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Phylogeny and species delimitation within the moss genus *Dicranum* Hedw.

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Phylogeny and species delimitation within the moss genus *Dicranum* Hedw.

Annick Séverine Lang

Naturalis Biodiversity Center (Sector Botany),
Universiteit Leiden
2014

To my family

Annick S. Lang

Phylogeny and species delimitation within the moss genus *Dicranum* Hedw.

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Phylogeny and species delimitation within the moss genus *Dicranum* Hedw.

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Chapter 1

General Introduction

Dicranum Hedw. (Dicranaceae, Bryophyta) is a large genus essentially found in the Holarctic. It mainly grows on soil in forest and mountain habitats forming dense, tomentose tufts or cushions (Crosby *et al.* 1999; Crum & Anderson 1981). Because of their morphological plasticity and broad distribution range, members of the genus *Dicranum* Hedw. (Dicranaceae, Bryophyta) are often difficult to identify, especially at the species level, hence many morphological species boundaries remain poorly understood. Furthermore, no comprehensive and complete study of this genus is available. This study focuses on the phylogeny and species delimitation within *Dicranum*, a Holarctic genus. Morphological and molecular species circumscription of species complexes are first studied, forming the basis for a phylogenetic reconstruction of the genus.

MEETING BRYOPHYTA

Bryophyta (mosses) is a highly diverse group of non-vascular land plants. With c. 12,500 species, bryophyta represents the second most diverse phylum of land plants and is found in every terrestrial ecosystem (Frey & Stech 2009; Crosby *et al.* 1999). Together with Marchantiophyta and Anthocerotophyta, Bryophyta have a haplodiplobiontic life cycle, with a haploid vegetative and dominant gametophyte, while the ephemeral diploid sporophyte remains attached to the maternal plant. Bryophyta display great morphological diversity, although they are characterized by few morphological synapomorphies such as multicellular rhizoids, leafy gametophytes and sporophytes with a capsule possessing a columella and stomata but lacking elaters (Frey & Stech 2009). They are generally small but their size can range from few millimetres (e.g. *Ephemeropsis*) up to 70 centimetres (e.g. *Dawsonia superba*). The more complex structures and positions of the sporophyte has been particularly important for the classifications of mosses, leading to a division between nematodontous and arthrodontous mosses based on the origin of peristome teeth, with the latter being further divided into acro- and pleurocarpous according to the position of the perichaetia on the stem, and into haplo- and diplolepidous mosses based on the tissue forming the teeth of the peristome (Goffinet & Shaw 2009).

Haplolepidids (Dicranidae) represent the second largest subclass of mosses, after the diplolepidous-alternate mosses (Bryidae) (Stech *et al.* 2012). They are usually characterized by a peristome with a single row of arthrodontous teeth around the opening of the capsule (La Farge 2002; Hernández- Maqueda *et al.* 2008; Stech *et al.* 2012). Currently, Dicranidae are divided into six recognized subclasses (Pottiales, Dicranales, Archidiales, Grimmiales, Bryoxiphiales, Scouleriales; Goffinet & Shaw 2009; Frey & Stech 2009) with two additional subclasses included in Frey & Stech (2009) (Mitteniales, Catoscopiales). Together with Grimmiales and Pottiales, Dicranales is one of

the largest order of the subclass and is characterized by usually smooth lamina cells, differentiated alar cells, a strong costa and trabeculate and striate peristome teeth (Goffinet *et al.* 2009; Frey & Stech 2009). The family Dicranaceae is complex family of the order Dicranales, which morphological concept has been redefined many times, counting up to 55 genera (e.g. Vitt 1984; Goffinet & Shaw 2009). The advances in molecular phylogenies allowed a clearer circumscription of Dicranaceae and reduced the number of genera to 24 (Stech & Frey 2008; Frey & Stech 2009).

MOLECULAR AND BARCODE MARKERS

During the past twenty years, DNA sequences of bryophytes have been increasingly used in systematic studies and taxonomical revisions giving new insights in bryology (Stech & Quandt 2010). While the first studies including DNA data utilized only one or two markers, the number of available markers, especially mitochondrial ones, has increased rapidly in the last ten years. Nevertheless, most of the studies still rely on four principal markers, namely *trnL-F*, *rps4*, *rbcL* and ITS (Stech & Quandt 2010). Recently, much effort has been placed in finding universal barcode markers. In addition to these traditional markers, *atpF-atpH*, *matK*, *psbK-psbI*, *rpoB*, *rpoC1* and *trnH-psbA* have been suggested as potential bryophyta barcode markers (CBOL Plant Working Group 2009). Of these ten potential loci, only six showed features that are suitable to delimit moss species (*trnL-F*, *rps4*, *trnH-psbA*, *rbcL*, *matK* and ITS; Liu *et al.* 2010, 2011). However, no consensus has been reached yet in finding the optimal combination of barcoding markers that are suitable for delimiting closely related species (Liu *et al.* 2010; Stech & Quandt 2010).

MOLECULAR SYSTEMATICS OF DICRANACEAE WITH EMPHASIS ON *DICRANUM*.

Although traditional morphological classification is often incongruent with modern systematics, which is based on molecular data, the monophyly of Dicranidae (arthrodonthous-haplolepidous mosses) is supported by all available phylogenetic reconstructions (e.g. Goffinet *et al.* 2001; Hedderson *et al.* 2004; La Farge *et al.* 2002; Stech *et al.* 2012). However, molecular studies have revealed that neither Dicranales nor Dicranaceae, as defined by Vitt

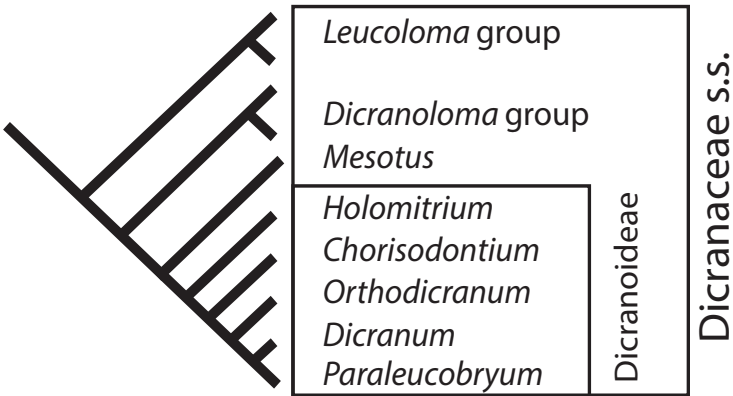


FIG. 1. Simplified cladogram of the Dicranaceae s.s. and relationships of its genera according to La Farge 2002 and Stech 1999.

(1984) and Goffinet & Shaw (2009), are a monophyletic group. While many taxa were clearly separated and thus placed in new lineages (Cox *et al.* 2010; Hedderson *et al.* 2004; La Farge *et al.* 2002; Stech 2008, 2012), the core of the Dicranaceae [Dicranaceae s.s. sensu La Farge (2002)] comprised four subfamilies: Dicranoideae, Mesotoideae, Dicranoloma group, and Leucoloma group, with Dicranoideae encompassing *Dicranum*, *Orthodicranum*, *Paraleucobryum*, *Chorisodontium*, *Eucamptodontopsis*, and *Holomitrium* (Fig. 1). Although molecular data gave new insights in the circumscription of the Dicranaceae, the relationships among genera remained ambiguous. Furthermore, the molecular species circumscription within the genera remained largely underexplored. The available molecular studies on *Dicranum* species complexes reveal ambiguous relationships among species due to their limited genetic variation (Ignatova & Fedosov 2008; Tubanova *et al.* 2010; Tubanova & Ignatova 2011) and is in need of further molecular studies.

TAXONOMICAL HISTORY OF *DICRANUM*

Dicranum is one of the largest moss genera, with more than 880 binomials originally given (van der Wijk *et al.* 1962; Tropicos.org). However, the genus as currently recognized, has ca. 90 accepted species (Frey & Stech 2009; Tropicos.org). Since the first nomenclatural description of the family, several revisions have been made, narrowing considerably the concept of its genera, and particularly the one concerning *Dicranum*.

Hedwig has described the genus in 1801 and considered *D. scoparium* as the type of the genus. A total of 34 other *Dicranum* species were simultaneously described in his *Species Muscorum Frondosorum* (1801). Of these 34 species, only three remained in the modern concept of the genus: *D. condensatum*, *D. scoparium* and *D. spurium* (Peterson 1979). Bruch, Schimper and Gümbel in 1847 (*Bryologia Europea*), worked on this family and described seven new genera within Dicranaceae and recognised 11 sections under *Dicranum*. Nearly simultaneously, Müller published his *Synopsis Muscorum Frondosorum* (1849), in which the treatment of the family encompassed six genera and five sections under *Dicranum*. Most of the genera and *Dicranum* species described by Bruch, Schimper & Gümbel and Müller are nowadays placed in other families, respectively other genera of the Dicranales (Goffinet & Shaw 2009; Peterson 1979). Until the beginning of last century, several other revisions of the Dicranaceae and *Dicranum* have been done on the country- or continental level (Japan, North America, Europe) leading to multiple rearrangements of the genus and subdividing it into different subgenera or sections (Table 1; Brotherus 1906, 1924; Mönkemeyer 1927; Nyholm 1953, 1954, 1987; Peterson 1979 Sakurai 1951, 1952; Takaki 1964). However, in the most recent treatments available (Hedenäs & Bisang 2004; Ireland 2007; Gao & He 1999), neither subgenera nor sections were taken into account.

It is evident that the conceptual disharmony and multiple re-classifications at the genus level is the expression of the difficulties to understand this taxon and to find unique morphological characters capable of providing clear species circumscriptions.

MORPHOLOGY AND CHARACTERISTICS OF *DICRANUM*

Dicranum is a dioicous genus that is characterised by densely tomentose acrocarpus stems. The leaves are generally falcate-second and lanceolate with a distal subula that varies from keeled to tubulose. They possess a strong costa that is percurrent to slightly excurrent. In cross-section, the costa has one row (sometimes two) of guide cells that are surrounded by stereid bands. The

TABLE 1. Taxonomic treatments of Dicranum according to the main European authors. The names of subgenera and sections within Dicranum are given.

Author	Brotherus (1906)	Brotherus (1924)	Mönkemeyer (1927)	Sakurai (1951)	Nyholm (1954)	Takaki (1964)	Nyholm (1986)
subgenera	Arctoa Chorisodontium Crassidicranum Dicranum Holodontium Leiodicranum Orthodicranum Paraleucobryum	Crassidicranum Eudicranum Pseudo-chorisodontium	Crasscostata Crispifolia Fragilifolia Fulvella Scoparia Strumifera Undulata	Falcati- Fragili- Elongati- Nippono- Pseudo-chorisodontium Scoparia- Undulati-		Crassidicranum Dicranum	
sections					Crassidicranum Fuscescentia Scoparia Spuria	Crassinervia Dicranum Elongata Fuscescentiforma Montana Muehlenbeckia Spuria	

dorsal side of the costa can be smooth or ornamented with lamellae, furrows or mamillae (Fig. 2). The leaves have a well differentiated double-layered alar region. The lower lamina cells are elongated and generally porose, while the upper lamina cells are either prosenchymatous (elongated and porose) or parenchymatous (short and smooth) (Fig. 2). The seta is mostly solitary, erect and twisted. It possesses an inclined capsule (Fig. 3A) with 16 bifid peristome teeth and a long-rostrate operculum (Goffinet & Shaw 2009; Ireland 2007). Morphological characters of *Dicranum* are very plastic and descriptions are usually based on few stable characters, such as sporophytic or costal characters. Some other discriminant characters are largely overlooked, especially in closely related species, as observed in *D. bardunovii*, *D. septentrionale*.

As in many bryophytes, vegetative reproduction plays an important role in *Dicranum*. While most species can easily propagate from gametophyte fragments, some species produce specialised structures such as flagelliform branchlets (*D. leioneuron* Kindb.), or have easily breakable leaf apices (*D. tauricum* Sapjegin). *Dicranum* can also reproduce sexually. Males can be either as large as female (Fig. 3B-C) or dwarf and growing on female stems (Fig. 3D), something called pseudomonoicy (Crawford et al. 2009). Sexual dimorphism in *Dicranum* is rather frequent. It was reported to occur in 20% of the species (Pichonet & Gradstein, 2012). Only few studies on dwarfism in *Dicranum* are available (Bisang & Ehrlén 2002; Briggs 1965; Ehrlén et al. 2000; Hedenäs & Bisang 2011; Sagmo Solli et al. 1998, 2000) and little is known about the mechanisms that triggers dwarfism, its consequences on populations structure and the possible hybridisation between species.



FIG. 2. Plant habit, leaf apex and leaf cross- section at base and upper part of A) *Dicranum scoparium* Hedw. and B) *D. tauricum* Sapjegin.

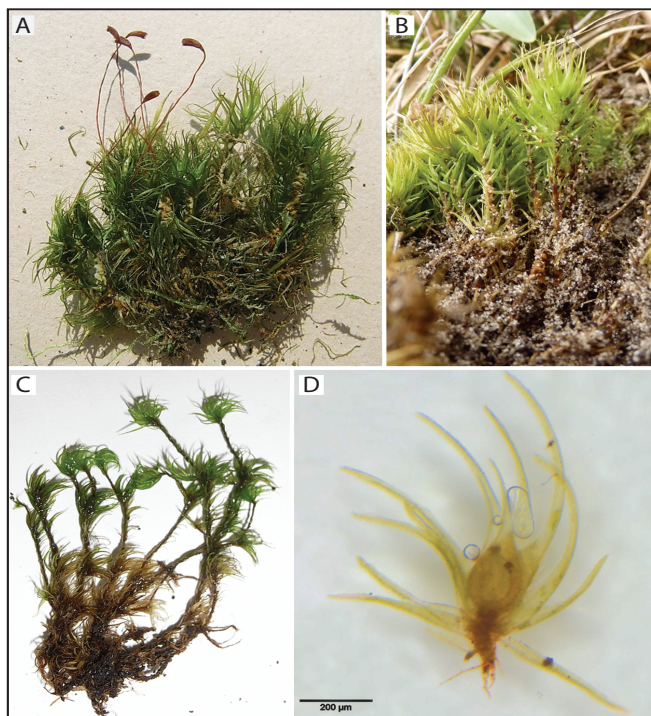


FIG. 3. Habitus of *Dicranum scoparium*. Female cushion with mature **A** sporophytes and **B** without sporophyte. **C** Normal sized males and **D** dwarfed male. Photo D by L. E. van Dijk

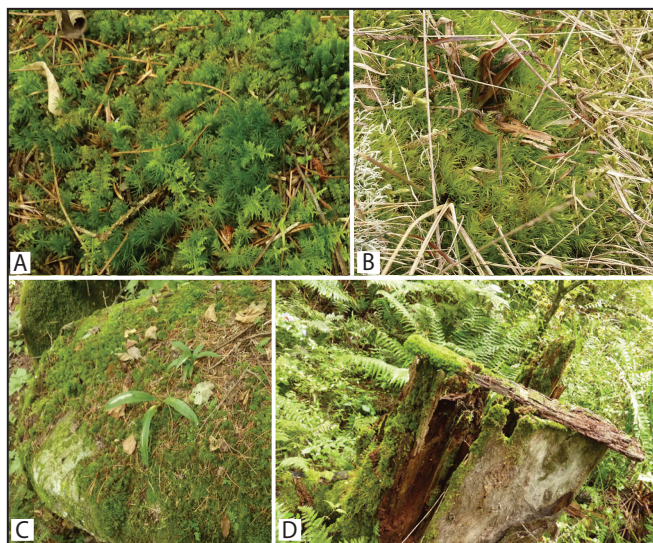


FIG. 4. Habitat type of *Dicranum*. **A** forest soil, **B** open sandy soil, **C** humus on boulder, **D** rotten tree bark.

DISTRIBUTION/ ECOLOGY IN THE HOLARCTIC

The majority of *Dicranum* species is found in the Northern hemisphere. About 30 species are counted for Europe and Asia (Gao & He 1999; Hedenäs & Bisang 2004) and 26 in Northern America (Ireland 2007; Lawton 1971). Generally, *Dicranum* species have large distributions covering more than one continent. One species with a particularly wide distribution is *D. scoparium*. It is typically found in the Holarctic, as defined by Schofield (1992). However, recently, this species has been reported in Australia and New-Zealand (Klazinga 2012). Nonetheless, few endemics exist in the Himalaya (*D. himalayanum*, *D. assamicum*, *D. kashmirensis* and *D. orthophyloides*; Chopra 1998; Dandotiya *et al.* 2011), in Japan (*D. leiodontum* and *D. setifolium*; Takaki 1972) and in the west coast of North America (*D. howellii*; Ireland 2007; Lawton 1971), or Hawaii (*D. speirophyllum*; Staples *et al.* 2004).

The distribution of species in the Holarctic is strongly associated with the vegetation zones, the degree of continentality and the altitudinal belts [e.g. *D. scottianum* and *D. canariense* occurring both in oceanic areas (Dierssen 2001; Hedenäs & Bisang 2004)]. Most of the species show a preference for acidic environments. They are found on all kinds of substrate: rocks, decomposed wood, sand, humus, bark, fen and bogs (Fig. 4 A-D). However, complete information about the distribution of many species, especially in species complexes, is still incompletely known, due in part to a poor morphological understanding and confusions between closely related species. Many species are considered as morphologically plastic and consequently as occurring in a broad spectrum of habitats. Recent molecular studies on the *D. acutifolium* species complex, however, revealed that this species complex contained multiple lineages that can be identified by few but distinct characters and occurred in different habitats (e.g. Otnyukova 2007; Tubanova *et al.* 2010; Tubanova & Ignatova 2011). This suggests that other *Dicranum* species with morphological plasticity might encompass several taxa with more restricted habitat preferences.

AIMS AND OUTLINE OF THE THESIS

This thesis aims at disentangling species circumscriptions of *Dicranum* species based on phylogenetic inferences. Phylogenetic reconstructions have been done sequencing five chloroplast (*rps4-trnT*, *trnL-F*, *psbA-trnH*, *rps19-rpl2*, *rpoB*) and one nuclear (nrITS) region. Besides molecular approaches, morphological studies were carried out in order to redefine the most suitable combination of characters for identifying species within complexes. A barcoding approach was also used on selected taxa, in order to evaluate the identification power of the markers on closely related species and we further tested the validity of automated species delimitation methods (GMYC and PTP) by comparing the estimated species with morphological and phylogenetic species circumscriptions.

