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Phylogenetic relationships in the Lejeuneaceae (Hepaticae) inferred using ITS sequences of nuclear ribosomal DNA

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Abstract

Sequences of the ITS1–5.8S–ITS2 region of nuclear ribosomal DNA were generated for 12 species from 9 genera of Lejeuneaceae and a single species of Jubulaceae (outgroup). The taxon sampling of Lejeuneaceae included representatives of the two widely recognized subfamilies, Lejeuneoideae and Ptychanthoideae. The molecular dataset was analysed independently and in combination with a morphological dataset. The nrITS dataset and the combined dataset resulted in identical topologies. The genus *Bryopteris*, sometimes treated as a separate family Bryopteridaceae, is nested within the Lejeuneaceae subfamily Ptychanthoideae. Lejeuneaceae subfamily Lejeuneoideae proved to be paraphyletic with the tribe Lejeuneeae sister to Ptychanthoideae, albeit without significant bootstrap support. The tribes Brachiolejeuneeae and Cheilolejeuneeae of Lejeuneoideae, established recently based on morphological evidence, are well supported in bootstrap analyses both of the ITS and the combined molecular–morphological datasets. The results support classifications of Lejeuneaceae based on morphological data and demonstrate the usefulness of the ITS region for phylogenetic studies within or among closely related genera of Lejeuneaceae.

Keywords: nrITS; DNA; Molecular phylogeny; Morphology; Bryophytes; Liverworts; Lejeuneaceae

Introduction

The family Lejeuneaceae is the largest family of the liverworts (Hepaticae) and contains almost 1000 species in about 90 currently accepted genera (Gradstein et al. 2003). Most of the species are tropical epiphytes. Attempts to classify the genera into subfamilies and tribes have been numerous (e.g., Mizutani 1961; Schuster 1963, 1984, 2001; Grolle 1964, 1983; Gradstein 1994; Weis 2001), but results were conflicting due to the limited number of phylogenetically informative morphological characters. At least nine subfamilies and many tribes and subtribes were proposed, but a strict consensus tree of the genera based on total morphological evidence, including 50 generic characters of sporophytes and gametophytes, was largely unresolved (Gradstein et al. 2003). The study nevertheless supported the recognition of two broad subfamilies, Ptychanthoideae and Lejeuneoideae, accepted by most previous authors, and some smaller clades. The poor resolution reflected the low morphological complexity of the gametophyte and sporophyte generations of this liverwort lineage, and indicated that additional characters are needed to obtain a better resolution and more phylogenetic hypotheses with statistical support.

Phylogenetic studies of liverworts using DNA sequence data were usually focused on the global relationships of the liverwort lineages (Capesius 1995; Capesius and Bopp 1997; Lewis et al. 1997; Beckert et al. 1999;

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Fig. 1. The nrITS region (ITS1–5.8S–ITS2), with indication of the binding positions of the primers used.

Nickrent et al. 2000; Pruchner et al. 2001; Stech and Frey 2001). Only a few papers explore relationships within families or genera of liverworts (Long et al. 2000; Boisselier-Dubayle et al. 2002; Heinrichs et al. 2002a, b; Groth et al. 2003). Ahonen et al. (2003) published a first molecular–phylogenetic analysis of 12 genera of Lejeuneaceae using partial cp *rbc*L and *trn*L-*trn*F as well as nrITS2 sequences. An important result of this study was the exclusion of *Nipponolejeunea* S.Hatt. from Lejeuneaceae. Ahonen et al. (2003, p. 301) also suggested the possible usefulness of the nrITS region for clarifying relationships between closely related taxa of Lejeuneaceae, but were not able to generate sequences of ITS1.

Here, we establish a protocol for the complete nrITS region (ITS1–5.8S–ITS2) and explore the usefulness of this marker for clarifying relationships within Lejeuneaceae (Fig. 1).

