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A molecular phylogeny of bryophytes based on nucleotide sequences of the mitochondrial *nad*5 gene

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Abstract. In contrast to animals, the slowly evolving mitochondrial nucleotide sequences of plants appear well suited to investigate phylogenetic relations between old taxonomic groups. Analysis of *nad*5 gene sequences in 47 bryophytes, the living representatives of very early land plants, confirm this assessment. Statistically reliable phylogenetic trees are obtained with different mathematical approaches. A group I intron sequence conserved in the *nad*5 gene of all 30 mosses and 15 liverworts investigated supports a sister group relationship of the two classes. The intron sequence adds phylogenetic information for fine resolution on top of the conserved exon sequences down to the level of classically defined orders or families, respectively. This intron is not present in the hornworts *Anthoceros husnotii* and *A. punctatus*. The results allow statements on diverging taxonomic interpretations and support the monophyly of the liverworts, mosses, Jungermanniidae, Marchantiidae and Bryidae, and allow recognition of subclasses like Hypnanae and Dicrananae. Among the mosses, the derived orders (subclass Bryidae) are confidently set apart from the Sphagnales, Andreaeales, Polytrichales and Tetraphidales with Buxbaumiales occupying a mediating position. Among the liverworts, full support is found for the classic separation of simple (jungermanniid) and complex thalloid (marchantiid) species with a strikingly low mitochondrial sequence divergence among the latter.

Key words: Bryophyta. Mitochondria, *nad*5 gene, group I intron, molecular phylogeny, taxonomy.

Molecular sequence data are being used increasingly to analyze phylogenetic relationships among taxonomic groups of all living organisms. It is generally more straightforward to find molecular markers which are useful in the analysis of recent evolutionary events, but more difficult to establish datasets that have retained phylogenetic informations on ancient events in land plant evolution which date back more than 450 mio. years. The "bryophytes" are considered as living representatives of such early and primitive land plants.

Many different taxonomic treatments of the bryophytes can be found in the classic systematic literature (Schuster 1984, Frahm and Frey 1992, Fukarek et al. 1992, Walther 1983) and we have chosen examples to discuss their phylogenetic implications on the basis of the molecular phylogenies presented here. While the distinction of the three classes hornworts (Anthoceropsida), liverworts (Hepaticopsida) and mosses (Bryopsida) is mostly accepted, varying numbers of subclasses and orders have been suggested for

lower taxonomic levels, most notably within the Bryopsida. In this latter class at least twelve orders (or suborders, see Vitt 1984) are distinguished, some of which have gained the status of unique superorders, subclasses or even classes (Andreaeopsida, Sphagnopsida) in certain systematic treatments (e.g. Fukarek et al. 1992, Walther 1983). Other orders (e.g. Bartramiales, Timmiales or Neckerales) are only defined as such in some classic assessments or have moot affiliations (e.g. Buxbaumiales, Tetraphidales, Schistostegales) with the more clearly defined orders (Frahm and Frey 1992). Morphological data lack sufficient synapomorphies which can be used in a cladistic analysis to derive a reliably resolved phylogeny of bryophytes as a basis for a natural taxonomy (Mishler and Churchill 1984, Mishler 1986) and were recently complemented with molecular data from the nuclear and chloroplast genomes.

Among the molecular sequences most widely used to establish phylogenies among plants in general and also among bryophytes in particular are the chloroplast *rbc*L gene and the nuclear 18S ribosomal RNA gene. The largest number of bryophyte taxa has recently been included in a *rbc*L study focussing on liverworts (Lewis et al. 1997). Differing results have been obtained in studies of the 18S rRNA gene of bryophytes. Hedderson and colleagues (1996) find support for a monophyly of liverworts and mosses both individually and jointly as sister to the tracheophytes. Capesius and colleagues (Capesius 1995, Capesius and Bopp 1997) on the other hand find support for the paraphyly of liverworts in which the simple thalloid and leafy species (Jungermanniidae) are linked to the mosses to the exclusion of the complex thalloid liverworts (Marchantiidae). Most recently the internal transcribed spacers (ITS) of the chloroplast ribosomal RNA genes were alternatively used to analyze relationships between bryophytes on the molecular level (Samigullin et al. 1998).

We have now used the informative window of a mitochondrial gene sequence for phylogenetic reconstructions among the bryophytes. Short regions of the *cox*3 gene had earlier provided a promising dataset which suggested that the highly conserved mitochondrial sequences may have retained an evolutionary history reflecting early phylogenetic branchings among plants (Hiesel et al. 1994, Malek et al. 1996). To increase the number of informative sites available for analysis, we have now investigated a 1104 nt. coding region of the largest protein gene in the mitochondrial genome, the *nad*5 gene, which encodes subunit 5 of the respiratory chain complex I, the NADH dehydrogenase. The phylogenetic information of the coding region is complemented and extended by a group I intron sequence that we find conserved in all mosses and liverworts investigated. The combined data support the presumed usefulness of mitochondrial sequences in plant phylogeny reconstruction and allow judgements on monophyletic groups down to the level of taxonomically defined orders.

In this contribution we will discuss our phylogenetic and systematic findings based on novel mitochondrial sequence data of 47 bryophyte species in the light of different taxonomic treatments and the results of earlier molecular studies.

Materials and methods

Nucleic Acid Preparation. Plant material was either collected in the field or cultivated on sterile agar (Knoop 1984). Total nucleic acids were extracted in the presence of cetyltrimethylammonium bromide (CTAB) or alternatively with the Plant DNeasy kit (Qiagen). The CTAB extraction method (Doyle and Doyle 1990) was modified by adding 1% polyvinyl-pyrrolidon (PVP 40), incubating at RT for 15 min and extracting once with phenol-chloroform. DNA and RNA were differentially precipitated in the presence of 2 M lithium acetate.