In **chapter 2**, *Dicranum scoparium* species complex has been studied. The molecular analysis shows that *D. majus* is clearly separated from the *D. scoparium* species complex. However, the circumscription of *D. bonjeanii*, *D. nipponense* and *D. howellii* are less clearly distinct. Additionally, in contrary to its numerous phenotypes, *D. scoparium* is proven to be genetically very homogeneous. Nevertheless, a subclade including specimens from both northern America and Asia is revealed.

Chapter 3 explores the species boundaries of another species complex, the *D. acutifolium* complex. Recent molecular studies have shown that *D. acutifolium* and *D. brevifolium* are poorly circumscribed. Moreover, they revealed two new species, *D. bardunovii* and *D. septentrionale*, which

were further supported by morphological characters. In this chapter, additional molecular studies provide stronger support for the four above-mentioned species. Furthermore, it is shown that the current concept of *D. brevifolium* includes characters attributed to the new species *D. septentrionale*, known from Russia. Additionally, the distribution area of this latter species is extended to Scandinavia.

Chapter 4 investigates the identification capacity of molecular markers using arctic *Dicranum* species. Phylogenetic studies usually employ several barcoding markers. However, few studies have investigated the circumscription capacity of these barcoding markers in bryophytes. It is shown that none of the markers, taken independently, is sufficiently discriminative for species level identification. However, increasing the number of variable characters by combining several markers provides supported species delineation.

In **chapter 5**, 28 out of 30 European *Dicranum* species are included in a phylogenetic analysis and two methods of species delimitation are compared, namely the general mixed Yule coalescent approach (GMYC) and Poisson tree processes (PTP). In this chapter, we investigate the congruence between morphological and molecular species circumscriptions. In line with the results obtained in chapters 2, 3 and 4, supported species delineation was obtained using five chloroplast and one nuclear markers, but species relationships remained unresolved. The phylogenetic reconstruction reveals that six species are molecularly indistinguishable from closely related allies, reducing the number of species to 24. The GMYC and PTP methods tended to overestimate the number of phylogenetic entities, estimating between 34 and 58 species, and exposed several incongruences between morphological species concept and molecular phylogenetic species delineations. These differences might ensue from evolutionary processes that were so far undiscovered, but might also be linked to methodological issues.

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-second, 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 *rpoB* gene, *trnH_{GUG}-psbA* and *rps19-rpl2* intergenic spacers, and two parts of the *trnS-F* region, namely *rps4-trnT_{UGU}* spacer and *trnL-F* (*trnL_{UAA}* intron and *trnL_{UAA}-trnF_{GAA}* spacer), as well as one nuclear region (nrITS1-5.8S-ITS2) were amplified and sequenced. Except for *rpoB* and *rps19-rpl2*, 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 *rps19-rpl2* sequences of several

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

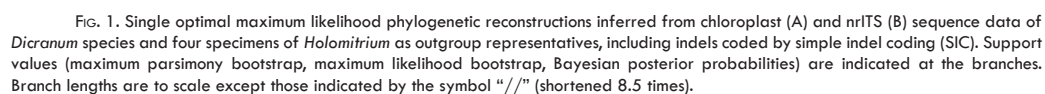
DNA Extraction, Amplification and Sequencing—Several leaves of a single stem apex taken from fresh or herbarium collections were carefully cleaned in demineralised water. DNA was extracted from the dried leaves using the DNeasy plant mini kit (Qiagen, Hilden, Germany) and eluted in 100 µl AE buffer. The PCR reactions were carried out in a final volume of 20 µl. The reaction mixture contained 1× buffer, 2.5 mM MgCl₂, 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.biportal.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 × 10⁶ generations for chloroplast and combined data and 11 ×



10⁶ 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.

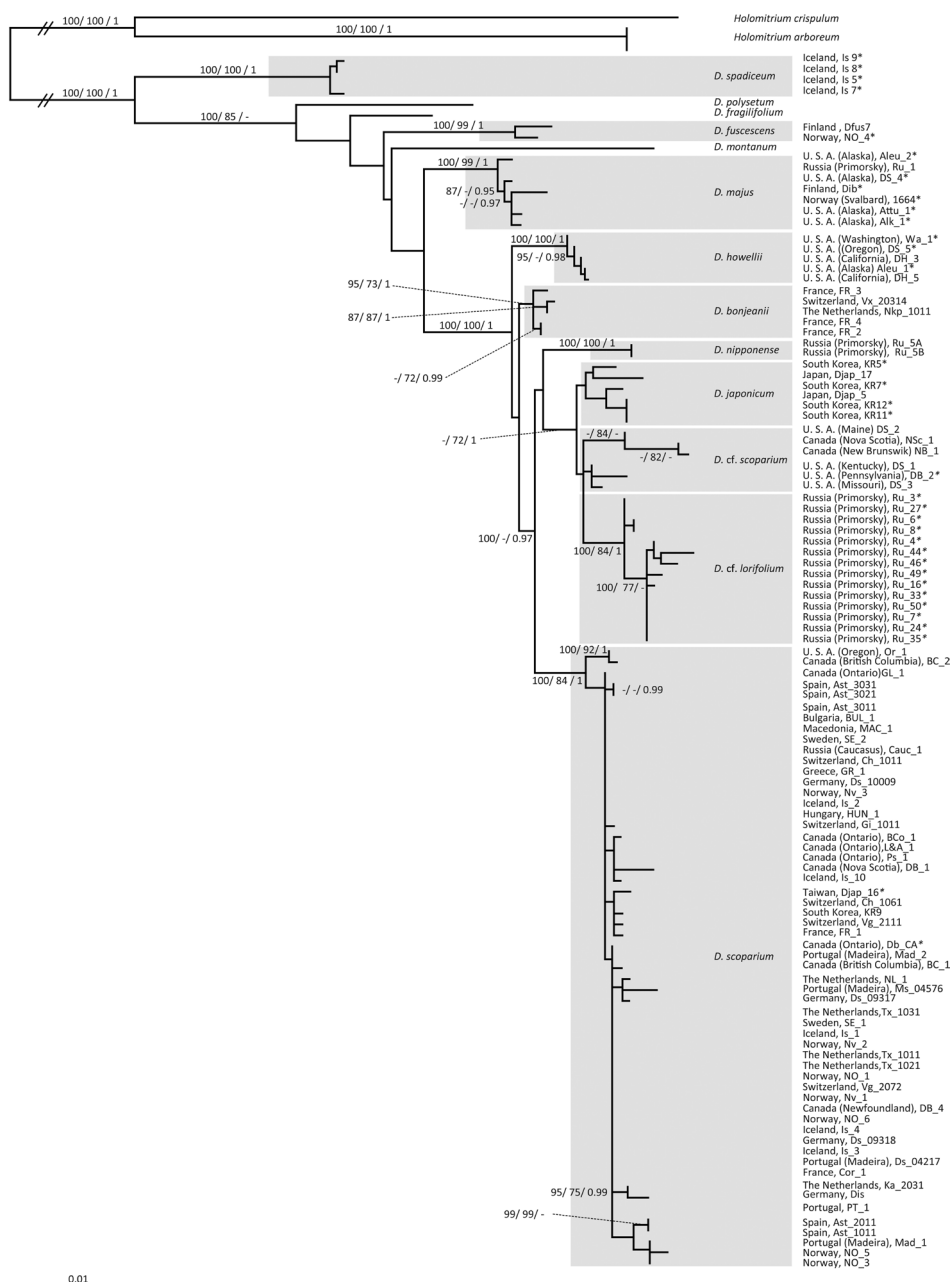


FIG. 2. Single optimal maximum likelihood phylogenetic reconstruction inferred from combined chloroplast and nrITS sequence data of *Dicanum* species and four specimens of *Holomitrium* as outgroup representatives, including indels coded by simple indel coding (SIC). Support values (maximum parsimony 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 $\geq 90\%$ 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 ($\geq 90\%$ BS except 84% for *D. cf. lorifolium* in Fig. 2, PP 1) but received lower (*D. majus*: $\sim 73\%$ 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; Hedén & Bisang 2004; Ireland 2007; Kiazanga 2012; present study).

	Gametophytic characters from the literature	Suggested diagnostic characters	Ecological and geographical range
<i>D. bonjeanii</i>	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.
<i>D. howellii</i>	Leaves falcate-second 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.
<i>D. japonicum</i>	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.
<i>D. lorifolium</i>	Leaves homomallous, falcate-second 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.
<i>D. majus</i>	Leaves long, strongly falcate-second, 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.
<i>D. nipponense</i>	Stems unevenly foliate, leaves erect-spreading or falcate-second, 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.
<i>D. scoparium</i>	Leaves straight to falcate-second, margins serrate, apex keeled, costa percurrent, 4 serrate ridges with several stered 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.

Chapter 3

Species delimitations in the *Dicranum acutifolium* complex (Dicranaceae, Bryophyta) using molecular markers

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ABSTRACT

Because of their morphological plasticity and broad geographic distribution, the taxonomy of *Dicranum* is difficult. The circumscription of the different species included in the *Dicranum acutifolium* complex is poorly understood and taxonomic confusions are frequent. The present study extends earlier ITS-based phylogenetic reconstructions of the *D. acutifolium* complex by analysing five additional chloroplast markers (*trnT-rps4*, *trnL-F*, *psbA-trnH*, *rps19-rpl2*, *rpoB*) together with ITS of a larger taxon sampling. The phylogenetic analyses delimit *Dicranum acutifolium* (Lindb. & Arnell) C.E.O. Jensen, *D. bardunovii* Tubanova & Ignatova, *D. brevifolium* (Lindb.) Lindb., and *D. septentrionale* Tubanova & Ignatova, which together form the *D. acutifolium* complex, and confirm that *D. pseudoacutifolium* is synonymous with *D. flexicaule*. *Dicranum septentrionale* was known so far from across Russia but also occurs in Scandinavia, where it was probably overlooked due to morphological resemblance with *D. brevifolium*. The problem of mixed collections for identification is exemplified by the holotype of *D. bardunovii*, which contains also individuals of the morphologically most similar *D. acutifolium* according to the molecular data. Morphometric analyses support the differentiation of the *D. acutifolium* complex. Furthermore, ordination analyses point to a continuous range of variation among species within the *D. acutifolium* complex, especially due to the larger variation of *D. septentrionale*.

INTRODUCTION

Dicranum Hedw. (including *Orthodicranum* (Bruch & Schimp.) Loeske) is a large moss genus of more than 90 accepted species (Frey & Stech 2009; Tropicos.org) with a predominantly Holarctic distribution. Several *Dicranum* species are known to be morphologically plastic (Hedenäs & Bisang 2004; Ireland 2007), and their circumscriptions remain ambiguous as neither a thorough worldwide revision nor a complete phylogenetic analysis of *Dicranum* is available yet. Nevertheless, a number of recent studies tackled certain groups of *Dicranum* species based on molecular data, namely

the *D. scoparium* Hedw. complex (Lang & Stech 2014), arctic *Dicranum* species (Lang *et al.* 2014), *Dicranum* species with fragile leaves (Ignatova & Fedosov, 2008) or the *D. acutifolium* (Lindb. & Arnell) C.E.O. Jensen and *D. fuscescens* Turner complexes (Tubanova *et al.* 2010; Tubanova & Ignatova 2011). The former two studies concluded that (closely related) *Dicranum* species can be best delimited based on combined analysis of five chloroplast markers and the nuclear ribosomal ITS region. Inferences on the *D. acutifolium* complex, in contrast, were based on ITS only and need to be re-evaluated based on a larger marker sampling.

The *Dicranum acutifolium* complex is part of *Dicranum* sect. *Spuria* Bruch & Schimp. (sensu Nyholm, 1987) and centred around two circumarctic – alpine species, *D. acutifolium* and *D. brevifolium* (Lindb.) Lindb. They were first described as varieties of *D. bergeri* Blandow (Lindberg & Arnell 1890) or *D. muehlenbeckii* Bruch & Schimp. (Lindberg 1865), respectively, the former being an erroneous name for *D. undulatum* (cf. Hedenäs & Bisang 2004). Otnyukova (2007) described a new species, *D. pseudoacutifolium* Otnyukova, which differed from *D. acutifolium* and *D. brevifolium* in morphological characters such as the absence of bulgings above cell walls, non-porose lower leaf cells, and inner perichaetial leaves abruptly contracted into a subula. However, molecular data revealed that the type specimen of this species corresponded to a weak form of *D. flexicaule* Brid., with whom it has been consequently synonymized (Tubanova *et al.* 2010), while other *D. pseudoacutifolium* specimens had identical ITS sequences with *D. acutifolium* (Tubanova *et al.* 2010). On the other hand, two newly identified molecular lineages were described as species, i.e. *D. septentrionale* Tubanova & Ignatova and *D. bardunovii* Tubanova & Ignatova (Tubanova *et al.* 2010; Tubanova & Ignatova 2011). These two species are morphologically very close to *D. brevifolium*, but also share several features with *D. acutifolium*. However, neither the newly described species nor *D. brevifolium* or *D. acutifolium* formed strongly supported clades based on ITS sequences only. The *Dicranum acutifolium* complex, in line with Tubanova *et al.* (2010), thus comprises four species, *D. acutifolium*, *D. brevifolium*, *D. bardunovii* and *D. septentrionale*. It is characterised by a combination of morphological characters including leaves that are keeled distally with the blade shaped like a pair of tongs in cross-section as well as thick-walled lamina cells that are subquadrate to short rectangular, sometimes irregularly shaped above and elongated and porose below.

This study extends the phylogenetic reconstructions of Tubanova *et al.* (2010) and Tubanova & Ignatova (2011) by analysing five chloroplast markers (*trnT-rps4*, *trnL-F*, *psbA-trnH*, *rps19-rpl2*, *rpoB*) in combination with ITS of a larger taxon sampling. Based on the molecular data and a re-evaluation of morphological characters, we aim to (i) add further molecular support to the circumscription of the *D. acutifolium* complex, (ii) clarify species circumscriptions in the complex, and in particular evaluate the taxonomic status of *D. bardunovii* and *D. septentrionale*, and (iii) investigate the value of morphological characters used to distinguish the species of the complex.

MATERIALS AND METHODS

Sampling— A total of 67 *Dicranum* specimens were sampled (Appendix 1). The sampling included 15 specimens of which ITS sequences had already been generated by Tubanova *et al.* (2010) and Tubanova & Ignatova (2011), 12 specimens newly sequenced for all six markers employed here (see below), and 40 specimens of which chloroplast and ITS sequences were generated for previous studies (Lang & Stech 2014; Lang *et al.* 2014; Stech, 1999; Stech *et al.* 2006). We studied 29 specimens of sect. *Spuria*: six originally identified as *D. acutifolium*, 13 *D. brevifolium*, two *D. septentrionale*, three *D. bardunovii* (including the holotype, from which two

plants were sequenced separately), one *D. drummondii*, and four *D. undulatum*. Eight *Dicranum* species were included as representatives of other sections than sect. *Spuria*: four specimens of *D. spadiceum* J.E. Zetterst. (sect. *Muehlenbeckia* Peterson), three *D. elongatum* Schleich. ex Schwägr. (sect. *Elongata* Hag.), five *D. fuscens* and 11 *D. flexicaule* (sect. *Fuscescentiformia* Kindb.), seven *D. majus* Turner, two *D. nipponense* Besch., two *D. scoparium*, and three *D. bonjeanii* De Not. (all sect. *Dicranum* Hedw.). *Dicranum muehlenbeckii* (sect. *Muehlenbeckia*) could not be included in the molecular analyses because of unsuccessful DNA amplification. However, two specimens were included in the morphological analyses. Previous studies resolved *Holomitrium* Brid. as sister group of *Dicranum* (La Farge *et al.* 2002; Stech *et al.* 2006). Therefore, four samples, one *H. crispulum* Mart. and three *H. arboreum* Mitt., were chosen as outgroup representatives (Appendix 1).

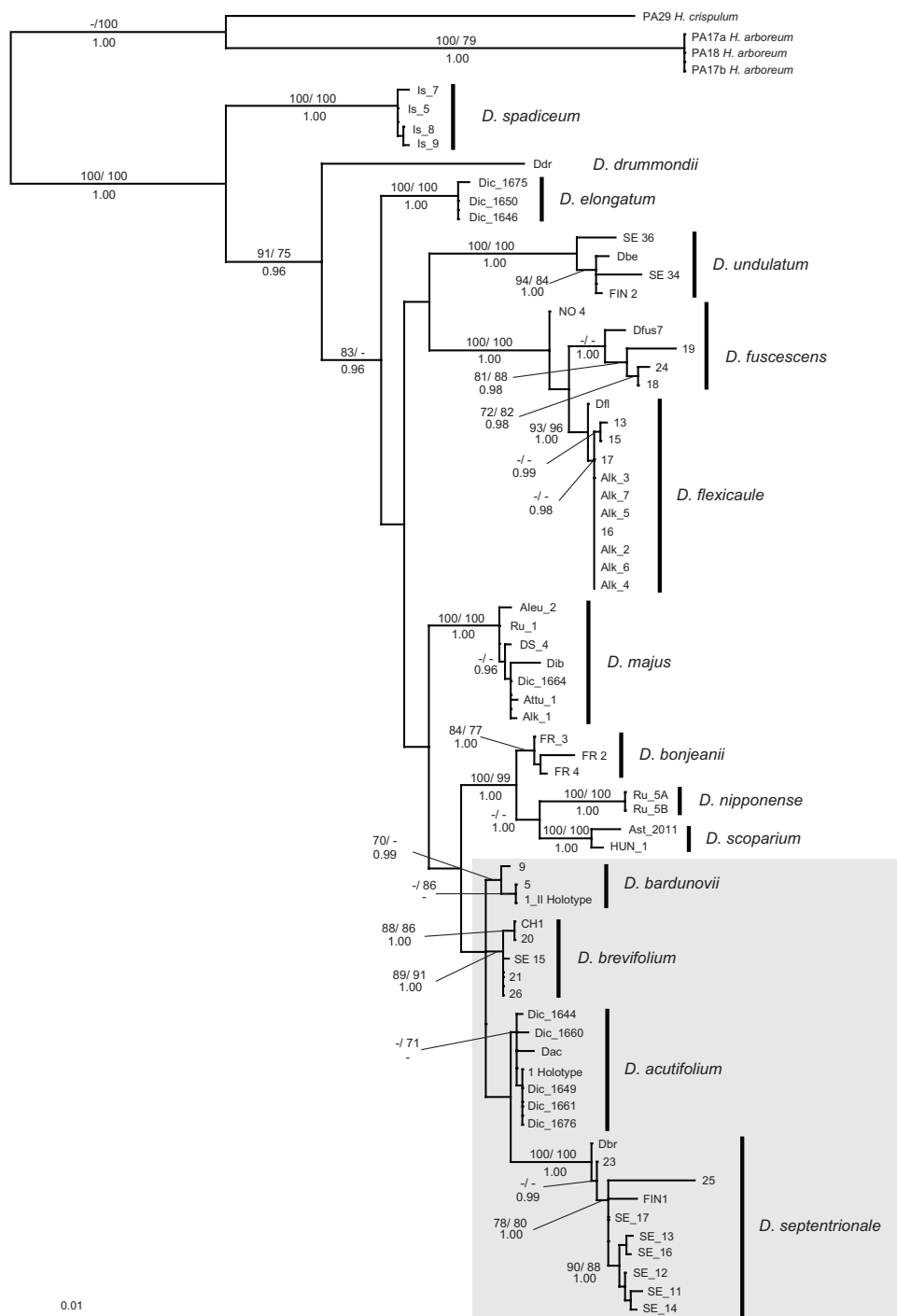
DNA extraction, amplification and sequencing— The greenest part of single gametophyte shoots was selected for DNA extraction. After cleaning the shoot under a binocular, total DNA was extracted using the NucleoSpin® Extract II Kit (Macherey-Nagel, Düren, Germany). Six markers employed to delimit closely related *Dicranum* species in Lang & Stech (2014) and Lang *et al.* (2014) were amplified and sequenced: five chloroplast regions (partial *rpoB* gene, *trnH_{GUG}-psbA*, *rps19-rpl2*, and *rps4-trnT_{UGU}* intergenic spacers, and *trnL_{UAA}* intron / *trnL_{UAA}-trnF_{GAA}* intergenic spacer) and the nuclear ribosomal nrITS1-5.8S-ITS2 region. PCR amplifications were performed as described in Lang & Stech (2014). All 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 v6.1.6 (Biomatters, available from www.geneious.com) using 65% similarity matrix costs, and manually adjusted. One short hairpin-associated inversion in the *trnH-psbA* spacer, which can flip at the population level and may significantly reduce phylogenetic structure if undetected (Quandt & Stech 2004; Borsch & Quandt 2009; Whitlock *et al.* 2010), was positionally separated in the alignment and not coded as indels.

