# Chapter 2

## What's in a Name? Disentangling the *Dicranum scoparium* species complex (Dicranaceae, Bryophyta)

A. S. Lang and M. Stech



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ABSTRACT

*Dicranum* is a large (ca. 90 species) and taxonomically complex moss genus. Circumscriptions and relationships of many *Dicranum* species remain ambiguous due to the absence of a worldwide revision and comprehensive phylogenetic analyses. In this study, we address species circumscriptions and relationships of presumed close allies within *Dicranum* sect. *Dicranum*. Molecular phylogenetic reconstructions based on five chloroplast regions and nrITS suggest a close relationship between *D. bonjeanii*, *D. howellii*, *D. nipponense*, *D. japonicum*, *D.* cf. *lorifolium*, and *D. scoparium*, which can be regarded as the *D. scoparium* species complex. In contrast, *D. majus* and *D. polysetum*, as well as *D. fuscescens* and *D. spadiceum* (former varieties of *D. scoparium*), are separated from the complex. Molecular data are generally congruent with the morphological species concept, but the circumscriptions of *D. bonjeanii*, *D. japonicum*, *D.* cf. *lorifolium*, and *D. scoparium* need further study. Most analysed *D. scoparium* specimens from across its Holarctic distribution are contained in one clade (*D. scoparium* s.s.), but a number of North American specimens are resolved as closely related to *D. japonicum* and *D.* cf. *lorifolium*. Costa cross sections and characters of the leaf apex (shape, serrulation of margins) are most relevant for identifying the studied *Dicranum* species morphologically.

INTRODUCTION

In bryophytes, polyphyly of (morpho)species may be the rule rather than the exception, due to a limited number of available morphological characters, the focus on a few key characters with recurrent homoplastic transitions of character states, and the influence of the environment on character variability (Vanderpoorten & Goffinet 2006). Insufficient knowledge of the spatial distribution of morphological variation has furthermore led to the description of high numbers of species and intraspecific taxa, especially in morphologically highly variable genera. In fact, incongruence between morphological species circumscriptions and molecular phylogenetic

reconstructions is increasingly being reported (for review see Heinrichs *et al*. 2009; Vanderpoorten and Shaw 2010). On the other hand, molecular inferences in bryophytes are often based on a single or few molecular markers (Stech & Quandt 2010), and genetic processes such as rapid diversification and incomplete lineage sorting, in particularly in recently diverged species (cf. Rittmeyer and Austin 2012) as well as cryptic speciation (e.g. Bickford *et al*. 2007), have not yet been well studied. Consequently, further comparative analyses of molecular versus morphological characters are necessary to better understand species circumscriptions in bryophytes.

*Dicranum* is a genus of haplolepideous mosses (Dicranidae) with a predominantly Northern Hemisphere, cool-temperate distribution (Frey & Stech 2009). More than 880 binomials were originally described in *Dicranum* (van der Wijk *et al*. 1962; Tropicos.org). To cope with this diversity, several regional taxonomic revisions and systematic treatments have been carried out (e.g. Sakurai 1951; Nyholm 1954, 1987; Takaki 1964, 1972; Peterson 1979; Crum & Anderson 1981; Noguchi 1987; Bellolio-Trucco & Ireland 1990; Otnyukova 2001; Hedenäs & Bisang 2004; Ireland 2007). However, with more than 90 currently accepted species, *Dicranum* (including *Orthodicranum*) is still one of the largest and taxonomically most complex genera of Dicranaceae and the Dicranidae in general (Frey & Stech 2009; Tropicos.org). Morphological species circumscriptions and relationships remain difficult to assess in *Dicranum* as long as neither a worldwide revision nor comprehensive molecular phylogenetic analyses are available.

*Dicranum* species are characterized by falcate-secund, narrowly lanceolate to ovate-lanceolate, usually unistratose leaves; entire to serrate leaf margins; a subpercurrent to shortly excurrent narrow costa that is smooth or with serrate ridges at back; subquadrate to elongate, thick-walled, often porose laminal cells; well-developed alar cells and a haplolepideous, *Dicranum*-type peristome with a single row of teeth around the capsule mouth (e.g. Hedenäs & Bisang 2004; Ireland 2007; Frey & Stech 2009). The main difficulty is to find stable morphological characters at the species level. Gametophytic characters, such as serrulation of leaf margins, number of costal ridges, shape of upper laminal cells, and leaf length and shape, vary considerably depending on environmental conditions (e.g. Hagen 1915; Briggs 1965; Bellolio-Trucco & Ireland 1990; Ireland 2007). Perichaetial leaves and sporophytic characters, which have been considered more significant for species identification (e.g. Hagen 1915; Peterson 1979), are often not available.

The problem of morphological species delimitation is well exemplified in a number of species of section *Dicranum* (Hedw.) Sull. (sensu Nyholm 1987; Bellolio-Trucco & Ireland 1990), whose circumscriptions are unclear due to morphological variability and intergrading forms. These species (including the Holarctic *D. scoparium* Hedw., *D. bonjeanii* De Not. and *D. majus* Turner as well as more narrowly distributed species such as *D. lorifolium* Mitt., *D. japonicum* Mitt. and *D. nipponense* Besch. in Asia, and *D. howellii* Renauld & Cardot in North America), may form a complex of closely related species, or represent intraspecific taxa within one broadly circumscribed species, *D. scoparium* s.l. (e.g. Nyholm 1954, 1987; Lawton 1971; Peterson 1979; Crum & Anderson 1981; Noguchi 1987; Gao & He 1999; Hedenäs & Bisang 2004; Ireland 2007).