PCR Amplification, Cloning and Sequencing. The upstream primer K (5′– ata tgt ctg agg atc cgc ata g – 3′) and the downstream primer L (5′ – aac ttt ggc caa gga tcc tac aaa – 3′) were routinely

used for amplification of the *nad5* gene region (see Fig. 1). Primer K2 (5′ – agt agc ttr gty cat mtt tat tc – 3′) located 31 nt upstream of K was used as an alternative upstream primer in some cases when internal mispriming was observed with oligonucleotide K. The PCR amplification assays contained 1 µl template DNA (approximately 10 ng – 1 µg), 10 µl 10 × PCR-Buffer (100 mM Tris/HCl pH 8.85, 250 mM KCl, 50 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$), 250 mM of each dNTP, 0.25 µg of each primer, 2.5 U DNA polymerase and double distilled water to 100 µl. Different commercially available thermostable DNA polymerases were used, e.g. a mixture (90:1) of *Taq* DNA Pol (Gibco BRL) and *Pwo* DNA Pol (Boehringer Mannheim). The addition of 0.1% skim milk powder (1 µl of a 10% aequous solution) improved PCR results in some cases (De Boer et al. 1995). A typical amplification assay included an initial denaturation (5 min, 94 °C) followed by 35 cycles with 1 min denaturation at 94 °C, 1 min annealing at 50°–55 °C and 2 min 30 sec synthesis at 72 °C and a final step of synthesis for 6 min at 72 °C. PCR fragments were blunt-end ligated into pBlueskript II SK+ (Stratagene). Positive clones were sequenced with a Thermosequenase kit (Amersham) using Cy5-fluorescence labelled oligonucleotides and run on an Alf Express sequencer (Pharmacia). Sequencing primers were universal and reverse primers of the polylinker sequence and three primers matching internal sequences of the cloned *nad5* fragment (Li: 5′ – gct gca tga atc raa gcr gat act gg – 3′, Le: 5′ – cat atc ttg ctc atc cga cat ggc atg – 3′ and Ki: 5′ – act ygg tta ccy gat gca atg gag ggt – 3′).

Sequence analysis. Sequence handling was done with the UWGCG (Genetics Computer Group, University of Wisconsin) software package 9.0 for UNIX (Devereux et al. 1984). Alignments were obtained with the pileup program. Phylogenetic tree construction was done on Macintosh computers with the PHYLIP software package (Felsenstein 1995) and the beta test version (v4d64) of the PAUP program (D. Swofford, unpublished), kindly made available by the authors. Congruent results were obtained with both program packages for all branchings of significant statistic support.

Results

The *nad5* region under investigation. The *nad5* gene has a complex structure of exons and introns in angiosperms (Fig. 1) which requires the assembly of the functional reading frame by cis-splicing and trans-splicing events (Knoop et al. 1991). For this phylogenetic survey a region of the *nad5* gene was chosen to exclude the known interrupted trans-splicing introns of angiosperms (Fig. 1) to avoid possible complications in PCR analysis.

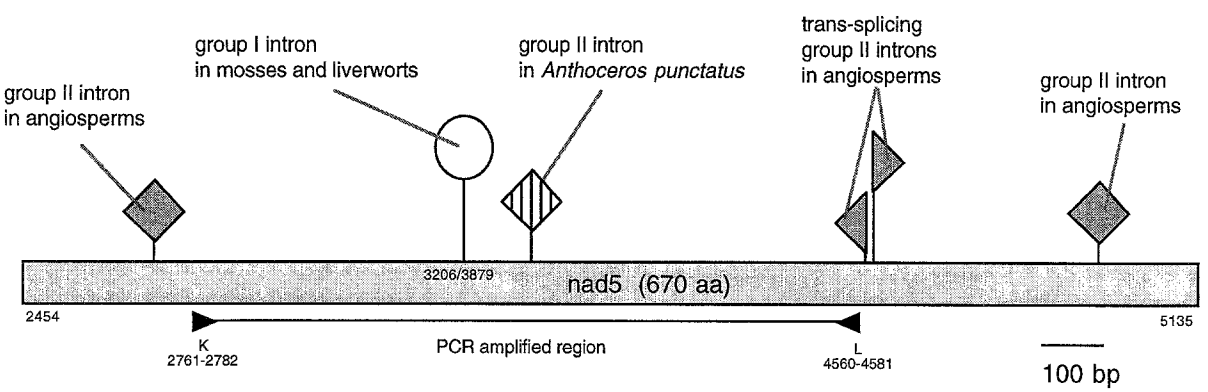


Fig. 1. Exon-intron structure of the *nad5* gene. The reading frame is interrupted by a single group I intron in *Marchantia polymorpha* which is positionally conserved in all bryophytes included in this study to the exclusion of hornworts. *Anthoceros punctatus* carries a group II intron at a different position. The coding sequence of angiosperms is interrupted by two cis-splicing and two trans-splicing group II introns. The indicated region of 368 codons under investigation in this study is bordered by the the two oligonucleotides K and L used for PCR amplification. Numbers indicate nucleotide coordinates of the *Marchantia polymorpha* chondriome sequence (M68929) for reference

Flanking PCR primers were designed for an *nad5* region encoding 368 amino acids, which is continuous in seed plants but includes the single group I intron in the *nad5* gene of the liverwort *Marchantia polymorpha*, the only bryophyte sequence previously available for this gene (Oda et al. 1992). The *nad5* gene regions were amplified and their sequences determined for 30 mosses (Bryopsida), 14 liverworts (Hepaticopsida) and two hornworts (Anthoceropsida) and the green alga *Lamprothamnium papulosum* (Charales). A list of species and corresponding database accessions is provided in Table 1.

Variability in exons and introns of *nad5*. The single group I intron in the *nad5* gene of *Marchantia* is conserved in all 14 liverwort and 30 moss species investigated but absent from the hornwort species *Anthoceros husnotii* and *A. punctatus*, the alga and the known seed plant sequences. An intron of 906 bp is found to interrupt the *nad5* coding region at a different location in *A. punctatus*. Remarkably, this is the first example of a plant mitochondrial intron that shows differential occurrence within a single genus, an observation that is even more striking as *A. punctatus* and *A. husnotii* have been combined as *Anthoceros agrestis* in a taxonomic treatment (Frahm and Frey 1992). Secondary structure modelling defines the novel intron in *Anthoceros punctatus* clearly as a member of the group II class of organellar introns (not shown).

The investigated exon regions of the *nad5* gene are highly conserved in all species and no length variation is observed in the alignment among the bryophytes. A single codon deletion is unique to the alga *Lamprothamnium papulosum* (Charales). The group I intron found in all bryophytes excluding *Anthoceros* shows considerable length variation (liverworts: 642–705 bp, mosses: 532–863 bp) which can be attributed to looped-out regions of the secondary structure, most notably L8 (see Fig. 3). The intron sequence is thus more variable and may yield phylogenetic information for resolution of close branches not resolved by the better conserved coding region. The *nad5* nucleotide alignment excluding introns (1104 nt.) allows on the other hand inclusion of species containing either introns at different positions or none at all and thus the definition of the algal sequence of *Lamprothamnium* as an outgroup for tree rooting.