The best substitution model was selected for each locus according to the Akaike information criterion (AIC) using MrModeltest (Posada & Crandall, 1998) executed through PAUP* 4.0b10 (Swofford 2002). Gaps were coded as informative by simple indel coding (SIC) (Simmons & Ochoterena 2000) as implemented in SeqState (Müller 2004). To check for incongruence, phylogenetic reconstructions based on chloroplast and nuclear sequences were visually compared. In addition, an incongruence length difference test (ILD, Farris *et al.* 1994) as implemented in PAUP* was performed with 100 replicates. As both visual inspections and the ILD test indicated that the plastid and nuclear tree topologies were congruent ($p=0.03$), the two datasets were combined for analysis in a total evidence approach.

Phylogenetic inferences were based on maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI) analyses, with and without indels coded by SIC included. Heuristic searches under parsimony were performed in PAUP* using simple sequence addition with 1000 replicates and tree bisection-reconnection (TBR) branch swapping. The nucleotide matrix was divided into three partitions for ML and BI, namely the non-coding chloroplast markers (*rps4-trnT*, *trnL-trnF*, *trnH-psbA*, *rps19-rpl2*), the chloroplast gene *rpoB*, and the nrITS region. Maximum likelihood analyses were carried out with RAXML v.7.2.6 (Stamatakis 2006) employing the graphical user interface raxmlGUI v.0.93 (Silvestro & Michalak 2012). As implemented in RAXML, the GTR model



0.01

of nucleotide substitution with Γ model of rate heterogeneity was used for all partitions. Bootstrap searches under ML were done using the thorough bootstrap heuristics algorithm with 20 runs and 1000 replicates. Bayesian analyses were run on the CIPRES science gateway (Miller *et al.* 2010). Bayesian posterior probabilities were calculated based on the Markov chain Monte Carlo (MCMC) method, using MrBayes v3.2.1 x64 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003), with MrModeltest best fit models HKY + Γ for the non-coding chloroplast markers and HKY + I for *rpoB* and *nrITS*. Nucleotide and indel data were treated as separate and unlinked partitions, employing the restriction site model ('F81') for the indel matrix as recommended by Ronquist *et al.* (2005). The a priori probabilities supplied were those specified in the default settings of the program. Two runs with four chains were run simultaneously (11×10^6 generations), with the temperature of the single heated chain set to 0.5. Chains were sampled every 1000 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 & 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%.

Morphological analysis—A total of 47 specimens were included in the morphological analyses: all six *D. acutifolium*, five *D. brevifolium*, three *D. bardunovii* and ten *D. septentrionale* as well as two *D. scoparium*, two *D. nipponense*, three *D. bonjeanii*, three *D. majus*, four *D. undulatum* and the four *D. spadiceum*, plus three additional *D. drummondii* and two *D. muehlenbeckii* specimens that could not be sequenced. Thirty-four gametophytic characters were scored according to their relevance for species identification (Nyholm, 1987; Hedenäs & Bisang 2004; Tubanova *et al.* 2010). As none of the examined samples carried sporophytes, sporophytic characters were not included in the statistical analyses. Presence or absence of character states was scored for each sample (Appendix 2). Morphological scoring was made under a light microscope on three branch leaves removed from the upper part of the stem, excluding the uppermost part. Three additional leaves were removed and used for scoring characters of costa cross-section. Multistate characters were artificially separated into binary characters for analytical reasons.

A multivariate approach was used to investigate the phenotypic affinities between the taxa of the *D. acutifolium* complex and other putatively closely related species. Morphological discontinuities were first explored through a hierarchical cluster analysis based on Jaccard distances and the complete-linkage method as clustering strategy using the *vegan* package (Oksanen *et al.* 2013) in R 2.15 (R Development Core Team 2013). Pearson's correlation coefficient was calculated to evaluate the optimal number of clusters. To further explore the morphological similarity of species, we performed an ordination with nonmetric multidimensional scaling (NMDS; Kruskal 1964) applying the *metaMDS* function of *vegan* with its default arguments. We used the Jaccard matrix to produce a five dimensional ordination (i.e., $k=5$) and plotted against species

← FIG. 1. Single optimal maximum likelihood phylogenetic reconstruction inferred from the partitioned matrix for the non-coding chloroplast loci (trnT-rps4- trnL-F- trnH-psbA- rps19- rpl2), the coding region *rpoB* and *nrITS*, including indels coded by simple indel coding (SIC). The default GTR+ Γ model was applied for all DNA partitions and F81 was employed for the indel matrix. Bootstrap analyses under ML were done using the thorough bootstrap heuristics algorithm with 20 runs and 1000 replicates. BI was obtained with the best fit models HKY + Γ for the first partition, and HKY + I for *rpoB* and *nrITS* and F81 for the indel matrix, after 11,000,000 generations with two runs and four chains and the temperature of the single heated chain set to 0.5. Trees were sampled every 1000 generations and a burnin was set to 25%. Four specimens of *Holomitrium* were used as outgroup representatives. Support values (MP and ML BS $\geq 70\%$, BI PP ≥ 0.95) are indicated at the branches. Grey boxes delimit species of the *D. acutifolium* complex.

groups. Differences between and among groups of the ordinations were tested using an analysis of similarities (ANOSIM; Clarke 1993; Chapman & Underwood 1999).

RESULTS

The total chloroplast alignment comprised 1901 positions, of which 122 were variable, and 90 of the variable characters were parsimony-informative. Of the 1077 positions in the ITS alignment, 124 ambiguous positions were removed from the subsequent calculations. The remaining 953 positions comprised 160 variable characters, of which 113 were parsimony-informative. Simple indel coding of the combined dataset yielded 155 additional characters (excluding three corresponding to an inversion in *psbA-trnH*), of which 99 were parsimony-informative.

Similar consistency indices resulted from the most parsimonious phylogenetic reconstructions with and without indels included (CI 0.7206 versus 0.7386), indicating a slightly lower amount of homoplasy in the indel characters. The single optimal ML tree of the combined markers is shown in Fig. 1, with bootstrap support values ($\geq 70\%$ BS) from the parsimony and likelihood analyses as well as posterior probabilities ($PP \geq 95$) from Bayesian inference indicated on the branches.

Regardless of the phylogenetic inference, *D. brevifolium* and *D. septentrionale* were resolved in well-supported clades ($\geq 89\%$ BS, PP 1; Fig. 1). *Dicranum bardunovii* was less strongly supported (70% ML BS, PP 0.99), whereas *D. acutifolium* only received 71% BS in the ML analysis. Relationships among these four lineages remained ambiguous. *Dicranum drummondii*, which shares common morphological characters with species of the *D. acutifolium* complex, was clearly separated from the latter as well as from *D. undulatum* (100% BS, PP 1). One of the two plants of the holotype of *D. bardunovii* corresponded to *D. acutifolium*. Furthermore, eight of the 13 samples identified as *D. brevifolium* corresponded to *D. septentrionale* and only five were attributed to *D. brevifolium*. These confusions were due to mis-identifications of the samples, as the species are readily confused morphologically.

The *D. acutifolium* complex was resolved as sister group to the *D. scoparium* complex, but relationships between these two complexes as well as *D. majus*, *D. elongatum* and the *D. fuscescens* complex (*D. flexicaule* and *D. fuscescens*) remained unsupported.

The cluster analysis of morphological characters (Fig. 2) divided the analysed specimens

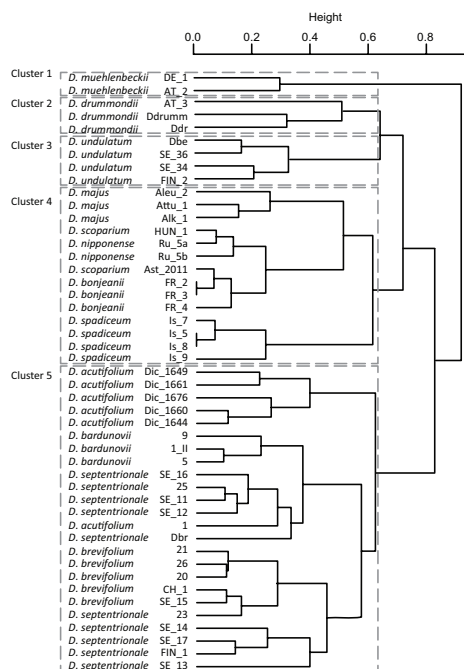


FIG. 2. Cluster dendrogram of 34 morphological characters and 47 specimens of *D. acutifolium*, *D. brevifolium*, *D. bardunovii*, *D. septentrionale*, *D. scoparium*, *D. bonjeanii*, *D. nipponense*, *D. majus*, *D. spadiceum*, *D. drummondii*, *D. muehlenbeckii* and *D. undulatum*, based on a Jaccard distance matrix and complete-linkage clustering strategy. The optimal number of clusters ($k=5$), delimited by the dashed rectangles, was calculated based on Pearson's correlation coefficient ($r=0.769$). Species names are based on the clades resolved in the molecular phylogenetic reconstructions.

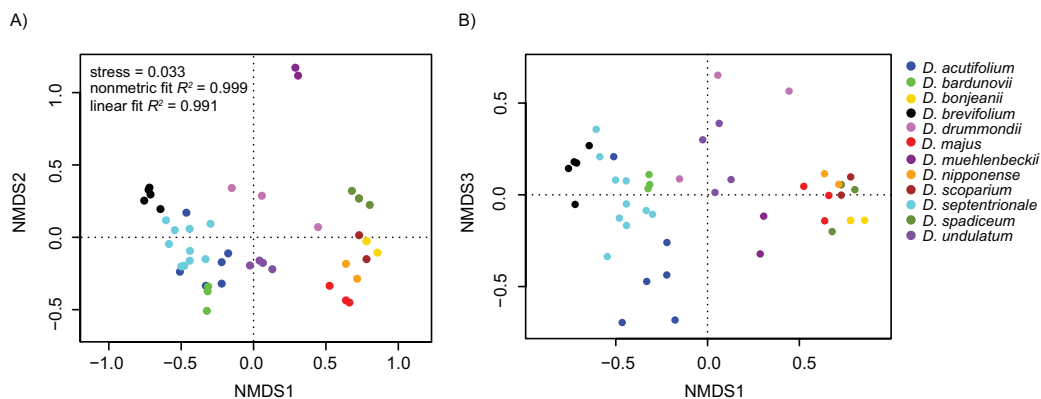


FIG. 3. Nonmetric Multidimensional Scaling (NMDS) ordinations of 47 *Dicranum* specimens using a Jaccard distance matrix and applying the metaMDS function of *vegan* with its default arguments ($k=5$). Scatterplots are showing the first and second (A), and first and third (B) dimensions. Species names are based on the clades resolved in the molecular phylogenetic reconstructions

into five optimal clusters (Pearson's correlation coefficient $r=0.769$). *Dicranum muehlenbeckii*, *D. drummondii* and *D. undulatum* were grouped in clusters 1, 2 and 3, respectively. Cluster 4 included all the specimens of the *D. scoparium* complex plus *D. majus* and *D. spadiceum*, while all four species from the *D. acutifolium* complex grouped in cluster 5. Within the cluster of the *D. acutifolium* complex, three subclusters corresponded to *D. acutifolium*, *D. bardunovii* and *D. brevifolium*, respectively, whereas *D. septentrionale* did not form a homogeneous group, in contrast to the molecular tree. SE_11, SE_12, SE_16 and 25 formed a group that was most similar to *D. bardunovii*, SE_13, SE_14, SE_17, and FIN_1 formed a group that was most similar to *D. brevifolium* and one sample (23) clustered with *D. brevifolium*. Furthermore, both samples from the holotype of *D. bardunovii* (1 and 1_II) were situated in different subclusters, but sample 1 was not part of the *D. acutifolium* cluster, in contrast to the molecular tree.

The five clusters identified in the cluster analysis were best distinguishable by the first three axes of the NMDS scatterplot (Fig. 3; stress value= 0.033, nonmetric fit $R^2=0.999$, linear fit $R^2=0.991$). While the specimens included in the *D. acutifolium* complex formed a first group with negative values on axis one, the specimens of the *D. scoparium* complex, plus *D. spadiceum* and *D. majus* formed a second group with positive values. *Dicranum undulatum*, as well as *D. muehlenbeckii* and *D. drummondii*, was plotted between group one and two, with the former in the vicinity of group one. While axis two further allowed a clear distinction of *D. muehlenbeckii* (Fig. 3A), axis 3 confirmed the separation of *D. undulatum* from the *D. acutifolium* complex and the distinction of *D. brevifolium*, *D. acutifolium*, *D. bardunovii* (Fig. 3B). Although *D. septentrionale* was differentiated by axis 1 and 2, its similarity with *D. brevifolium* and *D. bardunovii* was displayed by axis 3. The differentiation among species was supported by the ANOSIM ($R= 0.8867$; $p=0.001$, 999 permutations).

DISCUSSION

Both the present molecular phylogenetic reconstructions (Fig. 1) as well as the morphological analyses (Fig. 2) support the current circumscription of the *D. acutifolium* species complex, which comprises *D. acutifolium*, *D. bardunovii*, *D. brevifolium*, and *D. septentrionale*. Morphologically

TABLE 1. Table of characters for *D. acutifolium*, *D. brevifolium*, *D. septentrionale* and *D. bardunovii* based on the present study and the literature (Tubanová & Ignatova, 2011).

	Leaf orientation	Leaf margin	Leaf apex	Alar cells	Lamina	Basal lamina cells	Median lamina cells	Upper lamina cells	Costa
<i>D. acutifolium</i>	Generally straight or slightly second, slightly flexuose when dry. Not undulate.	Unistratose, entire.	Acuminate, serrulate, shaped like a pair of tongs in the middle and keeled to circular in apex.	Golden, bistratose.	Unistratose, no projecting cells, incrassate walls.	Rectangular to linear, not sharply different from median cells, porose.	Quadrate to elongated, generally porose.	Irregularly arranged, irregularly quadrate or elongated, generally eporose.	One row of guide cells, differentiated dorsal epidermis, smooth.
<i>D. brevifolium</i>	Generally second, curled or twisted when dry. Not or slightly undulate in distal half.	Unistratose, occasionally bistratose in apex with geminate teeth	Acuminate, serrulate, shaped like a pair of tongs.	Golden, bistratose	Occasionally projecting cells in upper part, bulging cell walls, incrassate.	Rectangular, different from median cells, generally eporose.	Quadrate, eporose.	Regularly arranged, quadrate, eporose.	One row of guide cells, differentiated dorsal epidermis, with rugosity on the abaxial side.
<i>D. septentrionale</i>	Falcate second, sometimes undulate, strongly curled when dry.	Unistratose, occasionally bistratose in apex with geminate teeth, serrulate.	Acuminate, serrulate, shaped like a pair of tongs, generally keeled in apex.	Generally golden, sometimes hyaline, bistratose.	Unistratose, occasionally with strongly projecting cells, especially in median cells, apex, bulging cell walls, incrassate.	Rectangular to linear, not sharply different from median cells, porose.	Quadrate to elongated, generally eporose.	Regularly arranged, irregularly quadrate, eporose.	One row of guide cells, sometimes with duplicated cells, differentiated dorsal epidermis, generally smooth.
<i>D. bardunovii</i>	Generally straight or slightly second, slightly undulate, slightly flexuose when dry.	Unistratose, serrulate, occasionally bistratose in apex with geminate teeth	Acuminate, serrate, mostly shaped like a pair of tongs, generally keeled in apex.	Hyaline, bistratose.	Unistratose, with projecting cells, bulging cell walls, incrassate.	Generally rectangular, porose.	Quadrate to elongated, porose or not.	Regularly arranged, irregularly projecting cells in upper half, eporose.	One row of guide cells, differentiated dorsal epidermis, projecting cells in upper half.

all four species together are characterised by incrassate lamina cells that are parenchymatous in the upper half of the leaf, acuminate and serrulate to serrate leaf apices, and a keeled upper leaf with incurved margins, resulting in a tong-shaped transverse section. The molecular data furthermore supports the conclusion of Tubanova *et al.* (2010) that *D. pseudoacutifolium* is synonymous with *D. flexicaule* (samples 13 and 15 in Fig. 1). Although supraspecific relationships in *Dicranum* remain largely unsupported based on the present molecular data (cf. also Lang & Stech 2014), and molecular relationships of *D. muehlenbeckii* await further study, no close relationship of the *D. acutifolium* complex with the other included species of sect. *Spuria*, *D. drummondii* and *D. undulatum*, nor with *D. fuscescens* (Fig. 1) and *D. muehlenbeckii* (Figs. 2, 3) are indicated. The latter two species share certain morphological characters with *D. acutifolium* and *D. brevifolium*, such as bulging cell walls, quadrate apical cells and slightly porose basal cells (e.g. Nyholm 1954, 1987; Ireland 2002), but are easily differentiated from the species of the *D. acutifolium* complex by their leaves not tong-shaped in cross section, thin lamina cell walls, and leaf margins serrate in the distal half. Despite its larger size, keeled leaf and the absence of bulging cell walls, *D. drummondii* is sometimes confused with *D. acutifolium* because of its flexuose leaves in dry state and irregularly shaped upper lamina cells (Ireland 2007; Nyholm 1987). *Dicranum undulatum* and *D. acutifolium* have both straight leaves that have projecting upper cells at back. However, the former has transversely undulate leaves that narrow into an obtuse apex, whereas the leaves of *D. acutifolium* end in a subulate point.