In this paper, we address species circumscriptions and relationships of *D. scoparium* and six presumed close allies (*D. bonjeanii*, *D. howellii*, *D. japonicum, D.* cf. *lorifolium*, *D. majus*, and *D. nipponense*), which together may form a species complex. Inferences are based on molecular phylogenetic reconstructions using chloroplast (*rpoB*, *trnH-psbA*, *trnL-trnF*, *rps4-trnT*, *rps19-rpl2*) and nuclear ribosomal ITS sequences. Implications of the molecular data for the suitability of gametophytic characters (e.g. number of costal ridges, serrulation of leaf margins) for species

identification are discussed.

## Materials and Methods

***Sampling—*** A total of 111 *Dicranum* specimens were sampled. The sampling included 17 specimens of which all or part of the sequences had been generated for earlier studies (Stech 1999; Stech *et al*. 2006; Stech & Frey 2008; Lang & Naciri 2010) and 94 newly analysed specimens. As initial molecular analyses showed that several specimens were probably misidentified, morphological re-identifications were carried out by the authors using identification keys for *Dicranum* in Japan (Noguchi & Iwatsuki 1987), China (Gao & He 1999), Europe (Hedenäs & Bisang 2004), and North America (Ireland 2007). These resulted in the following specimen counts and species names used in the final analyses (cf. Figs. 1, 2; Appendix 1): 63 specimens of *D. scoparium*, 40 of other species of section *Dicranum* (five *D. bonjeanii,* five *D. howellii*, six *D. japonicum*, 14 *D.* cf. *lorifolium*, seven *D. majus*, two *D. nipponense*, and one *D. polysetum*), and eight of species of other sections of *Dicranum* (one *D. fragilifolium*, two *D. fuscescens*, one *D. montanum,* and four *D. spadiceum*). Of the latter species, *D. fuscescens* (sect. *Fuscescentiformia*) and *D. spadiceum* (sect. *Muehlenbeckia*) (Chopra 1975; Nyholm 1987; Bellolio-Trucco & Ireland 1990) were former varieties of *D. scoparium*. *Dicranum fragilifolium* (sect. *Elongata*) and *D. montanum* (sect. *Montana*) were included for comparison as representatives of species that have never been associated with sect. *Dicranum*. The sampling of *D. scoparium* covered both the intraspecific morphological variation and distant parts of the species' Holarctic distribution range, i.e. North America (U.S.A., Canada), different parts of Europe (from Iceland to the Caucasus) and Macaronesia, and East Asia (Taiwan, South Korea). However, the sampling was biased towards Continental Europe due to limited availability of collections from other regions and misidentified collections. Besides, *D. scoparium* was recently included in the bryoflora of Australia (Klazenga 2012), but no material from Australia was available. Four samples of *Holomitrium*, one *H. crispulum* Mart. and three *H. arboreum* Mitt., were chosen as outgroup representatives based on the sister-group relationship of *Holomitrium* and *Dicranum* in earlier phylogenetic reconstructions (La Farge *et al*. 2002; Stech *et al*. 2006).

***Molecular Marker Selection—***Five chloroplast DNA regions described in Lang & Naciri (2010), i.e., partial *rpo*B gene, *trn*$H_{GUG}$-*psbA* and *rps*19-*rpl*2 intergenic spacers, and two parts of the *trn*S-F region, namely *rps*4-*trn*$T_{UGU}$ spacer and *trn*L-F (*trn*$L_{UAA}$ intron and *trn*$L_{UAA}$-*trn*$F_{GAA}$ spacer), as well as one nuclear region (nrITS1-5.8S-ITS2) were amplified and sequenced. Except for *rpo*B and *rps*19-*rpl*2, these regions are among the most frequently used phylogenetic markers in bryophytes (Stech & Quandt 2010). The regions rps19-rpl2 and rpoB presented, together with rps4-trnT, the highest sequence variation at the intraspecific level in *Dicranum scoparium* (Lang & Naciri 2010), and were included to overcome the problem of low sequence divergence in *Dicranum* as indicated in earlier phylogenetic studies (Stech 1999; La Farge *et al*. 2002; Stech *et al*. 2006). The rps19-rpl2 region has also been found in the mitochondrial and nuclear genomes of some species of green algae, bryophytes, and angiosperms (e.g. Turmel *et al*. 2002; Raubeson *et al*. 2007; Terasawa *et al*. 2007; Wang *et al*. 2008). Although the primers developed by Lang & Naciri (2010) were based on the chloroplast genome of the moss species *Physcomitrella patens* (Hedw.) Bruch & Schimp. (Sugiura *et al*. 2003), we performed a BLAST search (Altschul *et al*. 1990) and compared our sequences with chloroplast and mitochondrial *rps*19-*rpl*2 sequences of several

other land plants in GenBank, to assure that only orthologous copies from the chloroplast genome were used.

***DNA Extraction, Amplification and Sequencing*—**Several leaves of a single stem apex taken from fresh or herbarium collections were carefully cleaned in demineralised water. DNA was extracted from the dried leaves using the DNeasy plant mini kit (Qiagen, Hilden, Germany) and eluted in 100 µl AE buffer. The PCR reactions were carried out in a final volume of 20 µl. The reaction mixture contained 1× buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM of both forward and reverse primers, 0.0375 U (5 U/µl) Biotaq polymerase (Gentaur, Brussels, Belgium) and 1 µl DNA. The amplification reactions for the chloroplast markers were performed following Lang & Naciri (2010). Amplification of ITS followed Stech (2004) except for an annealing temperature of 45 °C. The PCR products were purified and sequenced at Macrogen Inc. (www.macrogen.com). GenBank accession numbers of all sequences are listed in Appendix 1.

