsis relaxa* and *Palustriella commutata*. Consequently, *Gradsteinia andicola* must be excluded from the Donrichardsiaceae and transferred to Amblystegiaceae. In contrast to the Hypnobartlettiaceae, which are clearly artificial and have to be rejected according to the position of *Hypnobartlettia fontana*, the Donrichardsiaceae should be maintained as long as no molecular data from *Donrichardsia macroneuron* are available. Nevertheless, rejection of Donrichardsiaceae seems most likely, as *Donrichardsia macroneuron* itself had been placed either in Amblystegiaceae as *Hygroamblystegium macroneuron* Grout or in Brachytheciaceae as *Eurhynchium macroneuron* (Grout) H. Crum, and was transferred back to Amblystegiaceae when Crum & Anderson (1979) established the genus *Donrichardsia*.

The molecular investigation of *Gradsteinia andicola* and the recent work of Ochyra & Bednarek-Ochyra (1999) on *G. torrenticola* reveal that the genus has so far been polyphyletic.

Due to the similarities between *Platyhypnidium mutatum* and *Gradsteinia torrenticola*, namely the broadly ovate, acute, sharply and distantly serrulate leaves with variously polystratose laminae *Gradsteinia torrenticola* was transferred to *Platyhypnidium* as *P. torrenticola* (Ochyra, C. Schmidt & Bültmann) Ochyra & Bednarek-Ochyra (Tab. 1). As a result, *Gradsteinia* now is a monotypic genus of Amblystegiaceae, with the single species *Gradsteinia andicola*.

As polystratose laminae obviously developed independently in Brachytheciaceae (*Platyhypnidium mutatum*, *P. torrenticola*) and Amblystegiaceae (*Gradsteinia andicola*, *Hypnobartlettia fontana*), this gametophytic character is not suitable to evaluate systematic relationships. The close sporophytic and molecular relationship of *Platyhypnidium mutatum* and *P. riparioides* led to the suspicion that species with polystratose laminae might have been developed from nearby growing species by mutations (Stech & Frahm 1999). However, in contrast to *Platyhypnidium mutatum*, the closest relatives of *Gradsteinia* and *Hypnobartlettia* have not yet been determined. *Cratoneuroopsis relaxa*, growing together with *Hypnobartlettia* in Waikoropupu Springs (New Zealand), is genetically more distant from *Hypnobartlettia* than *Amblystegium serpens* (Stech et al. 1999), and seems to be more closely related to *Gradsteinia* (Fig. 2). Material from other species from the type locality of *Gradsteinia andicola* was not available, and no accompanying species were reported by Ochyra (1990). If *Hypnobartlettia* and *Gradsteinia* derived from associated species by mutations, these species remain yet unknown, and molecular data from a broader range of species are needed to test this hypothesis. Like *Hypnobartlettia*, the genus *Gradsteinia* must be maintained for *Gradsteinia andicola* and at present cannot be synonymized with any other genus of Amblystegiaceae.

The circumscription of Amblystegiaceae itself is problematical (cf. Hedenäs 1995). The molecular data (Tab. 2, Fig. 2) indicate a well-supported „core group“ of *Amblystegium*, *Cratoneuroopsis*, *Gradsteinia*, *Hypnobartlettia*, *Cratoneuron* (Cratoneuraceae after Ochyra 1989)

Taxon	new name	new position
Donrichardsioideae		
<i>Donrichardsia macroneuron</i>	—	—
„ <i>Donrichardsia</i> spec.“	<i>Platyhypnidium mutatum</i>	Brachytheciaceae
<i>Gradsteinia andicola</i>	—	Amblystegiaceae
<i>Gradsteinia torrenticola</i>	<i>Platyhypnidium torrenticola</i>	Brachytheciaceae
<i>Richardsiopsis lacustris</i>	<i>Drepanocladus perplicatus</i>	Amblystegiaceae
Sciaromiopsioideae		
<i>Sciaromiella bartlettii</i>	—	—
<i>Sciaromiella longifolia</i> (fossil)	—	—
<i>Sciaromiopsis sinensis</i>	—	—

Tab. 1: Classification of the Donrichardsiaceae of Ochyra (1986a, 1990), with taxonomic and systematic changes according to recent analyses. Taxa in bold show the present circumscription of the family.

and *Palustriella* (Helodiaceae after Ochyra 1989), whereas *Hygrohypnum smithii* forms a separate clade together with *Rhytidium rugosum* (Fig. 2). Cladistic analyses of morphological data (Hedenäs 1995) resulted in a similar „core group“ of *Amblystegium*, *Cratoneurosis*, *Palustriella*, and also *Platylomella lescurii* (Sull.) Andrews, *Vittia pachyloma* (Mont.) Ochyra and *Thuidium glaucinum* (Mitt.) Besch. & Lac., with two species of Amblystegiaceae (*Calliergonella cuspidata* (Hedw.) Loeske, *Campylium stellatum* (Hedw.) C. Jens.) separated from this well-supported clade. The first molecular study of a larger number of Amblystegiacean species based on nrDNA ITS sequences also supported a close relationship of *Amblystegium*, *Cratoneuron* and *Palustriella* as well as a more separate position of *Hygrohypnum smithii* and *Calliergonella cuspidata* (Vanderpoorten 1999). However, some other species as *Hygrohypnum luridum* (Hedw.) Jenn. or *Campylium stellatum* were part of the „core group“ clade in the latter study. A reclassification of Amblystegiaceae is desirable after investigations using other molecular markers, e.g. from cpDNA, and a combined analysis of morphological and molecular data.

DNA sequence comparison obviously allows the resolution of the systematic position of the genera

of Donrichardsiaceae and Hypnobartlettiaceae and the evaluation of the systematic significance of gametophytic characters like polystratose laminae. All recent molecular and non-molecular analyses agree that the species of both families either belong to Amblystegiaceae or to Brachytheciaceae. Further molecular investigations are needed to define the systematic value of the Donrichardsiaceae and the systematic position of the species of Sciaromiopsioideae, which differ from *Donrichardsia* and *Gradsteinia* in their unistratose, but bordered leaves. A molecular systematic study of *Ochyraea tatrensis* is in progress to redefine its position after rejection of the Hypnobartlettiaceae.

Acknowledgments

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Tab. 2 (following pages): Alignment of the nrDNA ITS1/2 sequences of 17 species of the Hypnales and *Hookeria lucens* (ITS1: positions 1-365, ITS2 partial sequences: 366-650). The 5.8S gene and the 3' end of the ITS2 are omitted from the alignment. Unidentified bases are marked by N, small letters indicate ambiguous bases.