Plant organelle genes are subject to RNA editing. A recent systematic study has shown that DNA sequences may retain more phylogenetic information than cDNA sequences but most importantly that both types of sequences should not be mixed in alignments used for phylogenetic tree construction (Bowe and dePamphilis 1996). Trees shown here are based on the genomic sequence alignments, which are available from the WWW under http://www.biologie.uni-ulm.de/bio2/knoop/nad5.

A phylogeny based on the *nad5* coding sequences. Phylogenetic trees based on the exon sequence alignment were calculated with the three established methods (parsimony, maximum likelihood, distance matrix) and identical tree topologies were obtained for statistically significant branchings. A maximum likelihood tree constructed with DNAML of the PHYLIP package is shown in Fig. 2 which also includes the bootstrap values obtained with the parsimony method (DNAPARS). Only branchings supported with at least 60% bootstrap reliability are indicated. We will here consider only bootstrap values larger than 80% sufficiently reliable as a basis for the discussion of monophyletic groups. These groups generally turn out to be not only robust to variation of alignment parameters (see below) but also to the in- and exclusion of taxa in phylogenetic test caluclations.

The monophyly of liverworts (Hepaticopsida) and mosses (Bryopsida) is unequivocally supported. A sister group relationship to the exclusion of hornworts is suggested by the shared group I intron and the tree topology but only weakly supported (63%) by the bootstrap analysis. No significant sister group relationships of any bryophyte group with

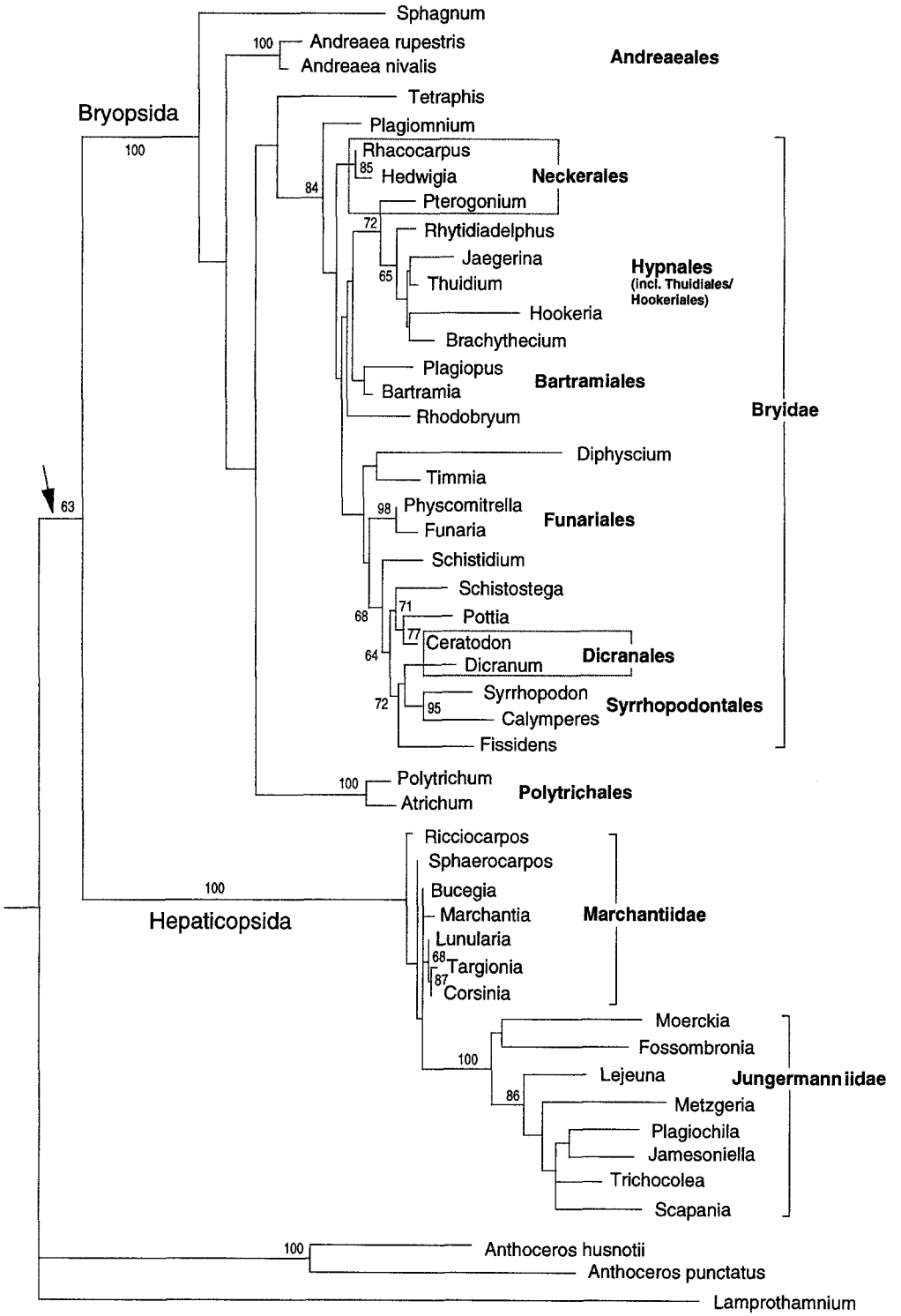
Table 1. A list of species investigated in this study. All *nad5* sequences except the one of *Marchantia polymorpha* are new accessions obtained during this study. The bryophyte species are grouped according to the taxonomy by Frahm and Frey (1992) which was chosen because it defines intermediate taxonomic levels for superorders and subclasses that can be discussed on the basis of the phylogenetic trees presented here. This taxonomy is also adopted by Sitte et al. (1998) with the exception that Marchantiopsida and Jungermanniopsida are considered at the level of classes. Buxbaumiales, Tetraphidales and Schistostegales have not been assigned to higher taxonomic levels by Frahm and Frey. Syrrhopodontales are tropical species not included in this taxonomy. Alternative taxonomic treatments are discussed in the text. As an example, other taxonomies (e.g. Fukarek et al. 1992) do not consider Bartramiales and Timmiales as unique orders but include these among Bryales, define Isobryales and Orthotrichales instead of Neckerales or place Thuidiales as a unique order. Alternatively, 15 suborders have been defined in a single order Bryales comprising the derived genera (Vitt 1984)