***Alignment and Phylogenetic Reconstruction*—**Sequences were aligned in Geneious v5.3.6 (Biomatters 2010) using 65% similarity matrix costs, and manually adjusted. Short hairpin-associated inversions in the *trnH-psbA* and *trnL-F* spacers, which can flip at the population level and may significantly reduce phylogenetic structure if undetected (Quandt and Stech 2004; Borsch and Quandt 2009; Whitlock *et al*. 2010), were positionally separated in the alignment and not coded as indels. The dataset used for phylogenetic analyses has been submitted to TreeBASE (study number 13703).

Phylogenetic inferences were based on maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI) analyses. Best-fit models of nucleotide sequence evolution were selected according to the Akaike information criterion in MrModeltest (Posada and Crandall 1998) executed through PAUP* 4.0b10 (Swofford 2002), namely HKY + Γ for the non-coding chloroplast markers (*rps4-trnT*, *trnL-trnF*, *trnH-psbA*, *rps19-rpl2*), and HKY + I for *rpoB* and nrITS. Gaps were coded as informative by a simple indel coding strategy (SIC) (Simmons and Ochoterena 2000) implemented in SeqState (Müller 2004). Sequence and indel data were treated as separate and unlinked partitions, employing the restriction site model ('F81') for the indel matrix.

To check for incongruence, phylogenetic reconstructions based on chloroplast and nuclear sequences (93 and 90 ingroup samples, respectively) were visually compared. In addition, an incongruence length difference test (ILD, Farris *et al*. 1994) as implemented in PAUP* was performed with 100 replicates.

Heuristic searches under parsimony were performed in PAUP* using simple sequence addition and tree bisection-reconnection (TBR) branch swapping. Bootstrap searches under parsimony were performed with 10,000 replicates using the fast bootstrap option. Maximum likelihood analyses were carried out with RAxML v.7.2.6 (Stamatakis 2006) employing the graphical user interface raxmlGUI v.0.93 (Silvestro and Michalak 2012). Bootstrap analyses under ML were done using the thorough bootstrap heuristics algorithm with 20 runs and 200 replicates. Bayesian analyses were run on the Bioportal server (www.bioportal.uio.no). Bayesian posterior probabilities were calculated based on the Markov chain Monte Carlo (MCMC) method, using MrBayes v3.0b4 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003). The a priori probabilities supplied were those specified in the default settings of the program. Two runs with four chains were run simultaneously ($30 \times 10^6$ generations for chloroplast and combined data and 11 ×