The existence of numerous intergrading forms occurring among the species of the *D. acutifolium* complex has caused much taxonomic confusion and led to frequent misidentifications. Their distinction is based on few subtle gametophytic characters (Table 1), and deviating forms render morphological identification difficult (Ireland 2002, 2007) as exemplified by the sample 1 of *D. acutifolium* or 23 of *D. septentrionale* (Figures 2, 3). Furthermore, herbarium collections of *D. brevifolium* were frequently found under different names, such as *D. drummondii*, *D. flexicaule*, or *D. undulatum* (Ireland 2002, Tubanova *et al.* 2010; Tubanova & Ignatova 2011). At the molecular level *D. acutifolium*, *D. bardunovii*, *D. brevifolium* and *D. septentrionale* seem more clearly distinguishable (Fig. 1), although the respective clades receive different statistical support, with *D. brevifolium* and *D. septentrionale* being well supported in all analyses, whereas *D. acutifolium* and *D. bardunovii* receive (lower) support only in part of the analyses. The molecular data also helped renaming misidentified specimens within the *D. acutifolium* complex, since a number of specimens identified as *D. brevifolium* were resolved in the clade of the *D. septentrionale* (Fig. 1). *Dicranum septentrionale* is a recently described species defined by few characters that were previously attributed to the morphological variation of *D. brevifolium* (Table 1). Nonetheless, the morphological characters frequently intergrade with *D. bardunovii* or *D. brevifolium* (Fig. 2, 3). According to the present results, *D. brevifolium* is characterised morphologically by tong-like leaf apices, elongated basal cells that are well differentiated from the median one and generally not porose, quadrate upper cells and rugose dorsal surface of the costa. In contrast, *D. septentrionale* is best differentiated by projecting lamina cells, especially at the leaf apex, elongated basal cell that gradually become quadrate, and irregularly shaped in the upper part of the leaf and a generally smooth dorsal epidermis. Furthermore, the distributions and ecological preferences of both *D. brevifolium* and *D. septentrionale* are incompletely known or misunderstood, considering that further *D. brevifolium* collections may appear to belong to *D. septentrionale*. While *D. septentrionale* was known so far from across Russia (Fig. 4), our study shows that its distribution range extends to

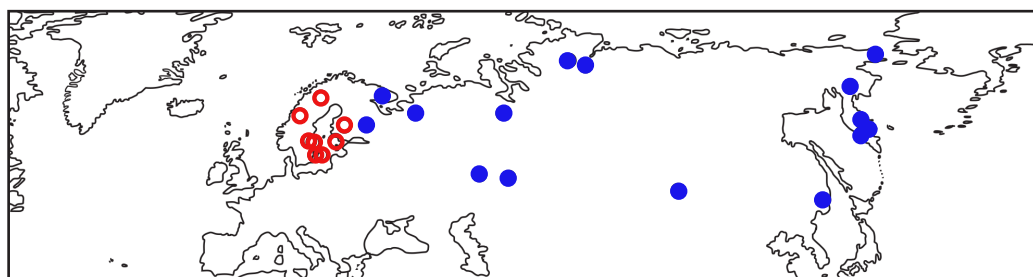


FIG. 4. Geographical distribution of *Dicranum septentrionale* according to collections studied in Tubanova et al. (2010) (filled blue dots) and the present study (empty red dots).

Scandinavia. This indicates that *D. septentrionale* has a Eurasian or possibly Holarctic distribution. In addition, the herbarium material we examined suggests that *D. septentrionale* could be a boreo-montane species, whereas *D. brevifolium* would be a (sub)arctic-alpine species, as suggested by previous studies (Amann et al. 1918; Dierssen 2001). A morpho-molecular re-identification of further specimens, including *D. brevifolium* from North America, is needed to delimit the distribution patterns and ecological preferences of *D. brevifolium* and *D. septentrionale* with more confidence.

Another part of the confusion between the species of the *D. acutifolium* complex may stem from the presence of mixed collections. A striking example is the holotype of *D. bardunovii*, which contains also individuals of the morphologically most similar *D. acutifolium* according to the molecular data (samples 1 and 1_II, Fig. 1). Not only are the morphological differences between *D. acutifolium* and *D. bardunovii* small (Table 1, Figs. 2-3), *D. bardunovii* also shows morphological variation departing from the holotype description. This is the case in the sequenced specimen 1, which resembles *D. septentrionale* by the presence of projecting cells in the lamina, the coloured alar cells and non porose, quadrate to elongated median lamina cells. Morphologically similar species growing in mixed cushions is not uncommon in *Dicranum*. For example, collections containing *D. scoparium* and *D. bonjeanii* have been found in locations where both species occurred also separately (own observations). Environmental conditions have a strong influence on morphological characters, especially in extreme conditions (Hedenäs et al. 2006), altering also typical characters. The distinction of closely related species is then even more difficult. Additionally, dwarf males growing on female stems (pseudomonoicy) are found both in *D. brevifolium* and *D. acutifolium* and have been seen in one *D. bardunovii* specimens (Tubanova & Ignatova 2011) a number of morphologically distinct specimens were revealed. They are similar to *D. acutifolium* (Lindb. & Arnell. Whether hybridisation, a process that affects also the morphology, occurs in mixed patches is still unknown. The present molecular data do not indicate any hybridisation for *D. acutifolium* and *D. bardunovii*, however, the absence of support for the *D. acutifolium* complex suggest that such a process might have occurred. The use of other molecular methods and more variable markers could be useful to understand the species dynamics at population level.

In line with a number of recent studies (e.g. Sukkharak et al. 2011; Carter 2012; Medina et al. 2012; Stech et al. 2013; Lang & Stech 2014) the present study displays the importance of molecular data for clarifying species circumscriptions, resolving taxonomical issues and for the re-evaluation of morphological characters in bryophytes and *Dicranum* in particular.

Chapter 4

DNA barcoding of Arctic bryophytes – an example from the moss genus *Dicranum* (Dicranaceae, Bryophyta)

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ABSTRACT

The identification of bryophytes from the Arctic is often difficult due to deviating morphologies under the extreme environmental conditions. This is especially true for species-rich and taxonomically complex genera, such as the moss genus *Dicranum*. DNA barcoding is expected to improve the identification of Arctic bryophyte species, but the optimal combination of barcoding markers for mosses in general, especially for delimiting closely related species, is still under discussion. In this paper, we test the discrimination capacity of six potential barcode markers (*rps4-trnT*_{UGU}, *trnL*_{UAA}-*trnF*_{GAA}, *trnH*_{GUG}-*psbA*, *rps19-rpl2*, *rpoB*, nrITS1-5.8S-ITS2) based on phylogenetic reconstructions of 30 *Dicranum* samples from Spitsbergen (Svalbard, Norway) and reference samples from all ten *Dicranum* species confirmed for the Svalbard archipelago and six additional Arctic *Dicranum* species. All 16 species (possibly except *D. fuscescens*), were distinguishable with bootstrap support >70% based on the combined sequence data, but none of the individual markers could delimit all included species. All Svalbard collections could be readily assigned to five species, *D. acutifolium*, *D. elongatum*, *D. laevidens*, *D. majus*, and *D. spadiceum*, respectively. It is concluded that DNA barcoding improves species identification of Arctic *Dicranum* plants, but that a combination of several markers is necessary in order to obtain reliable identification results, with the single loci ITS1, *trnL-F* and *rps4-trnT* being the most promising regions.

INTRODUCTION

Bryophytes comprise three different phylogenetic lineages of land plants, namely liverworts, hornworts, and mosses (e.g. Qiu *et al.* 2006). Of these, liverworts and mosses play an essential role in Arctic terrestrial ecosystems and constitute a major component of different types of tundra vegetation (e.g., Callaghan *et al.* 2004; Lang *et al.* 2012; Longton 1997). The Arctic bryoflora comprises a considerable diversity of approximately 700 species (Longton 1988; Frisvoll & Elvebakk 1996; Afonina & Czernyadjeva 1995; Konstantinova & Potemkin 1997).

Bryophytes, especially mosses, have been widely employed in studies of ecosystem processes and organismal interactions in (sub-)Arctic environments (e.g., Alsos *et al.* 1998; Gordon *et al.* 2001; Gornall *et al.* 2007; Jasmin *et al.* 2008; Krab *et al.* 2008; van der Wal & Brooker 2004). The Arctic has been divided into bioclimatic regions, where the High Arctic is characterized by an open herbaceous vegetation with some dwarf-shrubs on mineral soils, while the Low Arctic generally consists of a closer herbaceous vegetation, composed of dwarf and low shrubs on peat-rich soils (Walker *et al.* 2005). Biodiversity-based investigations of Arctic ecosystem processes, however, are still severely hampered by insufficient knowledge of bryophyte taxonomy and by the ability of species recognition based on morphological characters. In response to the extreme environmental conditions bryophytes display unusual growth forms and deviant gametophytic characters in the (High) Arctic. This plasticity makes morphological identification to species level difficult or even impossible (e.g., Buryová & Shaw 2005; Frisvoll & Elvebakk 1996; Hesse *et al.* 2012). This is especially true for species-rich and taxonomically complex genera such as *Bryum* Hedw., *Dicranum* Hedw. and *Schistidium* Brid. (e.g., Steere 1978; Hesse *et al.* 2012). Consequently, ecological studies have largely been limited to a few easily distinguishable species or genus-level identifications (e.g., Okitsu *et al.* 1998), or treated bryophytes as a single category without distinction of species (e.g., van der Wal *et al.* 2001). Sometimes bryophytes are even grouped with lichens and fungi as the outdated group of 'cryptogams' (e.g., Hudson & Henry 2010; Wahren *et al.* 2005; Epstein *et al.* 2004). The development of new identification tools to treat bryophytes in a more comprehensive way would surely increase the significance of ecological studies in the (High) Arctic, especially with respect to the potential of bryophytes for investigating the impact of global climate change (Tuba *et al.* 2011).

DNA barcoding is a molecular tool for species identification based on species-specific sequence differences in a short, standardized DNA region. In contrast to this original idea, however, barcoding in land plants (including bryophytes) is supposed to be based on one or two core markers plus additional information from other DNA regions where necessary (e.g., Hollingsworth *et al.* 2009, 2011). In bryophytes, especially mosses, the plastid markers recently proposed for barcoding of land plants (CBOL Plant Working Group 2009; Kress *et al.* 2005) either tend to be short (*psbA-trnH* spacer; Stech & Frey 2008; Stech & Quandt 2010), have different discrimination capacity at the species level (*rbcl*; Liu *et al.* 2010; Stech & Quandt 2010), or need more study concerning primer design and amplification strategy (*trnK/matK*, e.g., Bell *et al.* 2012). Although the optimal combination of barcoding markers for bryophytes is still under discussion (e.g., Liu *et al.* 2010; 2011; Bell *et al.* 2012; Hassel *et al.* 2013; Stech *et al.* 2013), several other molecular markers have already shown to be useful for inferring species delimitations and identifying species in bryophytes (e.g., Bell *et al.* 2012; von Cräutlein *et al.* 2011; Draper & Hedenäs 2009; Stech *et al.* 2011, 2013).

Dicranum (Dicranaceae) is a large genus belonging to the second largest subclass of mosses, Dicranidae. It comprises ca. 90 species essentially found in the Holarctic (Crosby *et al.* 1999; Ireland 2007), including about 30 species in the boreo-arctic region, of which ten species were accepted for Svalbard (Frisvoll & Elvebakk 1996). Several *Dicranum* species show a high morphological variability (Hedenäs & Bisang 2004; Hedenäs *et al.* 2006; Smith 2004; Lang & Stech 2014), which renders their identification challenging, in particular in plants (Hedenäs *et al.* 2006; pers. obs.). Previous phylogenetic studies revealed low sequence divergence in commonly employed plastid markers (La Farge *et al.* 2002; Stech *et al.* 2006). The nuclear ribosomal ITS region allowed

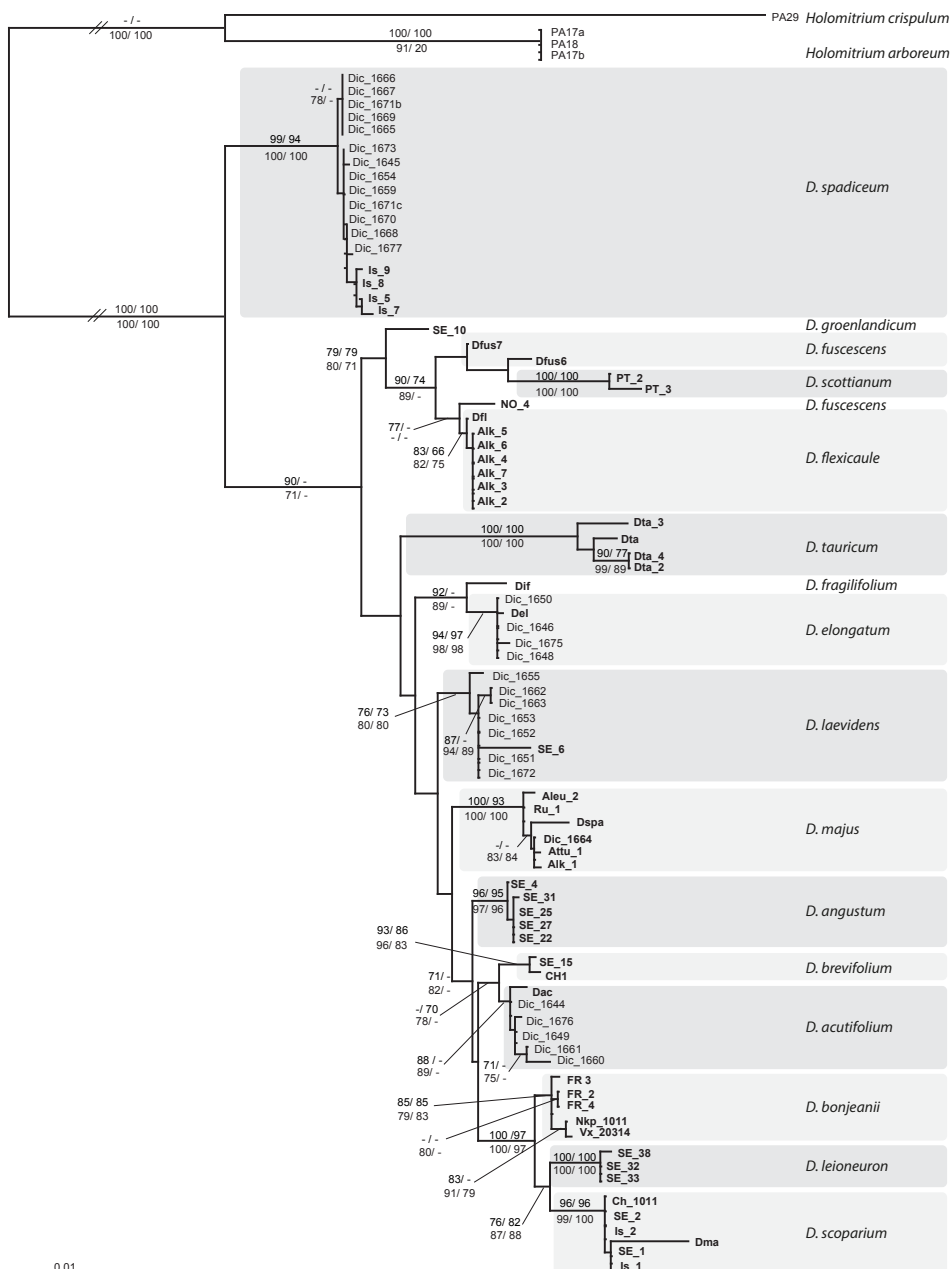


FIG. 1. Single optimal maximum likelihood phylogenetic reconstruction inferred from combined chloroplast and nrITS sequence data of *Dicanum* species, including indels coded by simple indel coding (SIC). Four specimens of *Holomitrium* were used as outgroup representatives and names in bold represent the reference samples. Numbers above branches indicate MP bootstrap support and numbers below the branch indicate ML bootstrap support, with and without indels. Branch lengths are to scale except those indicated by the symbol "//" (shortened 2 times).