Order	Species	accession
Anthoceropsida		
Anthocerotales	Anthoceros husnotii Steph.	AJ000697
	Anthoceros punctatus L.	AJ000698
Hepaticopsida		
Sphaerocarpidae		
Sphaerocarpales	Sphaerocarpos donnellii Aust.	AJ001033
Marchantiidae		
Marchantiales	Bucegia romanica Radian	AJ001031
	Corsinia coriandrina (Spreng.) Lindb.	AJ001034
	Lunularia cruciata (L.) Lindb.	AJ001002
	Marchantia polymorpha L.	M68929
	Targionia hypophylla L.	AJ001001
Ricciales	Ricciocarpos natans (L.) Corda	AJ001032
Jungermanniidae		
Jungermanniales	Jamesoniella autumnalis (DC.) Steph.	AJ000700
	Lejeuna cavifolia (Ehrh.) Lindb.	AJ000701
	Plagiochila asplenioides (L.) Dum.	AJ000704
	Scapania nemorea (L.) Grolle	AJ000706
	Trichocolea tomentella (Ehrh.) Dum.	AJ000707
Metzgeriales	Fossombronia pusilla (L.) Nees	AJ000699
	Metzgeria conjugata Lindb.	AJ000703
	Moerckia flotoviana (Nees) Schiffn.	AJ223717
Bryopsida		
Buxbaumiales	Diphyscium sessile (Schmid.) Lindb.	Z98972
Tetraphidales	Tetraphis pellucida Hedw.	AJ224855
Schistostegales	Schistostega pennata (Hedw.) Web. and Mohr	AJ224856
Syrrhopodontales	Calymperes erodes Müll.	Z98952
	Syrrhopodon spec.	Z98953
Sphagnidae		
	Sphagnum fallax Klinggr.	AJ001225
Andreaeidae		
	Andreaea nivalis Hook.	AJ001226
	Andreaea rupestris Hedw.	AJ001227
Bryidae		
Polytrichanae		
Polytrichales	Atrichum undulatum (Hedw.) P. Beauv.	AJ001229

Table 1 (continued)

Order	Species	accession
	Polytrichum formosum Hedw.	AJ001228
Dicrananae		
Dicranales	Ceratodon purpureus (Hedw.) Brid.	Z98955
	Dicranum scoparium Hedw.	Z98956
Fissidentales	Fissidens cristatus Wils. ex Mitt.	Z98954
Grimmiales	Schistidium apocarpum (Hedw.) B.S.G. em. Poelt	Z98958
Pottiales	Pottia truncata (Hedw.) B.S.G.	Z98957
Bartramianae		
Bartramiales	Bartramia halleriana Hedw.	Z98961
	Plagiopus oederi (Brid.) Limpr.	Z98962
Timmiales	Timmia bavarica Hessl.	Z98963
Funarianae		
Funariales	Funaria hygrometrica Hedw.	Z98959
	Physcomitrella patens (Hedw.) B.S.G.	Z98960
Bryanae		
Bryales	Rhodobryum roseum (Hedw.) Limpr.	Z98964
	Plagiomnium cuspidatum (Hedw.) Kop.	Z98965
Hypnanae		
Neckerales	Hedwigia ciliata (Hedw.) P. Beauv.	Z98966
	Pterogonium gracile (Hedw.) Sm.	Z98968
	Rhacocarpus purpurascens (Brid.) Par.	Z98967
Hookeriales	Hookeria lucens (Hedw.) Sm.	Z98969
Hypnales	Brachythecium rutabulum (Hedw.) B.S.G.	Z98970
	Rhytidiadelphus triquetrus (Hedw.) Warnst.	Z98971
	Jaegerina stolonifera Müll.	AJ224854
	Thuidium tamariscinum (Hedw.) B.S.G.	AJ004809
Alga		
Charales	Lamprothamnium papulosum (Wallr.) J. Gr.	AJ000702

Fig. 2. Phylogenetic tree based on the *nad5* exon sequence alignment determined with the maximum likelihood method (DNAML of the PHYLIP program package using default parameters). The *nad5* sequence of the green alga *Lamprothamnium* is used to root the tree (ln likelihood −7110). Bootstrap resampling (100-fold) values larger than 60 derived for trees alternatively constructed with the parsimony (DNAPARS) method are indicated in the maximum likelihood topology. Minor differences in topology between the methods are restricted to statistically unsupported branchings. Mosses (Bryopsida) and liverworts (Hepaticopsida) are represented as monophyletic groups. A sister group relationship of the two classes is suggested by the presence of a common group I intron (arrow) but only weakly supported by bootstrapping. Boxes indicate paraphyly of the orders Dicranales and Neckerales sensu Frahm and Frey (1992). Names of orders sensu Frahm and Frey (1992) are indicated where at least two species are included.


Sphagnum
100
Andreaea rupestris
Andreaea nivalis
Andreaeales
Bryopsida
Tetraphis
Plagiomnium
100
Rhacocarpus
85
Hedwigia
Neckerales
84
Pterogonium
72
Rhytidiadelphus
Jaegerina
65
Thuidium
Hypnales
(incl. Thuidiales/
Hookeriales)
Hookeria
Brachythecium
Plagiopus
Bartramia
Bartramiales
Rhodobryum
Bryidae
Diphyscium
Timmia
63
98
Physcomitrella
Funaria
Funariales
Schistidium
Schistostega
68
71
Pottia
77
Ceratodon
64
Dicranum
Dicranales
72
Syrrhopodon
95
Calymperes
Syrrhopodontales
Fissidens
100
Polytrichum
Atrichum
Polytrichales
Ricciocarpos
Sphaerocarpos
100
Bucegia
Hepaticopsida
Marchantia
Marchantiidae
Lunularia
68
Targionia
87
Corsinia
Moerckia
Fossombronia
100
Lejeuna
Jungermanniidae
86
Metzgeria
Plagiochila
Jamesoniella
Trichocolea
Scapania
100
Anthoceros husnotii
Anthoceros punctatus
Lamprothamnium

angiosperms is identified when the available sequences of the latter are included in the analysis (not shown).

Within the **mosses (Bryopsida)** all genera to the exclusion of *Sphagnum, Andreaea, Tetraphis* and those of the Polytrichales (*Atrichum, Polytrichum*) form an additional monophyletic group of derived mosses in full agreement with classical taxonomy (e.g. the subclass Bryidae sensu Frahm and Frey (1992), but to the exclusion of Polytrichanae). The branching order of *Sphagnum*, Andreaeales, Polytrichales and *Tetraphis* remains unsupported by bootstrap values (<60) but is identical in the ML and parsimony analyses. Treating Polytrichidae and Tetraphididae as unique subclasses distinct from the Bryidae as in some taxonomic systems of the bryophytes (e.g. Walther 1983, Fukarek et al. 1992) thus appears justified.