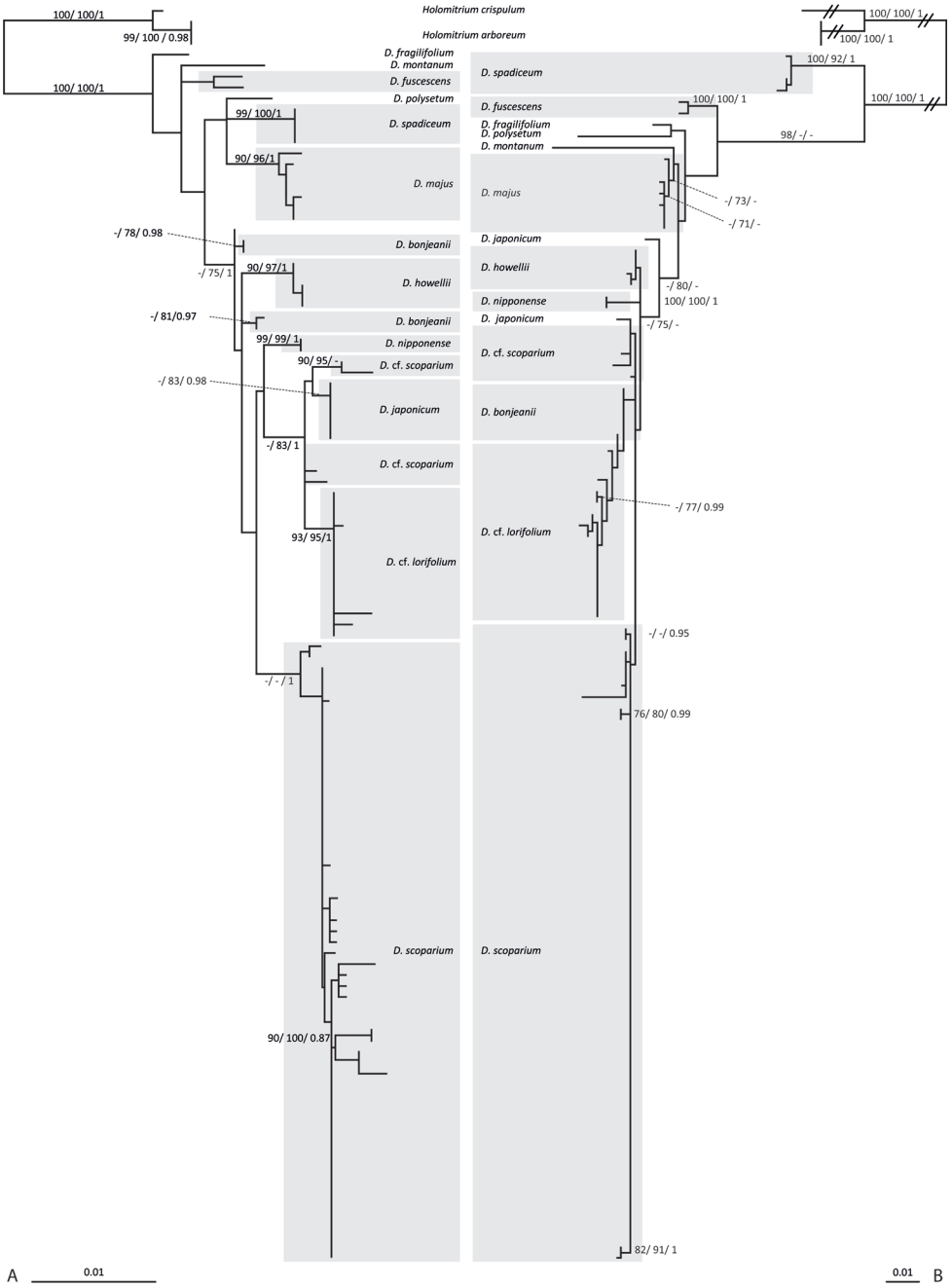


FIG. 1. Single optimal maximum likelihood phylogenetic reconstructions inferred from chloroplast (A) and nrITS (B) sequence data of *Dicranum* species and four specimens of *Holomitrium* as outgroup representatives, including indels coded by simple indel coding (SIC). Support values (maximum parsimony bootstrap, maximum likelihood bootstrap, Bayesian posterior probabilities) are indicated at the branches. Branch lengths are to scale except those indicated by the symbol "//" (shortened 8.5 times).

$10^6$ generations for ITS), with the temperature of the single heated chain set to 0.5. Chains were sampled every 1,000 generations and the respective trees written to a tree file. Fifty percent majority rule consensus trees and posterior probabilities of clades were calculated by combining the four runs and using the trees sampled after the chains converged. Trace plots generated in Tracer v1.5 (Rambaut and Drummond 2007) were used to check for convergence of the runs (plateaus of all runs at comparable likelihoods) and to infer the 'burnin', which was set to 25%.

RESULTS

Sequence lengths of nrITS ranged from 753–845 nucleotides (nt) in the ingroup (754–990 nt including *Holomitrium*). Corresponding length ranges of the chloroplast markers were 509–521 nt (509–521) for *rps4-trnT*, 449–462 (449–462) for *trnL-trnF*, and 139–140 (139–140) for *trnH-psbA*. No length variation was observed in *rps19-rpl2* (310 nt) or in the sequenced part of *rpoB* (457 nt). The combined chloroplast and ITS alignment comprised 2993 positions including *Holomitrium* (*rps4-trnT* positions 1–521, *trnL-trnF* 522–986, *trnH-psbA* 987–1135, *rps19-rpl2* 1136–1445, *rpoB* 1446–1902, ITS 1903–2993). Of the 2993 positions, 149 ambiguous positions in ITS were removed from the further calculations. Of the remaining 2843 included positions, 309 were variable, and 204 of the variable positions were parsimony-informative (*rps4-trnT* 40/27, *trnL-trnF* 26/23, *trnH-psbA* 17/10, *rps19-rpl2* 21/11, *rpoB* 20/13, ITS 185/120 variable/parsimony-informative positions). Coding gaps by simple indel coding (SIC) yielded a total of 145 indel characters (including *Holomitrium*), of which four corresponding to an inversion in *trnH-psbA* were excluded from phylogenetic analysis. Of the remaining 141 indels characters, 93 (*rps4-trnT* 1, *trnL-trnF* 3, *trnH-psbA* 1, ITS 88) were parsimony-informative.

Maximum parsimony analyses with or without indels included resulted in differently resolved most parsimonious phylogenetic reconstructions, but did not show incongruence with respect to significantly supported clades. Consistency indices of the reconstructions with or without indels included were similar (chloroplast: CI 0.6816 versus 0.6755, ITS: CI 0.9342 versus 0.8731), indicating only a slight increase in homoplasy due to the inclusion of the indel characters in either case.

The single optimal ML trees calculated from the combined chloroplast markers versus ITS including indels (lnL = -3,808.37 and lnL= -3,216.40, respectively), are shown in Fig. 1, with bootstrap support values (BS) from maximum parsimony and maximum likelihood analyses as well as Bayesian posterior probabilities (PP) indicated at the branches. Since both visual inspections of chloroplast versus ITS tree topologies and the ILD test ($p = 0.23$) indicated that the two datasets were congruent, combined analyses of all markers were performed as well, of which the optimal ML tree is shown in Fig. 2 (lnL = -7,367.38). Clade support in the respective analyses without indels was similar to the analyses with indels and therefore not indicated on the trees.

All trees displayed short branches within the ingroup (Figs. 1, 2). A clade comprising six species of sect. *Dicranum* (*D. bonjeanii*, *D. howellii*, *D. japonicum*, *D.* cf. *lorifolium*, *D. nipponense*, and *D. scoparium*) was recovered in both separate analyses (Figs. 1A, B) and with maximal support (100% BS, PP 1) in the combined tree (Fig. 2). *Dicranum majus* and *D. polysetum* were resolved outside this clade. Relationships between them and the other included *Dicranum* species remained largely unsupported. All species with more than one accession sequenced were resolved as monophyletic, except *D. scoparium* in all trees (Figs. 1, 2), *D. bonjeanii* in both separate trees (Figs.