Material and methods

Taxon sampling

The number of available collections of Lejeuneaceae not older than 2 years and allowing for sequencing of the ITS region was limited, but included species from the two subfamilies Ptychanthoideae and Lejeuneoideae, and from the main tribes with the exception of the Calatholejeuneeae (Gradstein et al. 2003). Lejeuneaceae (totalling approx. 90 genera) were represented by 12 species in 8 genera: subfamily Ptychanthoideae (18 genera) by 3 species in 2 genera, subfamily Lejeuneoideae (approx. 60 genera) by 9 species in 7 genera, Lejeuneoideae tribe Brachiolejeuneeae (8 genera) by 3 species in 3 genera, and Lejeuneoideae tribe Lejeuneeae (approx. 40 genera) by 6 species in 4 genera. Three genera, *Aureolejeunea* Schust., *Bryopteris* Nees, and *Lejeunea* Libert, were represented by two species each, and one species, *Bryopteris filicina* (Sw.) Nees, by two specimens (Table 1). The two specimens originated from the same locality and represented a more robust and more slender form of the species. The outgroup (*Frullania brasiliensis* Raddi, Jubulaceae) was selected following Weis (2001). All vouchers are deposited in the herbarium of the University of Göttingen (GOET).

DNA extraction, amplification, sequencing, and alignment

Total genomic DNA was extracted from herbarium specimens not older than two years, or from silica gel dried samples of field-collected material (Table 1), using the QIAGEN Plasmid Mini Kit, modified after Hellwig et al. (1999), or PUREGENE Genomic DNA Isolation Kit (Gentra Systems). Despite various difficulties throughout DNA preparation and PCR, likely due to high GC content and unknown cell components with putative inhibitor activity, several sequences were obtained. Highly denaturing PCR conditions through addition of dimethylsulphoxide (DMSO) increased the quality of the PCR products.

The upstream primers 1800f (Friedl 1996), alternatively Hep2 and Hep3 (Groth et al. 2002), and the downstream primer ITS4 (Friedl 1996) were used for amplification of the ITS1–5.8S–ITS2 region. The PCR amplification assays contained 1.5 µl template DNA (approx. 50 ng), 1 U Silverstar Taq Polymerase (Eurogentech), 5 µl 10 × PCR buffer for Silverstar Taq Pol, 1 µl dNTP mix (10 mM), 2 µl $MgCl_2$ (50 mM), 2 µl DMSO, 1 µl upstream primer (10 µM), 1 µl downstream primer (10 µM), and double-distilled water to 50 µl total volume. A typical amplification assay included an initial denaturation (2 min, 95°C), followed by 20 cycles with 40 s of denaturation at 94°C, 30 s plus 2 s increment of annealing at 51–55°C, 2 min plus 2 s increment of synthesis at 72°C, and a final step of denaturation (40 s at 94°C) followed by 2 min of synthesis at 72°C. After PCR, the DNA was purified with High Pure PCR product Purification Kit (Roche) or using sodium-acetate/isopropanol/ethanol purification. Sequencing primers were 1800f and ITS4, labeled with IRD 700 and IRD 800 for sequencing on a LiCor Long ReaDir 4200 using the Thermo Sequenase fluorescent-labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech). A sequencing assay included 10 ng template DNA per 1000 bp product. The 14 sequences created were manually aligned using Bioedit 5.0.9 (Hall 1999). ITS1, ITS2, the 5.8S sector, as well as the partial small subunit (SSU) and large subunit (LSU) sectors were identified by comparison with sequences from GenBank (AJ422240, *Lophocolea heterophylla*). Ambiguous regions were excluded from the phylogenetic analyses, resulting in an alignment including 418 putatively homologous sites (10 positions SSU, 70

Table 1. List of specimens analyzed for DNA, with taxon, voucher and source data, and GenBank accession numbers. *Bryopteris filicina* (2) is a slender form of *B. filicina* (1).

Species	Voucher	Source	GenBank Acc. No.
Lejeuneaceae			
Subfamily Lejeuneoideae			
Tribe Brachiolejeuneeae			
Brachiolejeunea laxifolia (Tayl.) Schiffn.	Gradstein 9900	Bolivia	AY466037
Dicranolejeunea axillaris (Nees & Mont.) Schiffn.	Heinrichs et al. AHH-0077	Costa Rica	AY466041
Odontolejeunea rhomalea (Spruce) Steph.	Gradstein 9936	Bolivia	AY466046
Tribe Lejeuneeae			
Aureolejeunea aurifera Schust.	Holz CR 99-0598	Costa Rica	AY466036
Aureolejeunea fulva Schust.	Holz CR 00-0823	Costa Rica	AY466035
Lejeunea laetevirens Nees & Mont.	Reiner-Dr. 4110	Tenerife	AY466043
Lejeunea flava (Sw.) Nees	Reiner-Dr. 4111	Tenerife	AY466042
Lepidolejeunea eluta (Nees) Schust.	Gradstein s.n.	Bolivia	AY466044
Omphalanthus filiformis (Sw.) Nees	Gradstein 9956	Bolivia	AY466034
Subfamily Ptychanthoideae			
Bryopteris diffusa (Sw.) Nees	Gradstein 9929	Bolivia	AY466040
Bryopteris filicina (Sw.) Nees (1)	Gradstein 9928	Bolivia	AY466038
B. filicina (2)	Gradstein 9926	Bolivia	AY466039
Marchesinia brachiata (Sw.) Schiffn.	Gradstein 9934	Bolivia	AY466045
Jubulaceae			
Frullania brasiliensis Raddi	Gradstein 9945	Bolivia	AY466047