Dichotomies are statistically supported in each case for two species of Funariales, Syrrhopodontales, Polytrichales and Andreaeales, respectively. Likewise all species of the Hypnales, including *Thuidium* and *Hookeria* which are treated in separate orders occasionally (Fukarek 1992), are found in a monophyletic group, although with low bootstrap support when based on exon data only. Similarly, two genera of Bartramiales (*Bartramia, Plagiopus*) are linked with all tree-building approaches but the dichotomy remains unsupported by bootstrap analysis.

Within the **liverworts (Hepaticopsida)** the Jungermanniidae (simple thalloid and leafy liverworts) appear as an advanced group comprising Jungermanniales and Metzgeriales. A monophyletic subgroup within the Jungermanniidae includes *Metzgeria* with the Jungermanniales. The monophyly of Jungermanniidae comprising these two orders is well supported in any case. Interestingly, all branches of Marchantiidae liverworts are significantly shorter than those of Jungermanniidae and preclude to consider the former subclass as monophyletic from the exon data alone.

Among the **hornworts (Anthoceropsida)** the two species studied are clearly linked but with terminal branches of significant lengths supporting *Anthoceros* as a divergent (and possibly evolutionary old) genus. Its placement in the overall topology remains ambiguous and the junction of the long hornwort branch to the rest of the tree can not be placed with confidence. These findings are supported by the group I intron conserved among the non-hornwort bryophytes and the presence of a different intron which is so far unique to *A. punctatus*.

A phylogeny including the group I intron in *nad5*. The group I intron sequence identified in all bryophytes investigated here with the exception of *Anthoceros* can be used as a source of additional phylogenetic information. The intron sequence is under a lower evolutionary constraint than the coding region and this is reflected in an increased sequence variability, most notably a much higher rate of indels vs. base exchanges, as typical for structural RNAs. The canonical group I intron secondary structure (shown examplarily in Fig. 3 for *Brachythecium rutabulum)* is, however, conserved in all 30 mosses and 15 liverworts. Large and non-overlapping gaps in loop L8 of the Funariales, *Schistostega, Hedwigia* and most importantly *Tetraphis*, and a non-homologous sequence stretch of liverworts vs. mosses make the intron alignment prone to individual judgement and the phylogenetic impact of single large sequence indels is easily overestimated, e.g. when gaps are considered as a fifth character state in parsimony analyses. However, the variation of alignment parameters or manual editing do not affect confidently identified groupings but rather alter bootstrap values. The full alignment can thus also be used for tree construction without manual editing (Fig. 4). Taxon subsets (e.g. on subclass level) allow more unequivocal alignments and result in higher bootstrap confidence for certain branches (Fig. 4). The intron-including tree topology confirms the clearly defined branchings derived

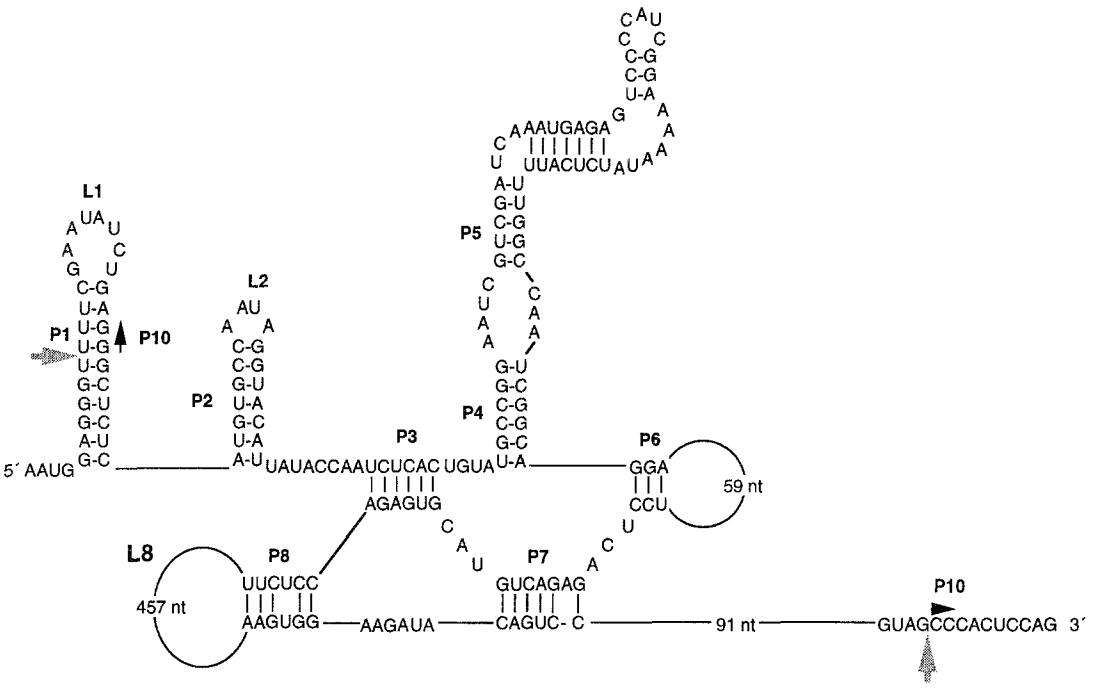


Fig. 3. The *nad5* group I intron secondary structure conserved in mosses and liverworts is shown as an example for *Brachythecium rutabulum*. Base pairing in the secondary and tertiary structure interactions P1 through P10 are indicated. Grey arrows indicate splice sites. Alignment of the intron sequences from the entire bryophyte spectrum is unambiguous when the most variable part of the intron, an internal region of loop L8, is excluded (see text)

from the exon-only data set (Fig. 2) without conflict and higher bootstrap values are obtained supporting detailed branchings not confidently identified before.

Phylogenetic relations among the mosses. The concept of Sphagnidae, Andreaeidae, Polytrichidae and Tetraphididae as subclasses parallel to Bryidae is fully supported by the intron data. More importantly, Buxbaumiales (*Diphyscium*) are now clearly placed with high bootstrap reliability as link between the four former orders (or subclasses) and the advanced mosses in the subclass Bryidae (Fig. 4).