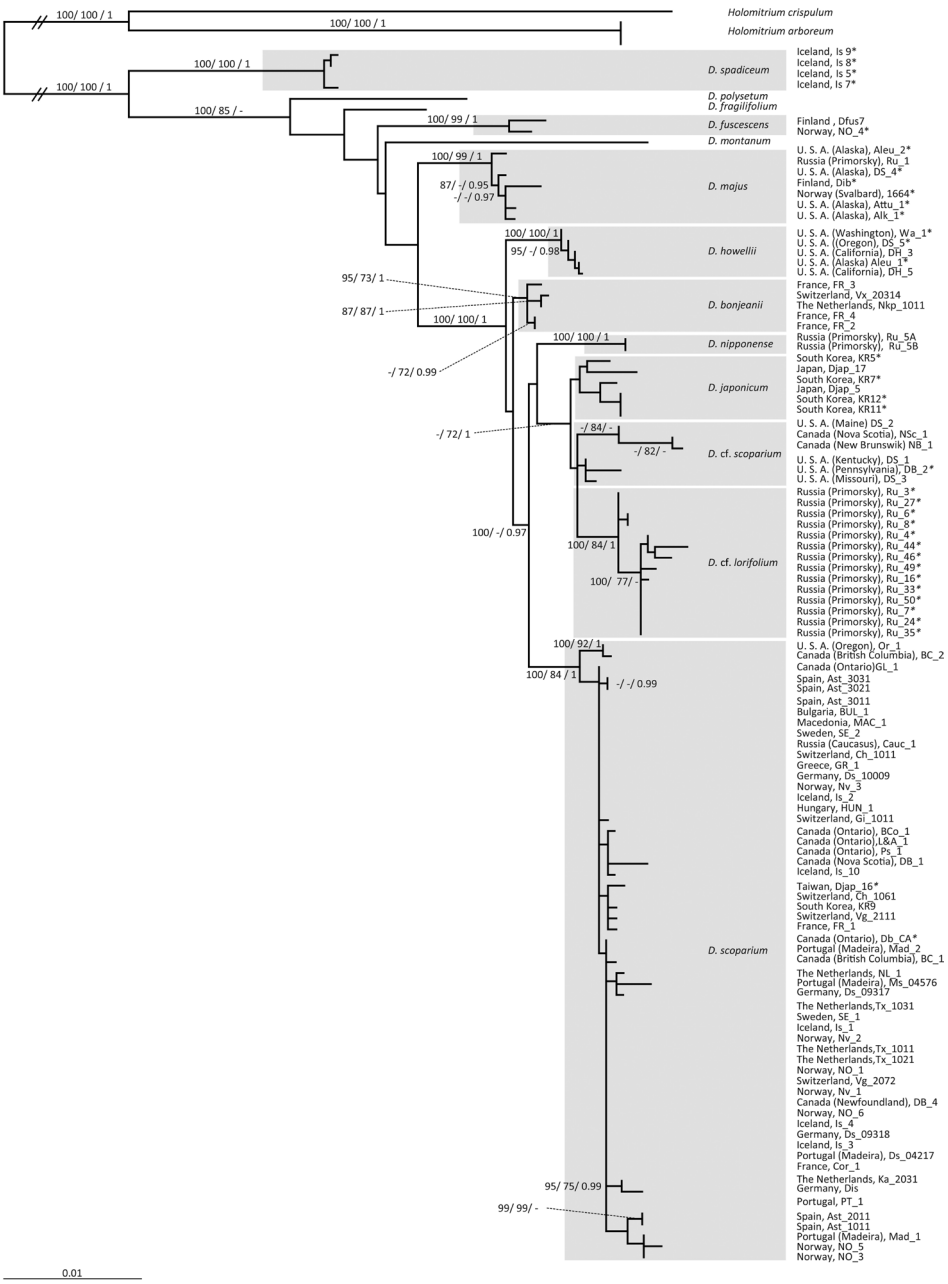


FIG. 2. Single optimal maximum likelihood phylogenetic reconstruction inferred from combined chloroplast and nrITS sequence data of *Dicranum* species and four specimens of *Holomitrium* as outgroup representatives, including indels coded by simple indel coding (SIC). Support values (maximum parsimony bootstrap, maximum likelihood bootstrap, Bayesian posterior probabilities) are indicated at the branches. Samples with an asterisk were re-identified morphologically (original identifications are indicated in Appendix 1). Branch lengths are to scale except those indicated by the symbol "//" (shortend 5.8 times).

1A, B), and *D. japonicum* in the ITS tree (Fig. 1B). The clades of *D. nipponense* and *D. spadiceum* received ≥ 90% BS and PP ≥ 0.99 in all analyses. The clades of *D. howellii*, *D.* cf. *lorifolium*, and *D. majus* were significantly supported in the chloroplast and combined trees (≥90% BS except 84% for *D.* cf. *lorifolium* in Fig. 2, PP 1) but received lower (*D. majus*: -/73% BS) or no support (*D. howellii, D.* cf. *lorifolium*), respectively, in the ITS tree. The clade of *D. fuscescens* received significant support in the ITS and combined trees but no support in the chloroplast tree. The specimens of *Dicranum bonjeanii* and *D. japonicum* were split into two clades in the chloroplast and ITS trees, respectively, and resolved as monophyletic with moderate ( *D. bonjeanii*: 95/73% BS, PP 1) or no support (*D. japonicum*), respectively, in the combined analysis. The majority of the sequenced *D. scoparium* specimens, including all European ones as well as ten from North America and two from East Asia, clustered in one clade in all analyses, with 100/84% BS and PP 1 in the combined analysis. Further putative *D. scoparium* specimens from North America and East Asia were part of a clade together with *D. japonicum* and *D.* cf. *lorifolium* (72/83% BS, PP 1 in the chloroplast and combined trees), but relationships within this clade remained unresolved.