positions ITS1, 153 positions 5.8S, 182 positions ITS2). The alignment is available from the second author upon request.

Phylogenetic analyses

Two different datasets were analysed. The first dataset consists of the above ITS alignment. The second dataset combines the ITS alignment with a morphological datamatrix of 50 gametophytic or sporophytic generic characters extracted from Gradstein et al. (2003). Maximum parsimony (MP) analyses were performed using PAUP* 4.0b10 (Swofford 2002) with the following options implemented: heuristic search mode with 1000 random-addition-sequence replicates, tree bisection-reconnection branch swapping (TBR), MULTrees option on, and collapse zero-length branches off. All characters were treated as equally weighted and unordered. Non-parametric bootstrap support (Felsenstein 1985) was estimated with full heuristic searcher, 1000 bootstrap replicates, and 10 random addition sequence replicates per bootstrap replicate, TBR swapping, MULtrees option on, collapse zero-branches off, and by saving all trees. In all analyses, *Frullania* was assigned as outgroup.

Results

Lengths of the ITS1 sequences of the ingroup species vary between 279 bp (*Lepidolejeunea eluta*) and 324 bp (*Bryopteris diffusa*), for ITS2 between 286–289 bp (*B. filicina*) and 332 bp (*Marchesinia brachiata*). ITS2 GenBank sequences of Lejeuneaceae of Ahonen et al. (2003) are longer (over 400 bp) than those of the present study due to the inclusion of part of the LSU. The ITS1 sequence of the outgroup taxon *F. brasiliensis* is slightly longer (333 bp) than those of the investigated ingroup species; ITS2 of this species could not be determined completely.

The 5.8S sequences of this study vary between 159 and 164 bp. They are nearly identical and phylogenetically uninformative with respect to the family Lejeuneaceae. In contrast, the ITS1 and ITS2 sequences are highly variable and phylogenetically informative. ITS1 proved to be more variable than ITS2, and a larger number of positions of this spacer had to be excluded from the analyses because of ambiguous positional homology. In total, 191 molecular and seven morphological characters are constant, 128 molecular and 36 morphological characters prove to be parsimony-informative, and 99 molecular and seven morphological characters are variable but parsimony-uninformative.

MP analyses of the ITS dataset and the combined ITS morphology dataset, respectively, result in a single most parsimonious tree that is fully resolved. Both topologies are largely uniform and lead to identical cladograms (Fig. 2).

Bootstrap support of the branches derived from the ITS and the combined dataset are similar.

The subfamily Lejeuneoideae emerges to be paraphyletic and the subfamily Ptychanthoideae is monophyletic, although with low bootstrap support. The tribes Brachiolejeuneeae and Lejeuneeae of the subfamily Lejeuneoideae are both recovered, the former sister to the remaining Lejeuneaceae and with 100% bootstrap support, the latter with moderate support. Two additional clades are found within Lejeuneeae with moderate to high bootstrap values: the *Aureolejeunea–Omphalanthus* clade with 93–97% BS, and the *Lejeunea–Lepidolejeunea* clade with 77% BS in the combined dataset. The genera *Aureolejeunea*, *Bryopteris*, and *Lejeunea*, each of them represented by two species in the analysis, are placed in well-supported monophyletic lineages.