Within the Bryidae a monophyletic formation that reminds of the superorder Hypnanae (see Table 1) *sensu* Frahm and Frey (1992) becomes discernible. This group, however, includes Bryales and Bartramiales which have been placed in superorders of their own, with the Bartramianae sensu Frahm and Frey also including the Timmiales. The *nad5* data do not suggest a classification of Hookeriales or Thuidiales in orders of their own as in some taxonomies (Fukarek et al. 1992) but their inclusion among Hypnales, which appear in a monophyletic group as the most derived taxa of this superorder. Among three species of Neckerales sensu Frahm and Frey only *Hedwigia* and *Rhacocarpus* (Hedwigiaceae) are linked in a well-supported dichotomy to the exclusion of *Pterogonium*, which is placed closer to the Hypnales. It is interesting to note in that context, that the order Neckerales is replaced by two orders Orthotrichales and Isobryales in alternative taxonomies (e.g. Fukarek et al. 1992). Bartramiales and Bryales occupy intermediate positions but their

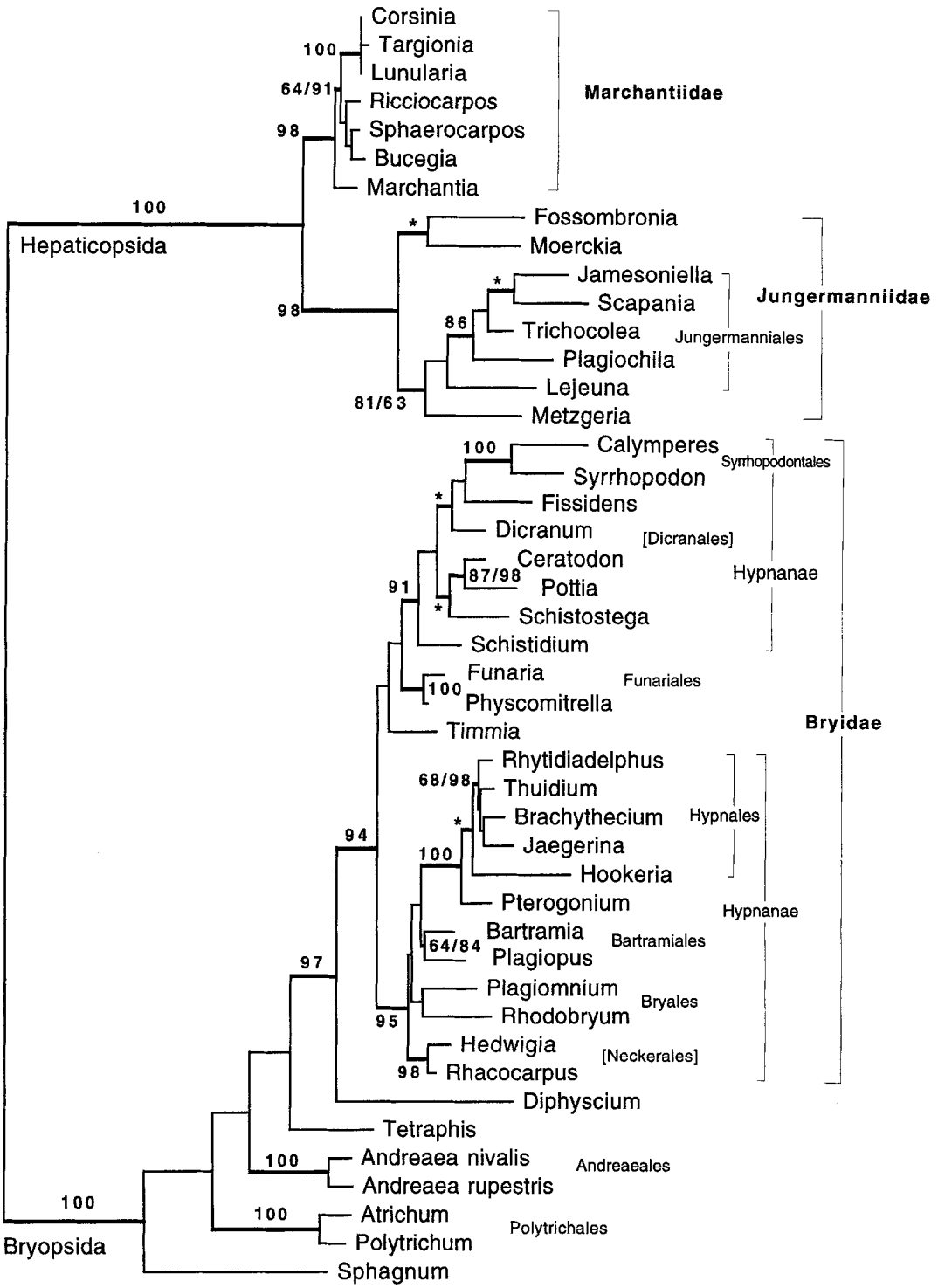


Fig. 4. The complete intron-including *nad5* sequence alignment (45 taxa, 2145 positions) obtained with PILEUP (gap penalty 1, gap extension penalty 0) has been used for tree construction without further editing. The potential for manual editing is obvious for this alignment, but like the variation of alignment

detailed branching order is not yet confidently resolved by the *nad5* data alone.

The Hypnanae cluster is confidently set apart from a second group of mosses in which the orders Dicranales, Fissidentales, Schistostegales, Syrrhopodontales and Pottiales are joined as monophyletic with *Schistidium* (Grimmiales) as a sister group. This cluster is in full agreement with the taxonomic proposal of a superorder Dicrananae including these orders (Frahm and Frey 1992). Like the Neckerales, however, the Dicranales themselves *(Dicranum* and *Ceratodon)* are rejected as a monophyletic group. *Dicranum* and *Ceratodon* are representatives of two different families (Dicranaceae and Ditrichiaceae) among the Dicranales and the divergence in this order may have been underestimated.

As stated above, the mitochondrial *nad5* sequences do not support a proposed superorder Bartramianae which comprises Timmiales and Bartramiales. Interestingly, both orders are not recognized at this taxonomic level in alternative taxonomies but included in the Bryales (Fukarek et al. 1992). *Timmia* is an early-branching genus in the subclass Bryidae according to the *nad5* data that may be less derived than previously thought. Its clear placement in relation to the Funariales and to the two potential superorders discussed above can, however, not yet be stated with confidence on the basis of the *nad5* sequences.