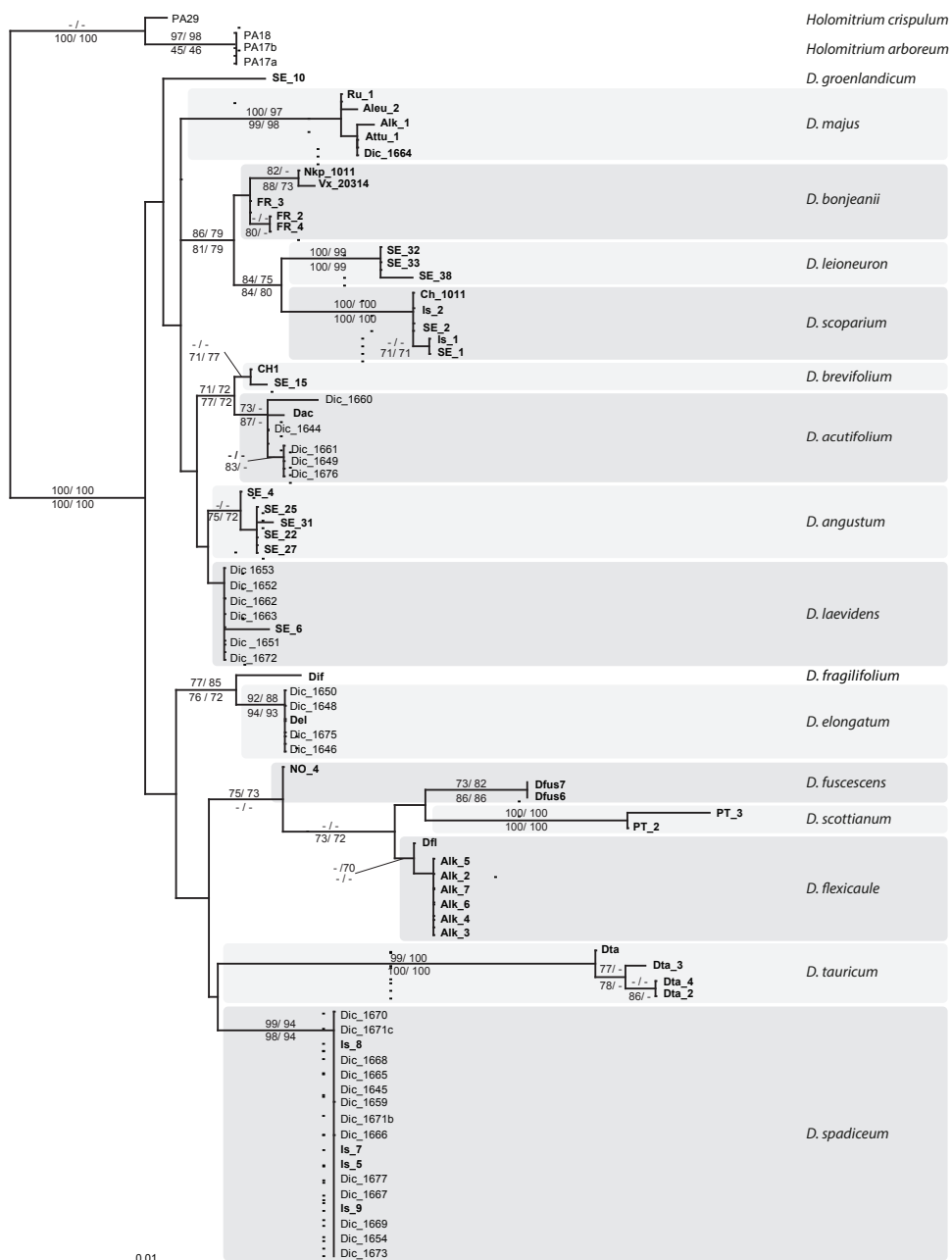


FIG. 2. Single optimal maximum likelihood phylogenetic reconstruction inferred from chloroplast sequence data of *Dicranum* species, including indels coded by simple indel coding (SIC). Four specimens of *Holomitrium* were used as outgroup representatives and names in bold represent the reference samples. Numbers above branches indicate MP bootstrap support and numbers below the branch indicate ML bootstrap support, with and without indels.



FIG. 3. Single optimal maximum likelihood phylogenetic reconstruction inferred from ITS sequence data of *Dicanum* species, including indels coded by simple indel coding (SIC). Four specimens of *Holomitrium* were used as outgroup representatives and names in bold represent the reference samples. Numbers above branches indicate MP bootstrap support and numbers below the branch indicate ML bootstrap support, with and without indels. Branch lengths are to scale except those indicated by the symbol "//" (shortened 2 times).

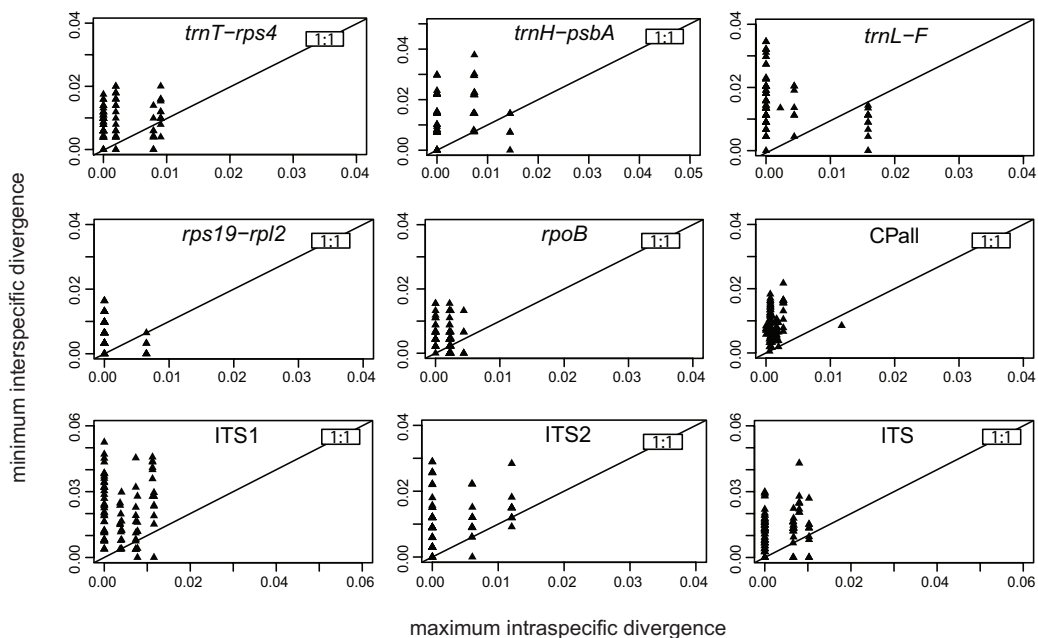


FIG. 4. Comparison of maximum intraspecific versus minimum interspecific divergence distances for *Dicranum* species pairs with more than one specimen sequenced. Genetic distances have been calculated using a K2P model of sequence evolution for *trnT-rps4*, *trnL-F*, *trnH-psbA*, *rps19-rpl2*, *rpoB*, all chloroplast markers combined (CPall), ITS and its partitions ITS1 and ITS2.

species identification in a study of Russian *Dicranum* species albeit with low support (Tubanova & Ignatova 2011; Tubanova *et al.* 2010). Most recently however, Lang & Stech (2014) showed that closely related species of the *Dicranum scoparium* Hedw. species complex were best resolved by combining five plastid regions and the nuclear ribosomal ITS region.

In the present study we aim to test how well *Dicranum* collections from the High Arctic archipelago of Svalbard can be identified to species level based on a DNA barcoding approach. Inferences are based on molecular phylogenetic reconstructions using chloroplast (*rpoB*, *trnH-psbA*, *trnL-trnF*, *rps4-trnT*, *rps19-rpl2*) and nuclear ribosomal ITS sequences from Svalbard and reference collections. The reference sequences were generated from well-identified, morphologically typical specimens from temperate to boreal regions. Furthermore, we examine the species discrimination efficacy of the six markers individually based on phylogenetic inference and comparison of maximum intraspecific versus minimum interspecific genetic distances.

MATERIALS AND METHODS

Sampling— According to the most recent checklist of the bryophytes of Svalbard (Frisvoll & Elvebakk 1996), 17 *Dicranum* species have been reported for the archipelago, of which ten were accepted in the checklist (*Dicranum acutifolium* (Lindb. & Arn.) C.Jens., *D. angustum* Lindb., *D. elongatum* Schleich. ex Schwägr., *D. flexicaule* Brid., *D. fuscescens* Sm., *D. laevidens* R.S. Williams, *D. majus* Sm., *D. scoparium* Hedw., *D. spadiceum* J.E. Zetterst., and *D. tauricum* Sapjegin), and seven were excluded due to erroneous identification (*D. bonjeanii* De Not., *D. brevifolium* (Lindb.) Lindb.,

D. fragilifolium Lindb., *D. groenlandicum* Brid., *D. leioneuron* Kindb., *D. muehlenbeckii* Bruch & Schimp., and *D. scottianum* Turner ex Scott). DNA sequences of 52 reference specimens comprising 16 out of the 17 species were compiled from earlier studies (Lang & Naciri 2010; Stech 1999; Lang & Stech 2014) or newly generated for this study (Appendix 1): *Dicranum acutifolium* (1 sample), *D. angustum* (5), *D. brevifolium* (2), *D. bonjeanii* (5), *D. elongatum* (1), *D. flexicaule* (7), *D. fragilifolium* (1), *D. fuscescens* (3), *D. groenlandicum* (1), *D. laevidens* (1), *D. leioneuron* (3), *D. majus* (6), *D. scoparium* (6), *D. spadiceum* (4), *D. scottianum* (2) and *D. tauricum* (4). The reference specimens originated from temperate to boreal regions, generally displayed morphologies typical for the respective species, and were mostly identified, or their identifications checked, by the authors or L. Hedenäs (Stockholm). *Dicranum muehlenbeckii*, however, could not be included in the analysis because of unsuccessful DNA amplification. Thirty *Dicranum* specimens from Svalbard (including one specimen already included in Lang & Stech, 2014) collected by the second and third author in Adventdalen (Longyearbyen area), Colesbukta, and Kongsfjorden (Ny-Ålesund area) in 2008–2010 were analysed (DNA numbers Dic_1644–Dic_1646, Dic_1648–Dic_1655, Dic_1659–Dic_1673, Dic_1675–Dic_1677). The collection strategy was to collect as many morphotypes as possible from the sampled areas. Four samples of *Holomitrium* Brid., one of *H. crispulum* Mart. and three of *H. arboreum* Mitt., were chosen as outgroup representatives according to earlier molecular phylogenetic reconstructions (La Farge et al. 2002; Stech et al. 2006). Voucher information and GenBank accession numbers of the DNA sequences generated from the 30 specimens are listed in Appendix 1. The nomenclature of *Dicranum* used in this study follows Hedenäs & Bisang (2004), which corresponds to the accepted species in Tropicos (Tropicos.org) and The Plant List (2013), except three species considered as synonyms in the latter two databases (*D. angustum* and *D. laevidens* as synonyms of *D. spadiceum* and *D. flexicaule* as synonym of *D. fuscescens*).

Molecular marker selection—For this study, we sequenced five chloroplast regions, i.e., partial *rpoB* gene, *trnH*_{GUG}-*psbA* and *rps19-rpl2* intergenic spacers, and two parts of the *trnS-F* region, namely *rps4-trnT*_{UGU} spacer and *trnL-F* (*trnL*_{UAA} intron and *trnL*_{UAA}-*trnF*_{GAA} spacer), and the nuclear ribosomal nrITS1–5.8S–ITS2 region. Amplification and sequencing success as well as haplotype diversity of the chloroplast markers were inferred for 54 *Dicranum scoparium* specimens by Lang & Naciri (2010). Subsequently these markers provided, together with nrITS sequences, valuable results in delimiting species of the *Dicranum scoparium* complex, based on a sampling of 111 *Dicranum* specimens (Lang & Stech 2014).

DNA extraction, amplification and sequencing—DNA was extracted from the dried leaves of a single plant using the NucleoSpin® Extract II Kit (Macherey-Nagel, Düren, Germany). Polymerase chain reaction (PCR) was performed following Lang & Stech (2014). 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 v6.1.6 (Biomatters 2010) using 65% similarity matrix costs, and manually adjusted. Gaps were treated as missing data or coded as informative by a simple indel coding strategy (SIC) (Simmons & Ochoterena 2000) as implemented in SeqState (Müller 2004). Short hairpin-associated inversions in the *trnH-psbA* spacer were positionally separated in the alignment and not coded as indels.

TABLE 1. Alignment length (Length), number of constant characters (Constant), variable characters (Variable), parsimony-informative characters (Parsi-info) and percentage of parsimony-informative characters (% parsi-info) for nucleotide and indel matrices. Values were calculated from alignments of each marker with outgroup and ingroup only (Ingr).

	<i>trnT-rps4</i>		<i>trnL-trnF</i>		<i>psbA-trnH</i>		<i>rps19-rpl2</i>		<i>rpoB</i>		ITS 1		ITS 2		ITS	
	ingroup		ingroup		ingroup		ingroup		ingroup		ingroup		ingroup		ingroup	
Species	15		16		16		14		16		16		16		16	
Length	522	471	471	471	149	149	310	310	457	457	360	304	427	371	966	854
Constant	485	498	432	436	136	138	292	299	441	443	287	274	369	348	821	798
Variable char.	37	24	39	35	13	11	18	11	16	14	73	30	67	23	145	56
Parsi-info	33	20	35	29	9	7	11	9	12	1	49	20	42	19	96	42
% parsi-info	6	4	7	6	6	5	4	3	3	2	14	7	10	5	10	5
#indels	3	3	6	6	5	5	1	1	0	0	64	34	59	32	123	66
Parsi-info	3	3	4	5	3	3	1	1	0	0	49	28	37	20	87	53
Total parsi-info	36	23	39	34	12	10	12	10	12	10	98	48	79	39	183	95

Numbers of constant, variable and parsimony-informative sites were calculated for each locus using PAUP* v4.0b10 (Swofford 2002).

Phylogenetic analyses using Maximum Parsimony (MP) optimality criterion and maximum likelihood (ML) were performed on every marker separately (including separate analyses of ITS1 and ITS2) and the combined markers (total evidence trees *sensu* Kluge 1989), both with and without indels included. Before combining markers, we tested for incongruence by visual inspection of the separate trees and by applying an incongruence length difference test (ILD, Farris *et al.* 1994) as implemented in PAUP* with 100 replicates.

MP analyses were performed using PAUP*. Heuristic searches were performed with 100 replicates using random sequence addition, one tree held at each step and tree bisection-reconnection (TBR) branch swapping, saving up to 10,000 trees. ML analyses were carried out with RAxML v. 7.3.0 (Stamatakis *et al.* 2006) employing the raxmlGUI v.0.93 interface (Silvestro & Michalak 2012). The default GTR+ Γ model was chosen for all markers. Bootstrap analyses under ML were done using the thorough bootstrap heuristics algorithm with 1,000 replicates.

Pairwise nucleotide distances between all sequences were calculated in PAUP* under the Kimura 2-parameter (K2P) model for the combined chloroplast dataset, ITS and all partitions. Maximum intraspecific distances were plotted against minimum interspecific distances for all possible species pairs with more than one specimen sequenced to infer the presence of a barcoding gap (cf. Stech *et al.* 2013).

RESULTS

Lengths of the sequenced chloroplast markers within *Dicranum* and *Holomitrium* ranged from 509-521 nucleotides for *rps4-trnT*, 449-471 nt for *trnL-F*, 136-140 nt for *trnH-*

psbA, 309-310 nt for *rps19-rpl2* and 457 nt for *rpoB*. The total plastid alignment comprised 1909 positions, of which 123 were variable, and 100 of the variable positions were parsimony-informative. Simple indel coding yielded a total of 15 additional characters, of which 11 were parsimony-informative. Hence, a total of 111 parsimony-informative characters resulted from the plastid markers. Sequences length of nrITS1-5.8S-ITS2 ranged from 747-900 nt (747-839 within *Dicranum*). The alignment comprised 1086 positions, of which 120 were removed from further calculations due to ambiguous alignment. Of the 966 remaining positions, 145 were variable and 96 of the variable positions were parsimony-informative. Simple indel coding yielded 123 characters of which 87 were parsimony informative. In total, 183 parsimony-informative characters resulted from nrITS. Respective numbers of parsimony-informative characters per plastid marker and for ITS1 and ITS2 separately are summarized in Table 1. The partitions with the most parsimony-informative characters were ITS1 (13.61%) and ITS2 (9.84%), followed by the chloroplast markers *trnL-F* (7.43%), *rps4-trnT* (6.32%), *trnH-psbA* (6.04%), *rps19-rpl2* (3.55%), and partial *rpoB* gene (2.63%).

Maximum parsimony analyses with or without indels included resulted in most parsimonious phylogenetic reconstructions with similar consistency indices (combined chloroplast: CI 0.7306 versus 0.7159, ITS: CI 0.7939 versus 0.8394), indicating only a slightly higher amount of homoplasy in the indel characters in ITS. Both visual inspections of plastid versus ITS tree topologies and the ILD test ($p = 0.29$) indicated that the two datasets were congruent and could be combined.

The single optimal ML tree of the combined analysis of all markers including indels is shown in Fig. 1 (lnL = -7188.5806). The optimal ML trees calculated from the combined chloroplast markers versus ITS (lnL = -3728.5203 and lnL = -3159.2803, respectively) are shown in Figs. 2 and 3, with bootstrap support values (BS) from maximum parsimony and maximum likelihood analyses. Separate clades of all 16 included *Dicranum* species were resolved in the combined tree (Fig. 1) and the plastid marker tree (Fig. 2) according to the positions of the reference specimens, except *Dicranum fuscescens* that was resolved as paraphyletic. The ITS tree (Fig. 3) was less resolved and most clades were weakly supported. The *D. leioneuron*, *D. majus*, *D. scottianum* and *D. spadiceum* clades received bootstrap support of $\geq 70\%$ in all three phylogenetic inferences. The *D. acutifolium*, *D. elongatum*, *D. scoparium*, and *D. tauricum* clades yielded high support in the chloroplast and combined analyses, whereas the *D. angustum*, *D. bonjeanii* and *D. brevifolium* clades were supported ($\geq 80\%$ BS) in ITS and the combined tree. The *D. flexicaule* and *D. laevidens* clades received high bootstrap support ($\geq 78\%$) only in the combined analysis (Table 2). High support in the combined trees was furthermore obtained for the sister group relationships of *D. elongatum* + *D. fragilifolium*, *D. flexicaule* + *D. fuscescens* + *D. scottianum*, as well as *D. bonjeanii* + *D. scoparium* + *D. leioneuron* of the *D. scoparium* complex. The Svalbard specimens clustered in five clades, namely the *D. acutifolium* (5 specimens), *D. elongatum* (4), *D. laevidens* (7), *D. majus* (1) and *D. spadiceum* (13) clades (Figs. 1-3).

Clades with more than one sequence were generally weakly supported in each of the seven single partitions (Table 2). Moreover, only six clades were recovered in at least four partitions, namely the *D. leioneuron*, *D. majus*, *D. scoparium*, *D. scottianum*, *D. spadiceum* and *D. tauricum* clades. ITS1 recovered the most clades with statistical support $\geq 70\%$ BS, whereas *trnH-psbA* recovered only the *D. elongatum* clade with strong bootstrap support (84/ 86%).