Discussion

The nrITS region is widely used to infer the evolution of various groups of organisms including angiosperms (Baldwin et al. 1995; Soltis and Soltis 1998; Hershkowitz et al. 1999). Among bryophytes, ITS variation is often large, sometimes making it impossible to align ITS sequences even at the species level (Shaw et al. 2002). Thus far, the ITS region has mostly been used to clarify relationships within the mosses (e.g. Shaw 2000; Vanderpoorten et al. 2001). Within leafy liverworts the ITS region was successfully employed in the vast genus *Plagiochila* (Dumort.) Dumort. (e.g., Groth et al. 2002, 2003; Heinrichs et al. 2002a, b; Renker et al. 2002). Major parts of both ITS1 and ITS2 of *Plagiochila* species could be aligned unambiguously, leading to well-resolved topologies at section level but not at deeper nodes.

The present study is a first appraisal of phylogenetic relationships within the family Lejeuneaceae using sequences of the complete nrITS region and combining molecular and morphological data. As in the study of Ahonen et al. (2003), the number of species and genera of Lejeuneaceae available for molecular analysis was very limited. Nevertheless, our results allow several important phylogenetic conclusions. As in *Plagiochila*, it appears that deeper nodes in Lejeuneaceae are not strongly supported by the ITS dataset, but generic clades receive significant bootstrap support. Contrary to the situation in *Plagiochila*, ITS1 of Lejeuneaceae is clearly more variable than ITS2. However, our results indicate that most parts of the two spacers can be aligned unambiguously at the generic level or between closely related genera of this family. Although difficult to sequence (Ahonen et al. 2003; own observations), the ITS region appears to be a powerful tool to resolve species relationships and to clarify affinities between the genera or tribes of the subfamilies of Lejeuneaceae.

The phylogeny of Lejeuneoideae

The subfamily Lejeuneoideae was recovered as a paraphyletic group in this study, with the tribe Lejeuneeae being sister to the monophyletic Ptychanthoideae. However, this topology is not well supported in bootstrap analyses of the two datasets. A study using less variable markers is needed to clarify the status of the two subfamilies. Ahonen et al. (2003) could demonstrate the monophyly of Lejeuneoideae using more conservative chloroplast markers in addition to ITS2.

Gradstein (1994) recognized two broad, morphologically defined tribes of Lejeuneoideae, Brachiolejeuneeae and Lejeuneeae. The two groups differed in the structure of the sporophyte stalk, which is composed of 20 rows of cells in Brachiolejeuneeae, of 16 rows in Lejeuneeae. Recent morphological–cladistic studies resolved Lejeuneeae as a monophyletic group, whereas Brachiolejeuneeae are paraphyletic or monophyletic, depending on the position of the genus *Symbiezidium* and on the application of equal or unequal weighting (Weis 2001; Gradstein et al. 2003). In the present study, which did not include *Symbiezidium*, the two tribes were recovered as monophyletic lineages in the molecular and combined trees.

The *Aureolejeunea–Omphalanthus* clade within the tribe Lejeuneeae corresponds to Cheilolejeuneeae of Schuster (2001) (= *Omphalanthus* complex of Gradstein et al. 1981; tribe "Omphalantheen" of Weis 2001). Morphologically, this clade stands out among members of Lejeuneeae by distal hyaline papillae, very large oil bodies, frequent possession of undivided underleaves, and numerous rosettes on spore surfaces (Gradstein et al. 2003). Our data indicate that Cheilolejeuneeae, to which 5–6 genera are attributed, are an important lineage within Lejeuneeae. The *Lejeunea–Lepidolejeunea* clade does not correspond to a previously recognized taxonomic group. According to Schuster (2001) the two genera probably belong in different tribes, although the phylogenetic position of *Lepidolejeunea* remains unclear. Morphologically, the two genera are far apart by their very different leaf areolation and innovation type. The status of this lineage obviously requires further study using a wider range of taxa.