## Discussion

***Tackling Species Circumscriptions and Relationships in Dicranum*—**Earlier molecular phylogenetic analyses indicated low sequence divergence within a clade comprising species of *Dicranum* (including *Orthodicranum*) and the closely related genera *Chorisodontium* and *Paraleucobryum*, contrary to higher sequence divergences in other species-rich genera of Dicranaceae such as *Dicranoloma* and *Leucoloma* (Stech 1999; La Farge *et al.* 2002; Stech *et al.* 2006). However, the respective analyses were based on limited taxon sampling and use of only one or two markers, rendering inferences about the suitability of DNA sequence data to resolve species circumscriptions and relationships in *Dicranum* difficult. Recently Tubanova and Ignatova (2011) analysed Russian *Dicranum* species based on nrITS sequences from a larger taxon sampling. Their study revealed some well-supported clades consisting of a single species or a few closely related species each, but the overall resolution and support of the phylogenetic reconstruction remained low. In cases of little molecular variation and phylogenetic structure, the best strategy would probably be to combine the information of several suboptimal markers to collect a small number of synapomorphic sites from each of them, until well-resolved phylogenetic trees can be produced (e.g. Edwards *et al.* 2007; Leaché and Rannala 2010; Stech & Quandt 2010; Dong *et al.* 2012), provided that issues such as evolutionary model selection per marker, possible incongruence between markers, or incomplete lineage sorting are taken into account (e.g. Holland *et al.* 2004; Kubatko and Degnan 2007; Liu and Pearl 2007). In the present study, trees generated separately from five chloroplast markers and ITS (Fig. 1) displayed an overall similar topology despite less resolution in ITS. Combined analyses of all six markers generally increased statistical support for clades at the species level in *Dicranum* (Fig. 2). Furthermore, all species with more than one accession sequenced (except possibly for *D. scoparium*, see discussion below) were resolved as monophyletic in the combined analysis, indicating that molecular lineages were generally congruent with the morphological species concept in *Dicranum*. However, the respective clades representing species were partly unsupported and their relationships remained largely unresolved. Probably an even higher number of molecular markers, including variable mitochondrial and single-copy nuclear markers, needs to be combined to infer supraspecific relationships in *Dicranum*.

***Molecular Characterisation of the D. scoparium Species Complex*—**The present molecular phylogenetic reconstructions (Figs. 1, 2) suggest a close relationship between *D. bonjeanii*, *D. howellii*, *D. nipponense*, *D. japonicum*, *D.* cf. *lorifolium*, and *D. scoparium*, which can be regarded as the *D. scoparium* species complex. These species were all considered to belong to section (or subgenus) *Dicranum* (e.g. Sakurai (1951) [as subgenus *Scopario-Dicranum*]; Takaki 1964; Nyholm 1987, Bellolio-Trucco & Ireland 1990), except *D. nipponense*, which was placed into a new subgenus *Nippono-Dicranum* by Sakurai (1951) based on peristome characters. As these characters turned out to be insignificant, however, and no other characters supported this segregation, *D. nipponense* was placed close to *D. scoparium* and allies again (Takaki 1964), which is supported by the molecular data. In contrast, other species of section *Dicranum* sensu Nyholm (1987), namely *D. majus* and *D. polysetum*, are separated from the *D. scoparium* complex based on the molecular inferences (Figs. 1, 2). The same holds for the species that were formerly treated as varieties of *D. scoparium* but placed in other sections than sect. *Dicranum* in the more recent literature, i.e., *D. fuscescens* (sect. *Fuscescentiformia*) and *D. spadiceum* (sect. *Muehlenbeckia*) (e.g. Nyholm 1987; Bellolio-Trucco & Ireland 1990). Whether additional *Dicranum* species that are putatively closely related to *D. scoparium* (e.g. *D. crassifolium* Sérgio, Ochyra and Seneca and *D. leioneuron* Kindb.) fall into the *D. scoparium* complex as defined here remains to be tested. Besides, at present no morphological synapomorphies are known that delimit the *D. scoparium* complex from *D. majus* and other *Dicranum* species.

***Morpho-molecular Species Circumscriptions*—**Distinction of the species here comprised as the *D. scoparium* complex and *D. majus* has been considered difficult for a long time due to their phenotypic plasticity. The gametophyte of *D. majus* is typically characterized by large plants with longer and more strongly falcate-secund leaves than in *D. scoparium*, leaf margins serrate in distal half, strongly porose lamina cells, and in particular a costa with a double row of guide cells (single row in *D. scoparium*) and an irregularly furrowed abaxial surface instead of with four continuous longitudinal ridges. However, large plants of *D. scoparium* may resemble *D. majus*, whereas small plants of *D. majus*, especially plants from arctic-alpine regions, which often show only one layer of costa guide cells, are easily confused with *D. scoparium* (e.g. Nyholm 1954; Takaki 1964; Hedenäs & Bisang 2004; Hedenäs *et al*. 2006). The morphological variation of *D. majus* led to the description of multiple varieties, of which two relate to the arctic morphotype: var. *orthophyllum* A. Braun ex Milde was commonly used in North America (Grout 1937; Steere 1978), while var. *condensatum* I. Hagen was recognized in the European and Russian Arctic (Hagen 1915; Brotherus 1923; Abramova *et al*. 1961). Wahlenberg (1814), in contrast, did not consider *D. majus* a separate species with intraspecific variation, but rather a form of *D. scoparium*. The present molecular data support the species status of *D. majus* and its separation from *D. scoparium*. Morphologically, the presence of furrows on the costa and the double row of guide cells, or at least few double guide cells that are always present at the leaf base, can be regarded as diagnostic characters for *D. majus* (Hedenäs *et al*. 2006; Table 1). Whether the distinction of two geographical morphotypes in Europe (Hedenäs *et al*. 2006) holds true at the molecular level remains to be tested.