Genetic distances were generally small in all markers. The ranges of intraspecific versus interspecific pairwise genetic distances overlapped for all markers, except in the combined chloroplast dataset

TABLE 2. Bootstrap values of MP and ML analysis including indels, for clades with more than one sequence. Values for each single markers as well as for the concatenated chloroplast markers (CPall), nrITS1-5.8S-ITS2 (ITS) and the combined sequence data (combined) are shown. Values $\geq 70\%$ BS are in bold. Dashes denote clades that were absent in the respective phylogenetic reconstruction.

species	<i>trnT-rps4</i>	<i>trnL-trnF</i>	<i>psbA-trnH</i>	<i>rps19-rpl2</i>	<i>rpoB</i>	ITS 1	ITS 2	CPall	ITS1	combined
<i>Dicranum acutifolium</i>	≤ 50	85/91						73/87	≤ 50	88/89
<i>Dicranum angustum</i>		83/85				72/82		67/75	81/88	96/97
<i>Dicranum borjaneii</i>	66/66	≤ 50	≤ 50			87/94		52/53	89/90	85/79
<i>Dicranum brevifolium</i>		≤ 50		≤ 50		80/72	76/64	55/71	92/92	93/96
<i>Dicranum elongatum</i>	≤ 50					59/61		92/94	62/64	94/98
<i>Dicranum fragilifolium</i> + <i>D. elongatum</i>	52/49				62/71	71/65	≤ 50	77/76	60/62	92/89
<i>Dicranum fuscescens</i>	81/86									
<i>Dicranum flexicaule</i>	≤ 50	56/53				75/93	≤ 50	64/63		83/81
<i>Dicranum scottianum</i>	86/85	100/100	-/66		60/62	84/92	52/56	100/100	86/90	100/100
<i>Dicranum fuscescens</i> + <i>Dicranum flexicaule</i>	≤ 50		-/54		61/64		56/60			
<i>Dicranum fuscescens</i> + <i>Dicranum flexicaule</i> + <i>Dicranum scottianum</i>	91/95	85/86						75/41	71/66	90/89
<i>Dicranum laevigens</i>		≤ 50		70/63	61/71	68/-		≤ 50		78/80
<i>Dicranum leioneuron</i>	57/77	53/74	-/53	≤ 50		55/73	62/62	100/100	83/90	100/100
<i>Dicranum majus</i>	59/87	89/94	51/65	≤ 50	≤ 50	77/60	72/67	100/99	77/81	100/100
<i>Dicranum scoparium</i>	62/70	91/88	≤ 50	82/85	65/63	60/70		100/100	≤ 50	96/99
<i>Dicranum spodiaceum</i>	-/64	64/87	56/65	≤ 50	52/58	100/81	100/97	99/98	99/92	99/100
<i>Dicranum tauricum</i>	100/99	99/99	≤ 50	92/100		82/78		99/100	69/61	100/100

(Table 3). Tables of all nucleotide distances measured are available on request. Furthermore, the comparison of maximum intraspecific versus minimum interspecific genetic distances (Fig. 4) showed greater intraspecific than interspecific distances for a number of pairwise comparisons in every partition (data points below the 1:1 line). Therefore, no clear barcode gap was obtained for all pairwise comparisons, i.e. none of the markers was powerful enough to discriminate all studied species.

DISCUSSION

DNA barcoding in *Dicranum* and implications for mosses in general— All *Dicranum* species included in this study except *D. fuscescens* were distinguishable based on the combined sequence data of five chloroplast markers and nrITS, with bootstrap support >70% for all clades of species represented by more than one sample (Fig. 1, Table 2). However, an increased sampling of the species represented in this study by one or few specimens would be necessary to confirm their monophyly and infer intra- versus interspecific sequence variation with more confidence. The present results support our earlier study focusing on closely related species within the *D. scoparium* complex (Lang & Stech 2014), namely the close relationship between *D. bonjeanii* and *D. scoparium* and the separation of *D. majus* from the *D. scoparium* complex. In addition, *D. leioneuron*, which was not included in Lang & Stech (2014), is resolved as a member of the *D. scoparium* complex here. As the molecular clades of these species coincide with the morphological species circumscriptions, we conclude that the sequenced entities are in fact separate species. *Dicranum flexicaule* is morphologically very similar to *D. fuscescens* and frequently regarded as a variety or a form of the latter (Ireland 2007; Mönkemeyer 1927; Podpěra 1954; Savicz-Lyubitskaya & Smirnova 1970; The Plant List 2013). However, several other authors accept *D. flexicaule* as a separate yet doubtful species (Bellolio-Trucco & Ireland 1990; Hedenäs & Bisang 2004). The present molecular data support a close relationship of *D. flexicaule* with *D. fuscescens*, and in addition *D. scottianum*, but analyses of a larger number of specimens or additional markers are necessary to resolve their relationships with confidence and conclude about the taxonomic status of *D. flexicaule*.

Each individual locus provided insufficient variability to distinguish all sequenced species (Table 2, Fig. 4). The ITS region as well as the chloroplast markers *trnL-F* and *rps4-trnT* showed the highest species discrimination capacity in terms of statistical support, in accordance with previous studies (e.g., La Farge *et al.* 2002; Hernández-Maqueda *et al.* 2008; Hollingsworth *et al.* 2009). However, the *D. elongatum* clade was only supported by *psbA-trnH* and none of the markers delimited *D. laevidens* (Table 2). The *psbA-trnH* spacer possessed a relatively high proportion of parsimony-informative characters in *Dicranum*, as in other moss species (Liu *et al.* 2010; Hassel *et al.* 2013), although the resulting tree was still poorly resolved (cf. Table 2). Neither the combined chloroplast loci nor the generally variable ITS region could discriminate all included *Dicranum* species with confidence. A recent study of the *Racomitrium canescens* (Hedw.) Brid. complex (Stech *et al.* 2013), another representative of subclass Dicranidae, showed that nrITS performed better in terms of species discrimination capacity than chloroplast data. In *Dicranum*, the concatenated chloroplast markers provided clades with generally better support, which may be due to the larger number of chloroplast markers included here than in the study by Stech *et al.* (2013), which employed solely the *rps4-trnT-trnL* region. Few studies have so far compared the performance of ITS1 vs ITS2 alone as barcoding markers for bryophytes. ITS2 was considered as universal barcode marker for plants and animals because of conserved regions in the adjacent genes, suitable for

TABLE 3 Pairwise Kimura 2-parameter (K2P) distances for ITS, the combined chloroplast markers and different partitions. The upper two rows indicate the ranges of intraspecific and interspecific distances for all *Dicranum* species. The last row indicates the overlap between the maximum intraspecific and minimum interspecific distances.

	trnT-rps4	trnL-trnF	psbA-trnH	rps19-rpl2	rpoB	ITS 1	ITS 2	ITS	CPall	Combi
intra- specific	0-0.0091	0-0.0159	0-0.0144	0-0.0065	0-0.0045	0-0.0116	0-0.0121	0-0.0103	0-0.0118	0-0.0089
inter- specific	0-0.0279	0-0.0391	0-0.0467	0-0.0165	0-0.0177	0-0.0638	0-0.0316	0-0.0627	0.0005-0.0296	0.0015-0.0346
overlap	0.0091	0.0159	0.0144	0.0065	0.0044	0.0116	0.0121	0.0103	0.0112	0.0073

primer design. Additionally, ITS2 had sufficient variability for identification of closely related species (Yao *et al.* 2010) and has proven to be conclusive for some bryophyte taxa, among them also Arctic species (Hassel *et al.* 2013). In contrast, other studies reported higher variation and species discrimination capacity of ITS1 in bryophytes (Liu *et al.* 2010; Stech *et al.* 2013), which was also the case in *Dicranum* (Table 2). As suggested by Stech *et al.* (2013), further analyses would be necessary in order to infer which part of the ITS region performs best as DNA barcode in bryophytes.

The present study is another example that a combination of several markers may be necessary to identify moss species with confidence based on molecular data. While one marker was sufficient to discriminate two closely related *Orthodontium* species (Rowntree *et al.* 2010), most complexes of closely related species needed several markers to be discriminated at species level (e.g., Carter 2012; Draper & Hedenäs 2009; Medina *et al.* 2012). However, finding the optimal combination of barcoding markers capable of delimiting closely related species is still a major concern in bryophytes and no consensus has been reached yet (Hollingsworth *et al.* 2011; Liu *et al.* 2010; Stech & Quandt 2010). The combination of ITS1 and/or ITS2, *rps4-trnT-trnL*, and *psbA-trnH* seems to be suitable for moss genera with generally low sequence divergence such as *Dicranum* (compared, for example, with its southern Hemisphere sister genus *Dicranoloma* (Renauld) Renauld; Stech *et al.* 2006). Additional markers such as *rps19-rpl2* may be required for certain species. The *rpoB* gene, in contrast, does not provide any additional resolution.

Molecular versus morphological identification of *Dicranum* specimens from Svalbard—

According to the molecular data, the 30 sequenced *Dicranum* specimens from Svalbard belong to five species, *D. acutifolium*, *D. elongatum*, *D. laevidens*, *D. majus*, and *D. spadiceum*. All of them are among the ten species accepted for the archipelago in the checklist by Frisvoll and Elvebakk (1996). The sequenced specimens represent most of the morphological variation of *Dicranum* in the respective sampled habitats and areas on Svalbard, and consequently, most, if not all, *Dicranum* species occurring there. Nonetheless, molecular analysis of an extended sampling across Svalbard would be necessary to assess whether the five other species accepted in the checklist are actually occurring on the archipelago or not, and to confirm the absence of the seven rejected species. Such an extended sampling would require extensive additional fieldwork or PCR amplification and sequencing of older herbarium material. The latter, however, seems to be difficult according to preliminary analyses (unpublished results) of collections from Edgeøya, eastern Svalbard, dating from the 1980s (cf. Hesse *et al.* 2012).

Dicranum species occurring in the Arctic are difficult to identify morphologically, especially in the field, but also microscopically. In few species the diagnostic characters seem to be stable, such as the strong costa and strongly incrassate, short and smooth upper lamina cells of *D. elongatum*, which could be recognized relatively easily. Most species present more variability in their diagnostic characters and are thus more often misidentified. For example, in temperate habitats, *Dicranum majus* is characterized by strongly falcate leaves, prosenchymatous and porose upper lamina cells, furrows on the costa and a double row of guide cells in the lower leaf. These typical characters are much less distinct in High Arctic specimens (Hedenäs *et al.* 2006). High Arctic specimens can therefore readily be mistaken for other species such as *D. scoparium* or *D. spadiceum* (Hedenäs & Bisang 2004; pers. obs.). The most difficult group of species to identify is comprised of *D. angustum*, *D. groenlandicum*, *D. laevidens*, and *D. spadiceum*. *Dicranum spadiceum* has long and narrow leaves ending in a tubular apex. Its leaf margins are slightly denticulate near the apex and the lamina cells are thin-walled and slightly porose. The basal cells are elongate, gradually becoming shorter and irregular, and lack pores, while the typical parenchymatous cells are sometimes restricted to the tip of the lamina. While *D. angustum* and *D. laevidens* are considered synonyms of *D. spadiceum* in The Plant List (2013), the former is distinguished by long, narrow, tubular and acuminate leaves as well as thin-walled and non-porose lamina cells. *Dicranum laevidens* is distinguished by an entire leaf margin as well as incrassate and porose, prosenchymatous lamina cells (Hedenäs & Bisang 2004). According to the molecular data, *D. angustum* and *D. laevidens* are clearly separated from *D. spadiceum* (Figs. 1-3), supporting their status as separate species. All respective Svalbard specimens belong to either *D. laevidens* or *D. spadiceum*, which corresponds well with the conclusions of Frisvoll and Elvebakk (1996) based on morphology that true *D. angustum* may be rare on Svalbard and further study is necessary to delimit *D. angustum* from *D. laevidens* or *D. spadiceum*. Again, additional fieldwork or molecular analysis of (old) herbarium specimens possibly representing *D. angustum* would be necessary. Species boundaries of *D. laevidens* and *D. groenlandicum* remained unclear because of their strong morphological similarities (Bellolio-Trucco & Ireland 1990; Hedenäs & Bisang 2004; Nyholm 1987; Steere 1978; Tuomikoski *et al.* 1973). In the absence of sporophytes, the distinction between the two species is essentially based on the different growth form, as *D. groenlandicum* grows in very dense and *D. laevidens* in looser tufts. Both the reference specimen of *D. groenlandicum* and one Svalbard specimen (Dic_1651) formed dense cushions, but the latter belonged to *D. laevidens* according to the molecular data, whereas the reference specimen was clearly separated molecularly (Figs. 1-3). This is a first indication that the habit may not always be reliable for identifying *D. groenlandicum*, and that in case no other gametophytic diagnostic characters can be found, sterile plants of *D. groenlandicum* can best be identified by DNA barcoding. Examination of supplementary material would be necessary to confirm this result.

Correct species identification is important in various fields of biodiversity assessments, ecology and conservation (Cornelissen *et al.* 2007; Dinnage *et al.* 2012; Steele & Pires 2011; Winter *et al.* 2012). Morphological identification of Arctic mosses requires taxonomic expertise and a combination of several stable characters. However, gametophytic characters often show deviating morphologies (Bellolio-Trucco & Ireland 1990; Hedenäs & Bisang 2004; Hesse *et al.* 2012). In *Dicranum* and many other genera, sporophytic characters are useful to distinguish gametophytically similar species, e.g., *D. groenlandicum* vs. *D. laevidens* and *D. fuscescens* vs. *D.*

flexicaule. Yet, sporophytes are rarely present in Arctic material. Therefore, species identification of Arctic plants of *Dicranum*, as well as of other complex moss species and genera, could greatly benefit from DNA barcoding. In *Dicranum*, identification can be best achieved using a combination of nuclear and plastid DNA sequences. Additional taxon sampling would, however, be necessary to better understand the relationships between morphological variability and genetic variation, solve taxonomic issues and build up a reference sequence database for molecular identification of unknown specimens by local BLAST searches in addition to molecular phylogenetic approaches.

Chapter 5

Phylogeny and species delimitations in European *Dicranum* (Dicranaceae, Bryophyta) inferred from nuclear and plastid DNA.

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ABSTRACT

DNA sequences are increasingly used for taxonomy, inferring phylogenetic relationships and identifying species boundaries. Many specific methods to define species delimitation have appeared recently, with the generalized mixed Yule coalescent (GMYC) method being the most popular. However, only few studies on land plants have been published so far and GMYC analyses of bryophytes are largely missing. *Dicranum* is a large genus of mosses whose (morpho-) species are partly ill-defined and frequently confused. To infer molecular species delimitations, we reconstructed phylogenetic trees based on five chloroplast markers and nuclear ribosomal ITS sequences from 28 out of 30 species occurring in Europe. We further applied GMYC and PTP species delimitation methods in order to compare their discriminatory power with species boundaries inferred from the molecular phylogenetic reconstructions and with the morphological species concept. Phylogenetic circumscriptions were congruent with the morphological concept for 24 species, while three taxa were molecularly indistinguishable from other closely related species. Phylogenetic relationships between *Dicranum* species remained largely unsupported. Automated species delimitation achieved similar results but tended to overestimate the number of potential species and exposed several incongruences between the morphological concept and inference from molecular phylogenetic reconstructions. It is concluded that GMYC and PTP methods potentially provide a useful and objective way of delimiting bryophyte species, but studies on further bryophyte data sets are necessary to infer whether incongruences might ensue from evolutionary processes and to test the suitability of these approaches.

INTRODUCTION

DNA sequence data are widely used for inferring species delimitations and phylogenetic

relationships. Specific methods to analyze species boundaries based on molecular phylogenetic reconstructions, however, have appeared only recently (cf. Carstens et al. 2013 for review), with the generalized mixed Yule coalescent (GMYC) method (Fontaneto et al. 2007; Pons et al. 2006) being most popular. This method estimates the point of transition from the level of species to population evolutionary processes, i.e. it detects species boundaries based on differences in branching rates at both species and population levels. Automated species delimitation methods are therefore considered especially useful in organisms with unclear species boundaries, due to poor taxonomy knowledge and because processes such as lineage sorting and introgression can obscure the species tree signal (O'Meara 2010 and references therein). Most GMYC studies so far focused on different animal groups (e.g. Poulakakis et al. 2012; Zaldívar-Riverón et al. 2010) and very few examples of analyses of other organisms such as algae (e.g. Leliaert et al. 2009), fungi (e.g. Parnmen et al. 2012) and land plants (e.g. Hernández-León et al. 2013) have been published. GMYC analyses of plant, and especially bryophyte, species are hence still largely missing.

Bryophytes are an important component of terrestrial ecosystems and count up to 18,000 known species (Goffinet & Shaw 2009). Nevertheless, because of the limited number of morphological characters available, the morphological plasticity of species and the generally broad geographical distribution, the taxonomy of many bryophyte lineages is still ambiguous. Molecular data can facilitate the circumscription of species, especially in taxa with extreme morphological similarities (e.g. Dong et al. 2012; Hedenäs & Eldenäs 2007; Heinrichs et al. 2009; Stech et al. 2013).

Species circumscription and identification in the Holarctic moss genus *Dicranum* (Dicranaceae, Bryophyta) has been notoriously difficult. The genus counts more than 90 species (www.Tropicos.org; Frey & Stech 2009), many of which are broadly distributed and display a great range of morphological plasticity, and only few species are habitat-specific (Hedenäs & Bisang 2004). Moreover, *Dicranum* and related genera display little molecular variation, as shown in previous studies (Cox et al. 2010; La Farge et al. 2002; Stech 1999; Stech et al. 2012). Assessing species delimitations in *Dicranum* is thus challenging both at the morphological and molecular level. Our recent studies on the *Dicranum scoparium* and *D. acutifolium* species complexes (Lang & Stech 2014; Lang et al. in press) as well as on boreal-arctic *Dicranum* species (Lang et al. 2014) showed that in several cases conclusive species delimitations could only be obtained from combined analyses of several chloroplast markers and nuclear ribosomal ITS sequences.

The present study aims to elucidate species boundaries within *Dicranum* on a broader geographic scale, including 27 of the 29 *Dicranum* species occurring in Europe (Hedenäs & Bisang 2004) plus *D. septentrionale* Tubanova & Ignatova, a newly recorded species in Scandinavia (Lang et al. in press). Molecular phylogenetic reconstructions based on five chloroplast markers (*trnH*_{GUC}-*psbA*, *rps4-trnT*_{UGU} and *trnL*_{UAA}-*trnF*_{GAA} intergenic spacers, *rps19-rpl2*, *rpoB*) plus the nrITS1-5.8S-ITS2 region will be used to test, for the first time in bryophytes, the congruence of two automated species delineation approaches, the general mixed Yule-coalescent (GMYC) models and Poisson tree processes (PTP). Sequence-based species delimitations will furthermore be compared with morphologically recognized species.