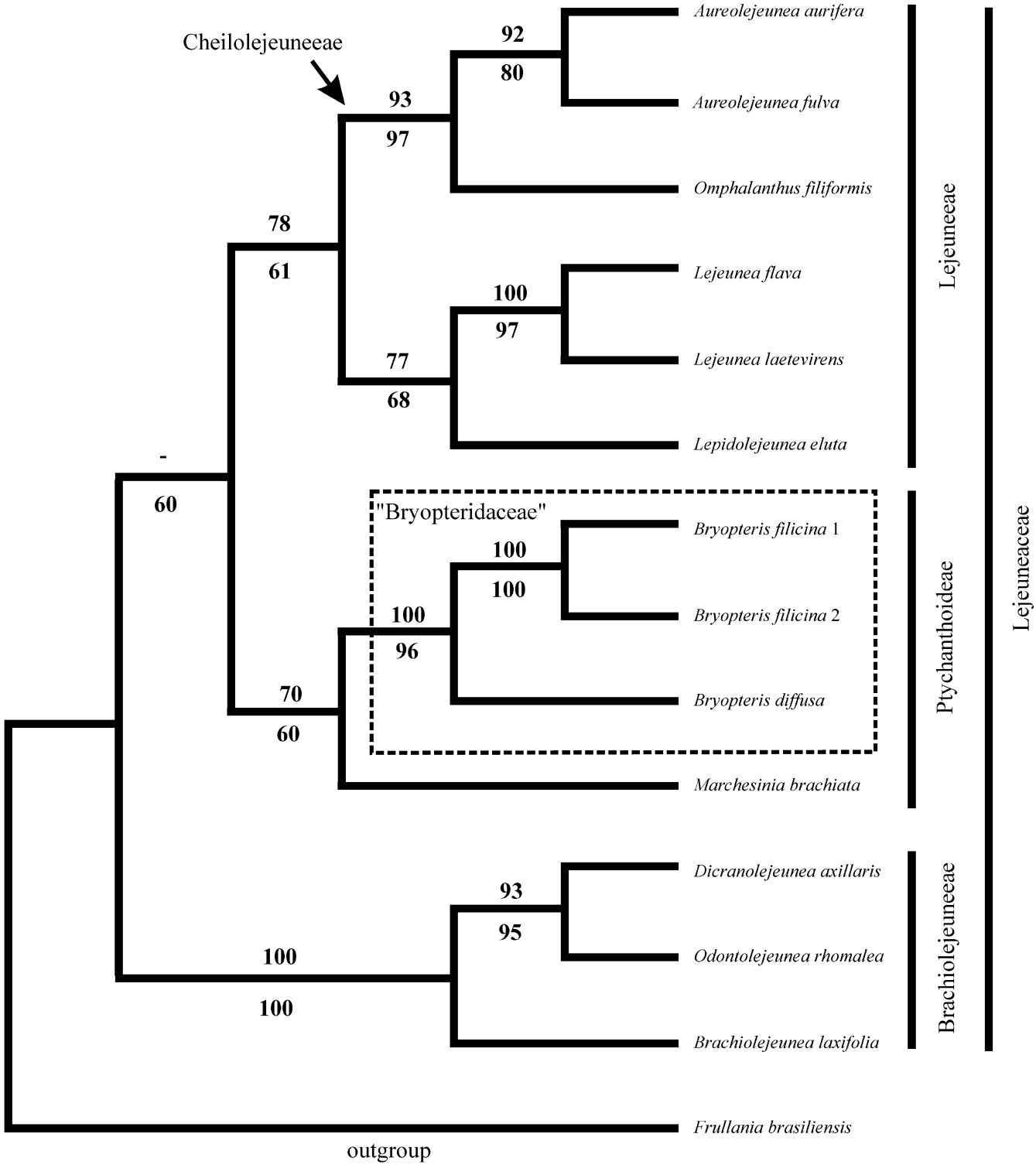

Fig. 2. Single most parsimonious tree found with sequences of the nrITS region (without ambiguously aligned areas) and with a combined morphological–molecular dataset. Numbers indicate bootstrap values (below branches: ITS dataset; above branches: combined ITS and morphological dataset).

The position of *Bryopteris*

The phylogenetic position of the genus *Bryopteris* has been discussed controversially for a long time. The genus contains three species and occurs disjunctly in tropical America and Madagascar, and doubtfully in Nepal (Gradstein et al. 2001). According to Stotler and Crandall-Stotler (1974) and Crandall-Stotler and Stotler

(2000), this genus should be removed from Lejeuneaceae and placed in a separate family Bryopteridaceae based on its different type of branching. Recent morphological–cladistic studies, however, recover *Bryopteris* as a member of Lejeuneaceae subfamily Ptychanthoideae (Gradstein 1994; Weis 2001; Gradstein et al. 2003). The results of our molecular and combined molecular–morphological analyses confirm the placement of *Bryopteris* in the Ptychanthoideae clade.

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