*Dicranum bonjeanii* can usually be recognized by its transversely undulate upper leaf portions, erect-patent leaves when moist, a narrow costa with two weak dorsal ridges that are serrate to nearly entire, and a rather broad leaf acumen. However, leaf shape and undulation have also been reported to be the most variable characters (Briggs 1965), and the taxonomic

TABLE 1. Recapitulation of gametophytic characters, ecological and geographical ranges for the species included in the *Dicranum scoparium* complex and *D. majus* (Gao & He 1999; Hedenäs & Bisang 2004; Ireland 2007; Klazenga 2012; present study).

| | Gametophytic characters from the literature | Suggested diagnostic characters | Ecological and geographical range |
|---|---|---|---|
| *D. bonjeanii* | Leaves erect-patent, upper portions transversely undulate, margins denticulate, acumen blunt or obtuse, costa narrow, 2 low dorsal ridges, weakly serrate, leaf lamina cells prosenchymatous, porose. | Leaf apex blunt or obtuse, costa narrow, with (usually 2) weakly developed ridges. | Moist soil or temporarily wet depressions in rich fens or mires. Holarctic. |
| *D. howellii* | Leaves falcate-secund to straight and erect, margins strongly serrate in the distal half, acumen acute, keeled or tubulose, costa percurrent to shortly excurrent, with 2 (- 4) toothed ridges, lamina cells prosenchymatous, porose. | Leaf apex acute, costa narrow, with two toothed ridges. | Soil, humus, humus over rock, rotten logs, tree trunks and tree bases. Western North America. |
| *D. japonicum* | Leaves erect-patent, sometimes undulate, margins coarsely serrate, costa slender, percurrent to shortly excurrent, ending in a short hairpoint, apex long subulate or keeled, costa with 2 serrate ridges, lamina cells oblong-rhomboidal in distal part, porose. | Leaf about 10 times as long as wide, leaf margins coarsely serrate, apex subulate or keeled. | Humic soil, rock, or rotten logs. China, Korea, Japan, Russian Far East. |
| *D. lorifolium* | Leaves homomallous, falcate-secund to erect-patent, narrowly lanceolate, margins sharply serrate near the apex, apex long, canaliculate, costa thin, percurrent, serrate distally, lamina cells rhomboidal to short-rectangular in distal part, porose. | Leaf about 10 times as long as wide, leaf margins sharply serrate distally, apex subulate. | Rotten wood or tree bases. China, Bhutan, Nepal, India. |
| *D. majus* | Leaves long, strongly falcate-secund, margins serrate in distal half, costa with 2 rows of guide cells, irregularly furrowed on abaxial surface, lamina cells prosenchymatous, porose. | Costa furrowed with 2 rows of guide cells at the base (or at least few double guide cells). | Humid to wet soil and rock, in forests and more open habitats. Holarctic. |
| *D. nipponense* | Stems unevenly foliate, leaves erect-spreading or falcate-secund, margins coarsely serrate, apex shortly acute to acuminate, costa subpercurrent, slender, 2 ridges, keeled, laminal cells elongate- rhomboidal distally, porose. | Stem apex crowded, leaves shorter at the base of the stem, falcate-lanceolate, margins dentate, apex keeled, obtuse. | Soil, rock, or rotten wood. China, Korea, Japan. |
| *D. scoparium* | Leaves straight to falcate-second, margins serrate, apex keeled, costa percurrent, 4 serrate ridges with several stereid bands proximally, lamina cells prosenchymatous, porose. | Leaf apex keeled, costa with 4 serrate ridges in upper part leaf. | Soil, humus, humus over rock, rotten wood, tree bases, dry and humid environments. Holarctic, Australia. |

rank of *D. bonjeanii* has often been questioned due to the presence of intergrading forms with *D. scoparium*. While in Europe and Asia *D. bonjeanii* is generally accepted as a separate species, North American bryologists preferred to consider it as one of the multiple forms of *D. scoparium,* probably induced by the environment (Grout 1937; Jennings 1951; Lawton 1971; Peterson 1979; Crum & Anderson 1981; but see Ireland 2007). *Dicranum bonjeanii* is mostly found in eutrophic fens, whereas *D. scoparium* s.str. is found on different substrates mainly in dry to mesic woodlands. Although the *D. bonjeanii* clade is only resolved in the combined chloroplast and ITS tree (Fig. 2), the present molecular data indicate that *D. bonjeanii* can, at least in Europe, be separated from *D. scoparium* s.str., which supports the ecological differentiation and morphological differences of *D. bonjeanii*, in particular the narrow costa ending into a broad apex as well as the (usually two) weakly developed costal laminae (Table 1). Whether this taxon occurs in North America as well remains to be tested, which seems to be complicated since most of the specimens collected under this name were wrongly identified (Ireland 2007).