MATERIAL AND METHODS

Sampling— A total of 202 *Dicranum* specimens were sampled (Appendix 1), representing 27 species of the 29 European species recognized by Hedenäs and Bisang (2004) and including the new European species record of *D. septentrionale*: six *Dicranum acutifolium* (Lindb. & Arnell)



FIG. 1. Single optimal maximum likelihood phylogenetic reconstruction of 28 European *Dicranum* species inferred from the partitioned (non-coding chloroplast, chloroplast and ITS) nucleotide matrix including indels coded by simple indel coding (SIC). Branch thickness and colours indicate bootstrap support and posterior probabilities from ML and respectively Bayesian analyses.

C.E.O. Jensen, nine *D. angustum* Lindb., six *D. bonjeanii* De Not., five *D. brevifolium* (Lindb.) Lindb., three *D. canariense* Hampe ex Müll. Hal., five *D. crassifolium* Sérgio, Ochyra & Séneca, one *D. dispersum* Engelmark, one *D. drummondii* Müll. Hal., four *D. elongatum* Schleich. ex Schwägr., three *D. flagellare* Hedw., 11 *D. flexicaule* Brid., four *D. fragilifolium* Lindb., six *D. fuscescens* Turner, two *D. groenlandicum* Brid., 11 *D. laevidens* R.S. Williams, three *D. leioneuron* Kindb., eight *D. majus* Turner, four *D. montanum* Hedw., four *D. polysetum* Sw., 65 *D. scoparium* Hedw., two *D. scottianum* Turner ex Robt. Scott, nine *D. septentrionale*, 15 *D. spadiceum* J.E. Zetterst., three *D. spurium* Hedw., four *D. tauricum* Sapjegin, four *D. undulatum* Schrad. ex Brid. and four *D. viride* (Sull. & Lesq.) Lindb. specimens. The sampling included 40 specimens newly sequenced for all six markers employed here, four specimens of which ITS sequences had already been generated by Tubanova *et al.* (2010) and Ignatova and Fedosov (2008) and 162 specimens of which chloroplast and ITS sequences were generated for previous studies (Lang & Stech 2014; Lang *et al.* 2014, *in press*; Stech 1999; Stech *et al.* 2006). As previous studies showed that *Holomitrium* is sister to *Dicranum* (La Farge *et al.* 2002; Stech *et al.* 2006), four samples, one *H. crispulum* Mart. and three *H. arboreum* Mitt., were chosen as outgroup representatives.

DNA extraction, amplification and sequencing— The greenest parts of single gametophyte stems were selected for DNA extraction and cleaned manually with demineralised water under a binocular. Total DNA extraction was carried out using the NucleoSpin® Extract II Kit (Macherey-Nagel, Düren, Germany). Six markers employed to delimit closely related *Dicranum* species in Lang and Stech (2014) and Lang *et al.* (2014 *in press*) were amplified and sequenced, i.e. five chloroplast regions (partial *rpoB* gene, *trnH*_{GUG}-*psbA*, *rps19-rpl2*, *rps4-trnT*_{UGU} and *trnL*_{UAA}-*trnF*_{GAA} intergenic spacer) and the nuclear ribosomal nrITS1-5.8S-ITS2 region. PCR amplifications were performed as described in Lang and Stech (2014). All 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* spacer, which can flip at the population level and may significantly reduce phylogenetic structure if undetected (Borsch & Quandt 2009; Quandt & Stech 2004; Whitlock *et al.* 2010), were positionally separated in the alignment and the corresponding indels were excluded.

Phylogenetic inferences were based on maximum likelihood (ML) and Bayesian inference (BI) analyses. Gaps were coded as informative by a simple indel coding strategy (SIC) (Simmons and Ochoterena 2000) implemented in SeqState (Müller 2004). To check for incongruence, phylogenetic reconstructions based on chloroplast and nuclear sequences were visually compared. In addition, an incongruence length difference test (ILD, Farrise *et al.* 1994) as implemented in PAUP* 4.0b10 (Swofford 2002) was performed with 100 replicates. As both visual inspections and the ILD test indicated that the plastid and nuclear tree topologies were congruent ($p=0.06$), the two datasets were combined.

Three nucleotide partitions were used in ML and BI, namely the non-coding chloroplast markers (*rps4-trnT*, *trnL-trnF*, *trnH-psbA*, *rps19-rpl2*), the coding chloroplast region *rpoB* and the nuclear spacer nrITS. ML analyses were carried out with RAXML v.7.2.6 (Stamatakis 2006) employing the graphical user interface raxmlGUI v.0.93 (Silvestro & Michalak 2012) with the default GTR model

of nucleotide substitution and Γ rate heterogeneity for all partitions. Bootstrap analyses under ML were done using the thorough bootstrap heuristics algorithm with 20 runs and 1000 replicates. BI analyses were run on the CIPRES science gateway (Miller *et al.* 2010). Bayesian posterior probabilities were calculated based on the Markov chain Monte Carlo (MCMC) method, using MrBayes v3.2.1 x64 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003). The a priori probabilities supplied were those specified in the default settings of the program. Best-fit models of nucleotide sequence evolution were selected according to the Akaike information criterion in MrModeltest (Posada and Crandall 1998) executed through PAUP*, namely HKY + Γ for the non-coding chloroplast partition, and HKY + I for coding and nuclear partitions. Sequence and indel data were treated as separate and unlinked partitions, employing the restriction site model ('F81') for the indel matrix as recommended by Ronquist *et al.* (2005). Two runs with four chains were run simultaneously (11×10^6 generations), 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 two runs and using the trees sampled after the chains converged. Trace plots generated in Tracer v1.5 (Rambaut & 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%.

Sequence-based species delimitation— Species boundaries were estimated using the GMYC (Fontaneto *et al.* 2007; Monaghan *et al.* 2009; Pons *et al.* 2006) and the PTP (Zhang *et al.* 2013) approaches. As GMYC requires a fully resolved topology with branch length estimates, we reconstructed an ultrametric tree with a strict molecular clock using parameters specified in BEAUti v. 2 and implemented in BEAST version 2.1.1 (Bouckaert *et al.* 2014). Branch lengths were estimated under a Yule prior with HKY nucleotide substitution model for each data partition. We included a gamma rate heterogeneity and no invariant sites for the chloroplast partition and both *rpoB* and ITS partitions included no gamma rate heterogeneity but estimated invariant sites. In the absence of fossil records, we applied a plastid substitution rate of 5.0×10^{-4} SD of 2.0–8.0 $\times 10^{-4}$ subst./site/My following Villarreal and Renner (2014) for chloroplast and *rpoB* partitions and a substitution rate of 1.35×10^{-3} subst./site/My for ITS as used in Heinrichs *et al.* (2006). The MCMC chains were run with 20×10^6 generations, saving the results every 2000th generation. The convergence of the runs was examined in Tracer v1.5. The maximum clade credibility tree was built from the combined runs after eliminating 25% of the trees for burnin in TreeAnnotator v1.7.2. The GMYC approach was carried out in R 2.15 (R Development Core Team 2013) using the splits (Ezard *et al.* 2009) and ape (Paradis *et al.* 2004) packages. The number of clusters and singletons were estimated by running both single and multiple threshold optimisations and using a multimodel Akaike information criterion with a model cutoff of $\Delta\text{AICc} = 7$ (Monaghan *et al.* 2009; Pons *et al.* 2006; Powell 2012). On the contrary to the GMYC approach, PTP neither requires an ultrametric tree nor a sequence similarity threshold as input data because speciation rate is modelled by using the number of substitutions between branching and speciation events (Zhang *et al.* 2013). We therefore used the RaxML trees as input data, with 500,000 MCMC generations, thinning set to 100 and burnin at 25%. The calculations were conducted on the bPTP webserver (<http://species.h-its.org/ptp/>).

Unbalanced sampling can affect the estimates of haplotypes and thus might overestimate the number of potential species (Bergsten *et al.* 2012; Zhang *et al.* 2013). Therefore, GMYC and PTP

analyses were additionally conducted on a reduced alignment containing only unique sequences (haplotypes). This reduced alignment, automatically obtained from the raxmlGUI interface, contained 145 sequences, with the strongest reduction in *D. scoparium* sequences retaining 18 out of the initial 65 sequences. The ultrametric and RaxML trees were reconstructed following the above-mentioned methods.

RESULTS

Phylogenetic reconstruction— The total chloroplast alignment comprised 1914 positions, of which 222 were variable, and 132 of the variable characters were parsimony-informative. Of the 1142 positions in the ITS alignment, 124 ambiguous positions were removed from the subsequent calculations. The remaining 1019 positions comprised 217 variable characters, of which 139 were parsimony-informative. Simple indel coding of the combined dataset yielded 240 additional characters (excluding three corresponding to an inversion in *psbA-trnH*), of which 148 were parsimony-informative.

The single optimal ML tree of the combined markers is shown in Fig. 1, with bootstrap support ($\geq 75\%$ BS) from likelihood analyses and posterior probabilities (PP ≥ 95) from Bayesian inference indicated on the branches. The phylogenetic reconstruction resolved 23 clades that corresponded to morphological species, including the two species with only one sample (Fig. 1). While the clades of *D. acutifolium*, *D. angustum*, *D. bonjeanii*, *D. brevifolium*, *D. flagellare*, *D. fuscescens*, *D. laevidens*, *D. majus*, *D. montanum*, *D. polysetum*, *D. scoparium* s.l. (including *D. leioneuron*, *D. cf. scoparium*, and *D. scoparium* s.s., cf. Lang & Stech 2014), *D. septentrionale*, *D. spadiceum*, *D. spurium*, *D. tauricum*, *D. undulatum* and *D. viride* were strongly supported ($\geq 81\%$ BS, PP < 0.97), *D. flexicaule* was supported only in the Bayesian reconstruction (62% BS, PP 0.99).

Dicranum groenlandicum did not form a monophyletic clade. Six species were molecularly indistinguishable from other closely related species: *D. fragilifolium* and *D. elongatum* formed a highly supported clade (95% BS, PP 0.99). While *D. crassifolium* was intermingled with *D. scoparium* s.s., *D. leioneuron* clustered with North American specimens of *D. cf. scoparium* in a highly supported clade (92% BS, PP 1). Finally, *D. scottianum* and *D. canariense* formed a highly supported clade (100% BS, PP 1). However, the samples of both *D. scottianum* and *D. canariense* clustered in supported subclade (94, 97% BS, PP 1, respectively).

Sequence-based species delimitation— The lineage through-time plot (Fig. 2b, c) indicated an exponential increase in branching rate near the tip of the tree. The single threshold GMYC model using the ultrametric phylogenetic tree created in BEAST resulted in the identification of 24 *Dicranum* clusters with high probabilities (CI= 23-26, InL of null model= 741.079, ML of GMYC model= 748.162, $p = 0.00269^{**}$) and 10 additional lineages consisting of single sequences, resulting in a total of 34 entities, excluding the outgroup (Fig. 2 a, b). The multiple threshold method gave four threshold times, resulting in a total of 58 entities that consisted of 38 clusters (CI= 30-39, InL of null model= 741.079, ML of GMYC model= 752.849, $p = 0.000634^{***}$) and 20 singletons, excluding the outgroup (Fig. 2 c; Appendix 2). Although the multiple-threshold option was statistically preferred over the single-threshold option ($\Delta AIC = 2.944$), neither model was significantly different (Chi-square= 9.375, d.f.= 9, $p = 0.40339$). An inspection of the results obtained from both analyses revealed that the multiple-threshold GMYC model considered a higher number of clusters from samples that belonged to single lineages (Fig. 2a). Therefore, we

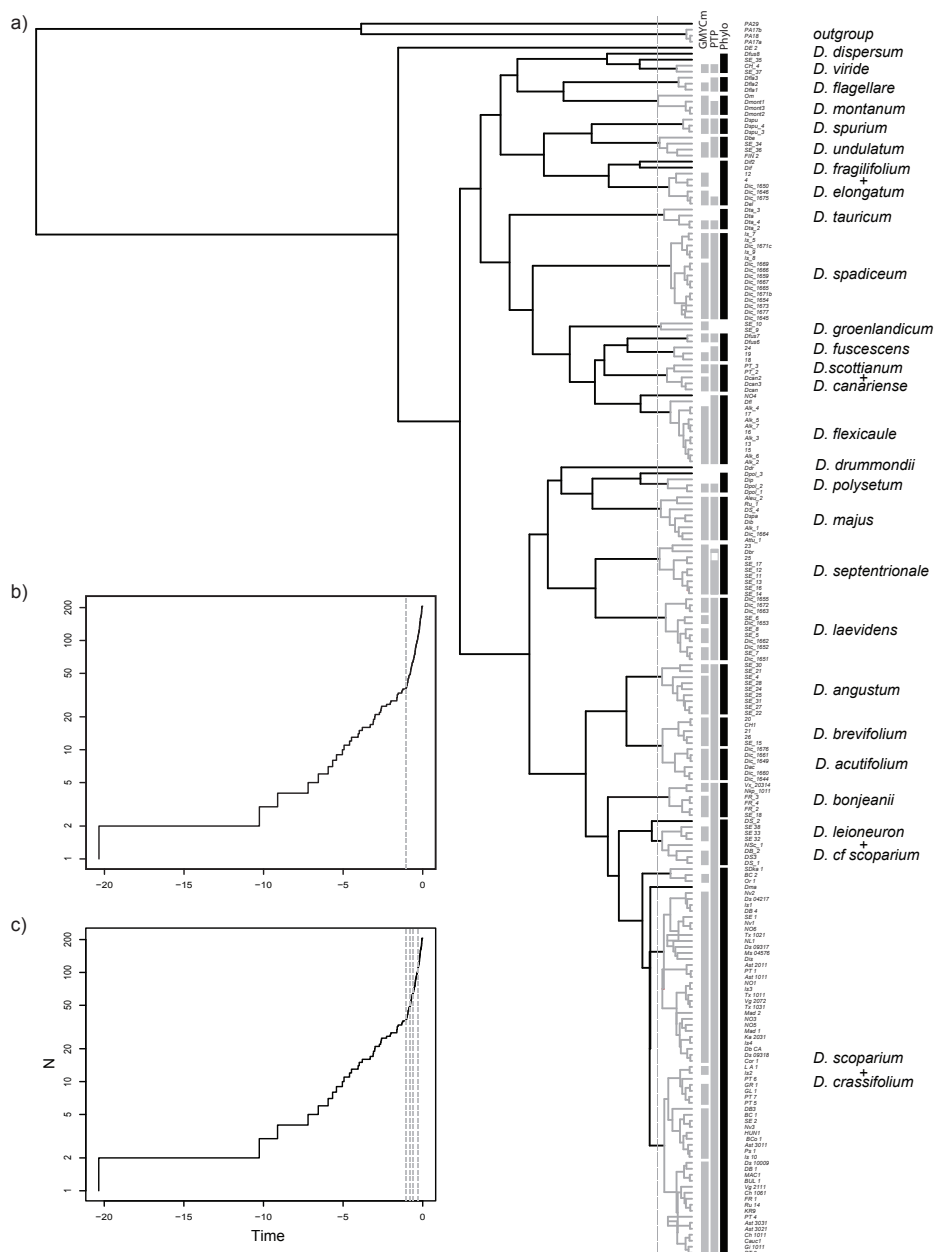


FIG. 2. Ultrametric tree depicting the relationship of *Dicranum* based on Bayesian analysis using a Yule model in BEAST and with fit of the general mixed Yules coalescent (GMYC) single threshold model from plastid and nuclear data. Branch length fitted a strict molecular clock (a). Lineages-through-time plot for single (b) and multiple (c) GMYC thresholds are illustrated. Estimated entities are indicated in grey. The vertical dashed line represent the timing of the earliest coalescent event. The two grey columns indicate the estimated entities from the multiple-threshold model (GMYCm) and the maximum likelihood solution of the Poisson tree processes (PTP), respectively. The black column indicates the phylogenetic boundaries of the *Dicranum* taxa (Phylo).

took a more conservative approach and discussed only the results of the single-threshold method.

The trees resulting from PTP gave similar results to GMYC (Fig. 2a). The number of estimated species varied between 25 and 116, excluding the outgroup (acceptance rate= 0.593), with 37 partitions supported by the ML search, excluding the outgroup (Fig. 2a, Appendix 3).

GMYC results based on the reduced alignment were similar to the results based on the extended alignment. The single-threshold model indicated the presence of 23 clusters and 31 entities while the multiple models resulted in four threshold times and resulted in 30 clusters and 48 entities, excluding the outgroup (CI= 9-40/ 38-68, lnL of null model= 259.1778/ 259.1778, ML of GMYC model= 263.0662/ 266.5076, $p=0.051/ 0.023^*$, respectively; Table 1). The number of estimated species obtained from PTP method ranged between 49-102 entities, excluding the outgroup (acceptance rate= 0.716), with 42 partitions supported by the ML search, excluding the outgroup (Appendix 4).

DISCUSSION

Phylogenetic reconstruction versus morphological species— The present study comprises the largest molecular dataset of *Dicranum* available so far, including all but two *Dicranum* species occurring in Europe following Hedenäs and Bisang (2004), plus *D. septentrionale*, recently described from Russia and newly identified in Scandinavia (Tubanov *et al.* 2010; Lang *et al.* 2014). The majority of the analysed species (23 out of 28, including two singletons), were molecularly recognisable based on the combined analysis of five chloroplast markers and nuclear ribosomal ITS sequences (Fig. 1), albeit not all with significant statistical support. The results support our recent phylogenetic studies on *Dicranum* species complexes and Arctic *Dicranum* species (Lang & Stech 2014; Lang *et al.* 2014; Lang *et al.* in press) in that a combination of molecular markers data can clarify species circumscriptions in *Dicranum*, and that the low resolution and clade support within *Dicranum* in earlier analyses (e.g. La Farge *et al.* 2002; Stech *et al.* 2006; Tubanov *et al.* 2010; Tubanov & Ignatova 2011) was a result of too few molecular markers analysed (cf. also Stech & Quandt 2010). Furthermore, the present study shows that, at least for Europe, the molecular data to a large extent support the morphological species concept, despite morphological confusions and subtle diagnostic characters in several species (e.g. Lang *et al.* in press; Tubanov *et al.* 2010).