Phylogenetic relations among the liverworts. The clear separation of Jungermanniidae and Marchantiidae is fully confirmed when the intron data are taken into account. As for the exon-based phylogeny very long terminal branches are obtained for the Jungermanniidae in stark contrast to the Marchantiidae. Consequently, resolution among the complex thalloid liverworts is low and significant bootstrap support is restricted to a group including *Corsinia, Targionia* and *Lunularia.* The few informative nucleotide differences preclude the identification of further branching details among the Marchantiidae, most notably on the placement of *Ricciocarpos* or *Sphaerocarpos* in unique orders or even higher taxonomic ranks as suggested by some taxonomists.

Within the Jungermanniidae the close association of *Metzgeria* (to the exclusion of the other two genera of Metzgeriales *Moerckia* and *Fossombronia*) with the Jungermanniales is supported from the intron data with higher bootstrap reliability than in the exon data set. A most derived group of the four Jungermanniales genera *Trichocolea, Plagiochila, Jamesoniella* and *Scapania* that was recognizable in the exon based tree is now clearly defined with bootstrap reliability.

Fig. 4 (continued)
parameters (e.g. gap penalty 2), is without influence on the statistically supported branches. Alignments are available from http://www.biologie.uni-ulm.de/bio2/knoop/nad5. The tree shown is the parsimony consensus topology obtained with PAUP*4d64 after 1000 bootstrap replicates. The single change of a significant branch in the distance matrix tree topology is the placement of *Tetraphis* as the basal-most branching genus of Bryopsida due to the large deletions in the intron. Artificial attraction of moss sequences with large deletions to the root of Bryopsida is e.g. also observed when gaps are counted as 5th character state in parsimony analyses. Bootstrap values are given for the parsimony/uncorrected distance matrix analyses only where at least one value is over 80 (for clarity, the arithmetic mean only is given when difference between the two values is less than 10). Bootstrap values over 80 are additionally obtained for some branches marked by asterisks when alignments of taxon subsets (e.g. on subclass level) are used for tree construction. Taxonomic suggestions of superorders (Dicrananae, Hypnanae) and subclasses (Jungermanniidae, Marchantiidae, Bryidae) supported by the trees are indicated

Discussion

Mitochondrial sequences as phylogenetic markers in the land plants. The slowly evolving mitochondrial sequences of plants may reveal a rich source of phylogenetic information, most notably for the analysis of early diverging groups in the land plant phylogeny. Only short regions of the mitochondrial *cox*3 gene had so far been analyzed with respect to its phylogenetic potential (Hiesel et al. 1994, Malek et al. 1996).

We have now investigated the suitability of exon and intron sequences from the mitochondrial *nad*5 gene to obtain phylogenetic trees for bryophytes as (one of) the presumptive earliest branching groups of land plants. Consistent tree topologies are obtained irrespective of the tree construction method used. The coding region allows a robust overall determination of phylogenetic relationships, supports mosses, liverworts and hornworts as monophyletic taxa and indicates the monophylies of younger phylogenetic separations within the former two classes. Liverworts and mosses are combined as sister taxa by a common group I intron to the exclusion of all other land plants. Closer phylogenetic relationships are defined in more detail when this conserved group I intron sequence in mosses and liverworts is taken into account for phylogenetic tree construction. Given the much higher variability of the intron, resolution reaches in some cases down to the level of taxonomically defined orders or even lower taxonomic levels, depending on the taxonomic system of reference. It is interesting to note that the different modes of evolution of a structural RNA molecule (the group I intron sequence) and the coding region largely result in concordant phylogenies and similar relative terminal branch lengths for this mitochondrial locus, at least when the impact of larger sequence indels in the intron is excluded.

Bryophyte taxonomy and phylogeny. Some of the taxonomic units proposed in systematic treatments of bryophytes are fully supported by the *nad*5 derived phylogenetic trees, others are to be considered as either moot or obsolete. Among the mosses the exclusion of Sphagnales, Andreaeales, Polytrichales, Tetraphidales and Buxbaumiales from the Bryidae sensu strictu is unambiguous and may justify their placement in separate subclasses. Buxbaumiales are clearly placed as the linking taxon between the former groups and the Bryidae according to *nad*5 data. This observation is in full accord with a cladistic analysis of morphological characters (Mishler and Churchill 1984). Likewise the recent investigation of chloroplast ITS sequences (Samigullin et al. 1998) also excludes these five orders from a subgroup of Bryidae according to our understanding.

Among the latter, the taxonomic groups Hypnanae and Dicrananae sensu Frahm and Frey (1992) are recognizable after modifications. A monophyletic group "Hypnanae" defined by the *nad*5 data would include the Bryales and Bartramiales. Superorders Bryanae and Bartramianae (Frahm and Frey 1992) can on the other hand not be recognized in the *nad*5 based trees, rather in line with the inclusion of Bartramiales among Bryales in alternative taxonomies (Walther 1983, Fukarek et al. 1992, Vitt 1984). The superorder Dicrananae finds so far support from the trees constructed here and should include the orders Schistostegales and Syrrhopodontales. The separation in the two superorders Dicrananae and Hypnanae also reminds vaguely of the dichotomy among 15 suborders in a single order Bryales sensu Vitt (1984), although Buxbaumiales have been included in the former and Funariales in the latter group in this system. The two superorders are likewise recognizable in the very recently published trees based on the chloroplast ITS sequences (Samigullin et al. 1998) but this is not fully conclusive as no species of Bartramiales, Timmiales, Funariales, Fissidentales and Pottiales had been included in that study. Timmiales and Funariales are placed as

unique orders near a dichotomy separating the two suggested superorders Hypnanae and Dicrananae according to our phylogeny, but their exact branching pattern can not be resolved at this time based on *nad5* alone. A study of 19 mosses on the basis of the 18S rRNA does not reveal any further reliable information other than support for an exclusion of Polytrichales and Sphagnales from the Bryidae in the sense outlined above (Capesius and Stech 1997). Considering the orders Neckerales (Frahm and Frey 1992) and Dicranales as monophyletic groups finds no support from the *nad5* based phylogenies and other molecular studies; similarly, members of the Neckerales are not placed jointly (Capesius and Stech 1997, Samigullin et al. 1998). Obviously the inclusion of further problem taxa is necessary in line with a further increase of data sets (or their fusion) to allow more definite statements.

Based on nuclear 18S rRNA genes of bryophytes Capesius and colleagues find a close affiliation between the Jungermanniidae and the Bryopsida (Bopp and Capesius 1996, Capesius and Bopp 1997). The *nad5* based trees do not support this observation but rather confirm the classic treatment of liverworts comprising Marchantiidae and Jungermaniidae. This classical view is also supported by the other molecular studies of bryophyte evolution (e.g. Samigullin et al. 1998), including those which are also based on the 18S rRNA data, albeit on different taxon samplings (Hedderson et al. 1996).