Other species within the *D. scoparium* complex cause identification problems in more restricted geographic areas. The western North American endemic *D. howellii* is characterized, when fertile, by the gradually acuminate inner perichaetial leaves (abruptly long-acuminate, convolute-sheathing in *D. scoparium*), whereas due to the plasticity of *D. scoparium*, the gametophytic characters of both species largely overlap. Besides, both *D. howellii* and *D. scoparium* occur on similar substrates (Table 1). Consequently, some authors considered *D. howellii* as a synonym (Grout 1937) or as a variety of *D. scoparium* (Peterson 1979). At the molecular level, however, *D. howellii* can clearly be distinguished from *D. scoparium* (Figs. 1A, 2). Gametophytically, the most reliable character to recognize *D. howellii* seems to be its narrow costa with two low ridges.

The East Asian *D. nipponense* resembles both *D. japonicum* in its larger forms (Noguchi 1987) and *D. bonjeanii* in its weaker forms (Otnyukova 2001). It is, however, distinguished from closely related *Dicranum* species by a combination of characters including unevenly foliate stems (lower leaves smaller than upper ones) with leaves crowded at the stem apex, falcate-lanceolate leaves that are broadest just above the base and keeled above with a broad and dentate point, and linear-rectangular and strongly porose laminal cells (Otnyukova 2001). Further differences with *D. bonjeanii* are the strongly dentate margins and the costa having usually two to three serrate ridges at the back (Table 1). Besides, *D. nipponense* grows mainly on rotten wood. Together with the degree of molecular divergence (cf. the significant support and comparatively long branches in Figs. 1 and 2), these characters indicate that *D. nipponense* is well characterized.

The circumscription of *D. scoparium* itself and its delimitation from *D. japonicum* and *D.* cf. *lorifolium* seems to be most difficult according to the present results. As the large clade of mainly European specimens (Figs. 1, 2) corresponds to the typical morphology of *D. scoparium* (cf. Hedenäs & Bisang 2004), we consider it to represent *D. scoparium* s. s. The fact that some specimens from North America and East Asia are included in this clade coincides with the wide distribution of *D. scoparium*. A larger sampling from outside Europe is, however, necessary for more solid conclusions on the total distribution and frequency of *D. scoparium* s. s. in the different continental areas.

The other specimens from North America and East Asia originally identified as *D. scoparium* (cf. Appendix 1; Fig. 2) differ from the plants in the *D. scoparium* s. s. clade in having generally narrower, linear leaves ending in an acuminate to setaceous apex. The distal abaxial side of the costa has at least four strongly serrulate lamellae, the leaf margins are strongly serrulate, and the lamina cells are finely porose. A BLAST search of the respective sequences did not correspond to

any North American or Asian accession available on GenBank, including *D. bardunovii* Tubanova & Ignatova, which was recently described from Buryatia, south-central Siberia, based on morphological characters and nrITS sequences (Tubanova and Ignatova 2011). The sequenced specimens from Japan, South Korea, and Russia morphologically resemble two closely related East Asian species, *D. japonicum* and *D. lorifolium*; however, morphological differences between these species and *D. scoparium* are rather subtle. The latter differs from the two Asian species mainly by a broader leaf base and a percurrent instead of excurrent costa (Gao & He 1999). In addition, Noguchi (1987) mentions that the main difference between *D. japonicum* and *D. scoparium* is found in their habitat preference, with the former growing on humus in shady habitats and the latter in sunnier and drier places. The morphological characters distinguishing the two Asian species from each other are mainly based on the sporophyte, namely inclination of the capsule as well as size and papillosity of the spores (Gao & He 1999). The only gametophytic difference is the canaliculate (*D. lorifolium*) versus keeled (*D. japonicum*) leaf apex (Table 1). Considering these slight differences and the fact that two further specimens from Japan were originally identified as *D. japonicum*, we provisionally conclude that the specimens from Japan and South Korea belong to *D. japonicum*. The Russian specimens probably represent *D.* cf. *lorifolium* as judged from the presence of suberect capsules in the two fertile specimens sequenced (Ru_6 and Ru_7). The sequenced North American specimens, in contrast, fall within the range of morphological variation of *D. scoparium*. Their taxonomic status and delimitation from *D. japonicum* and *D.* cf. *lorifolium* needs to be assessed based on further molecular and morphological analyses of a larger sampling of North American and Asian specimens, which may reveal further "cryptic" molecular lineages as well.

Because bryophytes have limited numbers of morphological characters, which are in addition under strong environmental constraints (e.g. Briggs 1965; Vanderpoorten & Goffinet 2006), it is difficult to identify key characters defining species. This is especially true in a genus with polymorphic species such as *Dicranum*. Molecular phylogenetic analyses may allow species to be circumscribed even when morphological characters are ambiguous (e.g. Vanderpoorten & Goffinet 2006; Stech *et al*. 2011; Stech *et al*. 2013). However, it is necessary to re-address the morphology in light of the molecular phylogenetic reconstructions to infer whether morphologically variable but molecularly well-supported species can be identified with certainty by morphological characters (e.g. Sukkharak *et al*. 2011). In the present study, molecular analyses provided useful support for defining part of the species of the *D. scoparium* complex as well as *D. majus*, which allowed reconsideration of the most reliable gametophytic characters to identify them (Table 1). On the other hand, the molecular data raised questions concerning the circumscription of *D. bonjeanii*, *D. japonicum*, and *D. lorifolium*, species for which the morphological definition was also limited. Further data are necessary to reach taxonomic conclusions for these species and to finally decide how many taxa should be distinguished in the *D. scoparium* complex. Nevertheless, the present approach is promising for the study of other taxonomically difficult complexes of closely related species in bryophytes.