The long terminal branches of Marchantiidae vs. Jungermanniidae in the 18S rRNA trees (Capesius and Bopp 1997) present just the opposite situation when compared to the mitochondrial data. It remains an interesting biological question to explain why the molecular clock apparently ticks so much slower in the mitochondrial but so much faster in the nuclear DNA of Marchantiidae. Consistently, the 18S data result in a more detailed resolution of the ten Marchantiidae species with *Sphaerocarpos* at their base while no significant branchings can be deduced for ten Jungermanniidae which strikingly are found in a well supported monophyly together with *Anthoceros* (Capesius 1995, Bopp and Capesius 1996). The 18S rRNA data set can result in an odd, albeit statistically insignificant, topology with Jungermanniidae branching after mosses and *Anthoceros* when Polytrichidae are included (Capesius 1995).

Monophyly of four Bryopsida species on the one hand and nine genera of Marchantiidae is observed in a recent study based on the *rbc*L gene (Lewis et al. 1997). Among the Jungermanniidae the only statistically supported branch contains advanced Jungermanniales to the exclusion of *Porella* which is grouped with *Metzgeria*. The peculiar association of *Metzgeria* with the Jungermanniales is thus observed both in the mitochondrial *nad5* trees presented here as well as with the chloroplast *rbc*L data.

In conclusion, the mitochondrial genes of plants and their introns appear to be a useful source of phylogenetic information in old plant groups. We will extend the findings based on the *nad5* gene both taxonomically and with data from further mitochondrial loci. Preliminary data show that the mitochondrial *nad2* gene may allow amplification of a similarly large region that includes different introns in mosses and liverworts. These data may also help to define the position of bryophytes in relation to vascular plants which is still unclear at present.

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References

Bopp M., Capesius I. (1996) New aspects of bryophyte taxonomy provided by a molecular approach. Bot. Acta 109: 368–372.

Bowe L. M., dePamphilis C. W. (1996) Effects of RNA editing and gene processing on phylogenetic reconstruction. Mol. Biol. Evol. 13: 1159–1166.

Capesius I. (1995) A molecular phylogeny of bryophytes based on the nuclear encoded 18S rRNA genes. J. Plant Physiol. 146: 59–63.

Capesius I., Stech M. (1997) Molecular relationships within mosses based on 18S rRNA gene sequences. Nova Hedwigia 64: 525–533.

Capesius I., Bopp M. (1997) New classification of liverworts based on molecular and morphological data. Plant Syst. Evol. 207: 87–97.

DeBoer S. H., Ward L. J., Li X., Chittarajan S. (1995) Attenuation of PCR inhibition in the presence of plant compounds by addition of BLOTTO. Nucl. Acids Res. 23: 2567–2568.

Devereux R., Haeberli P., Smithies O. (1984) A comprehensive set of sequence analysis programs for the VAX. Nucl. Acids. Res. 12: 387–395.

Doyle J. J., Doyle J. L. (1990) Isolation of plant DNA from fresh tissue. Focus 12: 13–15.

Felsenstein J. (1995) PHYLIP version 3.57c manual. University of Washington, Seattle.

Frahm J.-P., Frey W. (1992) Moosflora. UTB, Stuttgart.

Fukarek F., Schultze-Motel J., Siegel M. (1992) Moose, Farne, Nacktsamer. Urania, Leipzig.

Hedderson T. A., Chapman R. L., Rootes W. L. (1996) Phylogenetic relationships of bryophytes inferred from nuclear-encoded rRNA gene sequences. Plant Syst. Evol. 200: 213–224.

Hiesel R., von Haeseler A., Brennicke A. (1994) Plant mitochondrial nucleic acid sequences as a tool for phylogenetic analysis. Proc. Natl. Acad. Sci. USA 91: 634–638.

Knoop B. (1984) Culture methods for bryophytes. In: Vasil I. (ed.) Cell culture and somatic cell genetics of plants. Academic Press, New York, pp. 96–106.

Knoop V., Schuster W., Wissinger B., Brennicke A. (1991) Trans splicing integrates an exon of 22 nucleotides into the *nad*5 mRNA in higher plant mitochondria. EMBO J. 10: 3483–3493.

Lewis L. A., Mishler B. D., Vilgalys R. (1997) Phylogenetic relationships of the liverworts (Hepaticae), a basal embryophyte lineage, inferred from nucleotide sequence data of the chloroplast gene *rbc*L. Mol. Phyl. Evol. 7: 377–393.

Malek O., Lättig K., Hiesel R., Brennicke A., Knoop V. (1996) RNA editing in bryophytes and a molecular phylogeny of land plants. EMBO J. 15: 1403–1411.

Mishler B. D., Churchill S. P. (1984) A cladistic approach to the phylogeny of the "bryophytes". Brittonia 36: 406–424.

Mishler B. D. (1986) A Hennigian approach to bryophyte phylogeny. J. Bryol. 14: 71–81.

Oda K., Yamato K., Ohta E., Nakamura Y., Takemura M., Nozato N., Akashi K., Kanegae T., Ogura Y., Kohchi T., Ohyama K. (1992) Gene organization deduced from the complete sequence of liverwort *Marchantia polymorpha* mitochondrial DNA. A primitive form of plant mitochondrial genome. J. Mol. Biol. 223: 1–7.

Samigullin T. H., Valiejo-Roman K. M., Troitsky A. V., Bobrova V. K., Filin V. R., Martin W., Antonov A. S. (1998) Sequences of rDNA internal transcribed spacers from the chloroplast DNA of 26 bryophytes: properties and phylogenetic utility. FEBS Lett. 422: 47–51.

Schuster R. M. (ed.) (1984) New manual of bryology. Hattori Botanical Laboratory, Nichinan.

Sitte P., Ziegler H., Ehrendorfer F., Bresinsky A. (1998) Strasburger – Lehrbuch der Botanik für Hochschulen, 34th ed. Gustav Fischer, Stuttgart Jena New York.

Vitt D. H. (1984) Classification of the Bryopsida. In: Schuster R. M. (ed.) New manual of bryology. Hattori Botanical Laboratory, Nichinan, pp. 696–759.

Walther K. (1983) Kapitel V,2, Brophytina. Laubmoose. In: Gerloff J., Poelt J. (eds.) Syllabus der Pflanzenfamilien. Gebrüder Borntraeger, Berlin.

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