In contrast to these results, nine species showed discrepancies between their morphological concepts and their molecular circumscription, namely *D. groenlandicum*, *D. elongatum*, *D. fragilifolium*, *D. scottianum*, *D. canariense*, *D. leioneuron* and *D. cf scoparium*. *Dicranum groenlandicum* was resolved as paraphyletic but without significant statistical support (Fig. 1). This arctic species is morphologically very similar to *D. laevidens* and, in absence of sporophytes, both species are essentially differentiated based on the growth form. However, recent molecular studies on arctic *Dicranum* suggested that both species represent two separate entities (Lang *et al.* 2014). The present phylogenetic reconstruction confirms the separation of *D. groenlandicum* from *D. laevidens* and further confirms the delineation of the latter species. Nevertheless, additional sequences of *D. groenlandicum* are necessary to infer its delimitation. *Dicranum elongatum* and *D. fragilifolium* are morphologically different and occupy different habitats (Ireland 2007). Moreover, *Dicranum elongatum* is frequently confused with *D. groenlandicum*, while *D. fragilifolium* shares morphological similarities with *D. tauricum* (Hedenäs & Bisang 2004; Ireland 2007). Despite their clear morphological distinctions, the present molecular phylogenetic reconstruction indicates that both *D. elongatum* and *D. fragilifolium* belong to the same taxon (Fig. 1). The two Macaronesian-Atlantic European species *D. canariense* and *D. scottianum* were resolved in one well-supported clade.

Because of their morphological resemblance, *D. canariense* has been considered as a subspecies or variety of *D. scottianum* (Tropicos.org). In the current concept, *D. canariense* differs from the latter by its strongly denticulate margins and thick and denticulate costa (Hedenäs & Bisang 2004). The sampling included in this study confirms their close relationship and indicates that both taxa should be distinguished at subspecies level, however a larger sampling would be necessary to confirm these results. Morphological and ecological characters of *D. leioneuron* have been discussed several times, as it is frequently confused with either *D. bonjeanii* or *D. scoparium* (Ahti & Isoviita 1962; Corley 1991). Consequently, *D. leioneuron* has been sometimes considered as an ecotype of *D. scoparium* or a variety of *D. bonjeanii* (Ahti & Isoviita 1962), a hypothesis that is rejected by the present phylogenetic reconstructions (Fig. 1), which in turn confirm the observations of Corley (1991). Despite being molecularly separated from *D. bonjeanii* and *D. scoparium* s.str., the *D. leioneuron* specimens included in this study clustered in a well-supported lineage together with North American samples, named as *D. cf. scoparium* in Lang & Stech (2014). Morphology and habitat of these two groups are, however, clearly different: the North American specimens have falcate-second leaves that are serrate on the margins and a lamellate costa. The *D. leioneuron* specimens, on the other hand, have all the characteristics of this species, i.e. small and erect-patent leaves; very thin nerve and without dorsal lamellae. Additionally, flagellary shoots are common in this species. Although the present data does not indicate any hybridization processes, the use of other molecular methods or more variable markers could bring new insights in understanding the relationship between *D. leioneuron* and *D. scoparium*. Finally, *D. crassifolium* is a species that has been described recently (Sérgio *et al.* 1995) and that has been found only in few places in Europe. This species resembles *D. scoparium* but is most similar to *D. transylvanicum* (not included here) due to a bi- or even tristratose leaf lamina and denticulate leaf margins. The present molecular phylogenetic inferences, however, show that this species actually corresponds to *D. scoparium*. *Dicranum scoparium* is known to be very plastic morphologically and occurs in a very broad range of habitats (Hedenäs & Bisang 2004; Ireland 2007; Lang & Stech 2014; Smith 2004), including soil or humus, as well as on rocks or tree bases, in open and shady places where *D. crassifolium* grows as well (Sérgio *et al.* 1995). What triggers the deviating leaf lamina morphology of *D. crassifolium*, and how *D. transylvanicum* relates to *D. crassifolium* and *D. scoparium*, remains to be tested.

Various factors such as the environment or polyploidisation may account for the observed morphological variability of *D. crassifolium*, *D. scottianum* and *D. canariense*, for exemple. Deviating morphologies are frequently observed in bryophytes, especially in species growing in stressful environmental conditions (Buryová & Shaw 2005; Hedenäs *et al.* 2006; Pereira *et al.* 2013; Sæstad 1998; Sæstad *et al.* 1999; Spitale & Petraglia 2010). Most of the *Dicranum* species are widespread and found in a great range of habitats. Hence, local adaptation could partly explain the morphological differences of genetically similar taxa, such as observed in *D. fragilifolium* and *D. elongatum* or *D. leioneuron*. Although the present data does not indicate any hybridisation events, this genetic process is known to influence the morphology (Draper & Hedenäs 2009; Hedenäs 2008; Natcheva & Cronberg 2004; Sotiaux *et al.* 2009). Moreover, the consequences of the special sexual reproduction of *Dicranum*, i.e. dwarf males growing on the branch of a female plant (pseudomonocicy), are largely unknown and would deserve further investigations, in order to explain genetic relationship of closely related species.

TABLE 1. Type of alignment, species delimitation method and number of estimated entities obtained for *Dicranum*. LR and LR test of the GMYC single- threshold (GMYCs) and multiple-threshold (GMYCm) analyses are also mentioned. Significant values are indicated with an asterisk. The species delimitation results are compared with the number of supported phylogenetic entities (phylo) obtained from maximum likelihood analyses.

Alignment	method	Number of sequences	Number of estimated clusters	entities	LR	LR test
extended	GMYCs	206	24	33	9.723	0.021*
	GMYCm		35	58	19.778	0.011*
	PTP			37		
	phylo		21	23		
reduced	GMYCs	145	23	31	7.778	0.051
	GMYCm		30	47	14.660	0.023*
	PTP			42		
	phylo		20	22		

Species delineation using GMYC and PTP— The definition of boundaries between species clusters is essential, as it will influence the interpretation of the phylogenetic reconstructions (Powell 2012). However, one of the major drawbacks of molecular taxonomy is putting a non-arbitrary threshold for delineating species. The main advantage of general mixed Yule-coalescent (GMYC) or Poisson tree processes (PTP) methods is the objective estimation of phylogenetic entities and the circumscription of taxa based on branch length dynamics rather than sequences similarities (Monaghan *et al.* 2009; Pons *et al.* 2006). Although GMYC and PTP performances have been proven to be stable under a wide range of conditions, the accuracy of species delimitation methods will principally depend on the singularities of the data set and the initial species concept used (Talavera *et al.* 2013; Zhang *et al.* 2013). In this study, 34 species were recovered by GMYC single threshold methods, which corresponds generally well with the phylogenetic reconstruction.

However, disagreements were observed, such as in *D. scoparium* but also *D. viride*, *D. fragilifolium*- *D. elongatum*, *D. flexicaule* *D. fuscescens* and *D. polysetum*, where overestimations in the number of entities occurred compared to the molecular and morphological delimitations (Fig. 2a). Each of these species counted one additional entity when compared to the phylogenetic tree, except for *D. fragilifolium*- *D. elongatum* and *D. scoparium* which counted a total of three, respectively four entities. Simultaneously, GMYC calculations considered both sample of *D. groenlandicum* as one species and both *D. brevifolium* and *D. acutifolium* were considered as belonging to the same lineage. The number of ML estimates obtained from the PTP of the extended dataset were relatively similar to the results obtained from GMYC methods (Table 1). However, the number of PTP estimates based on the reduced dataset was slightly higher (Table 1). Simulations have shown that an unbalanced sampling are likely to increase the estimates of haplotypes of the oversampled species (Bergsten *et al.* 2012; Zhang *et al.* 2013) and each specimen of an undersampled species might be counted as separate entity (Zhang *et al.* 2013). In our study, the reduced sampling of *D. scoparium* did not decrease the number of potential species. On the contrary, most of the haplotypes or unique sequences, in particular within *D. scoparium*, were considered as single lineages (Appendix 4). The effect of unbalanced sampling in our dataset has probably less impact on the species delimitation due to the generally low variability in *Dicranum*. Indeed, weak signals and high levels of uncertainty can explain the large range of estimated

species in both PTP estimations (J. Zhang, pers. communication).

Overestimations in the GMYC have been observed in previous studies (e.g. Miralles and Vences 2013; Puillandre *et al.* 2012; Talavera *et al.* 2013) and were often related with errors in the GMYC methods or in the construction of the ultrametric, rather than to taxonomical knowledge gaps (Talavera *et al.* 2013; Zhang *et al.* 2013). As our PTP estimates, obtained from a RaxML tree, were relatively close to the phylogenetic clades and not substantially different from the GMYC results, we considered that errors in the ultrametric tree construction had little effects on the species delimitation. As for now, the GMYC and PTP analysis revealed multiple lineages within species in *Dicranum* that lack morphological and ecological support. Simultaneously, these methods showed an absence of DNA divergences between *D. acutifolium* and *D. brevifolium* as well as between *D. scottianum* and *D. canariense*, which indicates that these four morpho-species might belong to two single taxa.

CONCLUSIONS

Biodiversity assessments rely on the correct delimitation of species. The identification of bryophyte species is largely based on morphological characters, which are often subtle and difficult to apply, or prone to plasticity induced by environmental conditions. Phylogenetic species delimitations, on the other hand, also rely on a certain degree of subjectivity. Automated methods such as GMYC and PTP may provide a more objective approach to molecular species delineation based on maximum likelihood inferences, although inferred boundaries are only putative. Our results showed that DNA-based circumscriptions were generally congruent with morphological species delimitations. Nevertheless, GMYC and especially PTP methods exposed several incongruences between morphological concepts and inference from molecular phylogenetic reconstructions. These incongruences might ensue from evolutionary processes, but also display the need for further testing on other bryophyte data sets to infer the suitability of GMYC and PTP methods for species delimitation in bryophytes.

Chapter 6

Summary and conclusions

Dicranum is a large genus essentially found in the Holarctic (Crosby *et al.* 1999; Frey & Stech 2009). With more than 90 accepted species, *Dicranum* is one of the largest genera of Dicranaceae (Missouri Botanical Garden; Frey & Stech 2009) and about 30 species are recorded for Europe (Hedenäs & Bisang 2004). *Dicranum* species grow in a broad range of habitats, forming dense, tomentose tufts or cushions (Crum & Anderson, 1981), and are easily recognized in the field by their typical « *Dicranum*-look »: acrocarpous stems and leaves that are lanceolate, gradually acuminate and sometimes secund. When fertile, sporophytes have long-rostrate opercula, cucullate calyptrae and 16 peristome teeth that are divided to half-way (Nyholm 1987; Hedenäs & Bisang 2004; Smith 2004; Ireland 2007). However, the taxonomy of this genus is controversial (Allen 1998; Ireland 2007). It has been divided into seven sections (Peterson 1979; Nyholm 1987) whose characteristics are not always distinctive. Moreover, many species are difficult to distinguish due to their morphological plasticity. Intergrading forms are often found, leading to frequent confusions and unclear taxonomy.

In this thesis, species delimitations of temperate and arctic *Dicranum* lineages were investigated using molecular phylogenetic reconstructions and barcoding methods. Four potential barcode markers (*rps4-trnT*, *trnL-F*, *psbA-trnH*, *rnlTS*) and two additional chloroplast markers (*rps19-rpl2* and *rpoB*) were sequenced for 90% of the species known in Europe. Molecular data were analysed with maximum parsimony, maximum likelihood and Bayesian inferences for phylogenetic investigations. Furthermore, Bayesian approaches were used for testing automated species delimitation methods (generalised mixed Yule coalescent (GMYC) and Poisson tree processes (PTP)). Morphological characters were re-addressed in the light of the molecular phylogenetic inferences. Finally, gametophytic characters were re-examined and scored for statistical analyses in order to evaluate their relevance for distinguishing closely related species.

What is the *Dicranum scoparium* complex? What are the morphological characters of *Dicranum scoparium* and how is it related to its morphologically close species?

Bryophytes have a limited number of morphological characters that are strongly influenced by the environment (Briggs 1965; Vanderpoorten & Goffinet 2006). Therefore, it can be difficult to define stable characters that are distinctive for each species. In this thesis, the problem of species delimitations was first investigated in a number of species of section *Dicranum* (Hedw.) Sull. (sensu Nyholm 1987; Bellolio-Trucco & Ireland 1990) whose morphological forms intergrade into one another (Lang & Stech 2014; **chapter 1**), with a focus on the widespread and polymorphic *D.*

scoparium. Molecular phylogenetic reconstructions indicated that molecular lineages are generally congruent with morphological species concepts in *Dicranum*. They further suggested a close relationship of the Holarctic *Dicranum scoparium* Hedw. and *D. bonjeanii* De Not. with the more narrowly distributed *D. howellii* Renauld & Cardot (North America), *D. lorifolium* Mitt., *D. japonicum* Mitt. and *D. nipponense* Besch. (Asia), which together could be regarded as the *D. scoparium* species complex. However, other species of section *Dicranum*, namely *D. majus* Turner and *D. polysetum* Sw. were separated from the *D. scoparium* complex, although frequent morphological confusion with *D. scoparium* are reported. The large sampling of *D. scoparium*, including North American, European and Asian specimens and covering the high degree of morphological trait variation, revealed a monophyletic lineage, defined as *D. scoparium* s.s., that corresponds to the morphological concept of this species: the leaves are straight to falcate-secund ending in a keeled apex, margins are serrate, the costa is percurrent with four serrate ridges on its dorsal side and several stereid bands can be seen in cross-sections, lamina cells are prosenchymatous and porose. Nonetheless, *D. crassifolium* Sérgio, Ochyra & Séneca also corresponded to *D. scoparium* s.s. despite its bistratose lamina and dentate margins (**chapter 5**). Furthermore, several *D. scoparium*-looking specimens from North America (*D. cf. scoparium*) were separated from *D. scoparium* s.s. and cluster with *D. leioneuron* Kindb. (**chapter 5**).

What are *D. bardunovii* and *D. septentrionale*? Are they separate species? Are their morphological characters adequate for distinguishing them from the closely related *D. acutifolium* and *D. brevifolium*?

Two new species have been recently described from Russia: *D. bardunovii* Tubanova & Ignatova and *D. septentrionale* Tubanova & Ignatova. These two species resemble in many characters to *D. acutifolium* and *D. brevifolium* and are easily overlooked. Thus, the four mentioned species are considered to belong to a complex of species named the *D. acutifolium* species complex. The discovery of *D. bardunovii* and *D. septentrionale* is based on phylogenies using only the nuclear spacer nrITS (Tubanova *et al.* 2010; Tubanova & Ignatova 2011). Although this marker is often considered as sufficient for species delimitation (Chen *et al.* 2010; Liu *et al.* 2011), species circumscription in *Dicranum* lacked support and resolution (Lang & Stech 2014, **chapter 1**) due to the generally low genetic variability in Dicranaceae (Stech 1999; La Farge *et al.* 2002). Hence, several markers were necessary for clearer species circumscriptions as shown in the previous study (Lang & Stech 2014). Therefore, we studied the molecular relationship of the *D. acutifolium* species complex and its close allies by combining additional chloroplast markers to the existing ITS phylogeny and analysed the relevance of morphological characters. The combined molecular analyses corroborated the results obtained by Tubanova *et al.* (2010) and Tubanova & Ignatova (2011). While the delimitation of *D. septentrionale* became strongly supported, the circumscription of *D. bardunovii* remained less clearly defined. Nevertheless, both of them could be recognized as species and were distinct from *D. brevifolium* and *D. acutifolium*. The molecular circumscription of *D. bardunovii*, *D. acutifolium* and *D. brevifolium* and especially *D. septentrionale* was in sharp contrast with their morphological resemblance because the characters differentiating the four species are minute and may be easily overlooked. The recognition of these species was moreover hampered by the occurrence of mixed collections, as exemplified by the holotype of *D. bardunovii*, which contained also individuals of *D. acutifolium*. In line with previous morphological and phylogenetic

analyses (e.g. Sukkharak *et al.* 2011; Carter, 2012; Medina, 2012; Stech *et al.* 2013; Lang & Stech, 2014) the present study highlighted the importance of molecular data for clarifying species circumscriptions.

Is barcoding a method that can be used for identifying *Dicranum* species? How do barcode markers perform in terms of species identification?

Correct species identification is important in various fields of biodiversity assessments, ecology and conservation (Cornelissen *et al.* 2007; Dinnage *et al.* 2012; Steele & Pires 2011; Winter *et al.* 2013) but morphological identification of organisms with reduced sizes such as bryophytes can be difficult, especially of arctic bryophytes species, whose gametophytic characters show extreme deviating morphologies and sporophytes are mostly absent (Bellolio-Trucco & Ireland 1990; Hedenäs & Bisang 2004; Hesse *et al.* 2012). DNA barcoding method is an alternative approach to investigate species delimitation. In **chapter 4**, we demonstrated that molecular circumscription of arctic *Dicranum* species using a high number of barcode markers was possible. However, analyses of each individual marker indicated that they tended to possess little interspecific variability. Moreover, the performance of ITS1 and ITS2 was overrated and failed at discriminating all *Dicranum* species. Interspecific genetic distances were generally small and overlapped with intraspecific genetic distances. Used in combinations, the most commonly used markers, *trnT-trnF*, *psbA-trnH* and *rnlTS*, did not contain a natural “barcode gap” either, meaning that some species remain difficult to circumscribe (**chapter 5**). However, since the overlap was reduced and in line with other barcode studies (Liu *et al.* 2011; Stech *et al.* 2013; Hassel *et al.* 2013), these four markers could be considered as potential barcode markers in *Dicranum*.

Is automated species delimitation congruent with the morphological concept of European species?

Generalized mixed Yule coalescent approach (GMYC) or Poisson tree processes (PTP) are species delimitation methods based on likelihood (ML) phylogenetic inferences (Pons *et al.* 2006; Monaghan *et al.* 2009). While GMYC requires a time-calibrated tree, PTP also works on standard phylogenetic trees. Nevertheless, both methods calculate a number of entities that represent theoretical species. Based on a phylogenetic reconstruction that included 28 of the 30 *Dicranum* species found in Europe, the number of entities recovered by the GMYC methods varied between 34 and 58 and 37 estimated entities were recovered by PTP approaches (**chapter 5**). When considering the single threshold GMYC model and the ML tree obtained from the PTP, both methods were relatively congruent with 34 and 37 species, respectively. These estimations, however, were slightly higher than the number of morphological species. The overestimations concerned *D. scoparium* but also *D. viride*, *D. fragilifolium*, *D. fuscescens* and *D. polysetum*. Simulations have shown that an unbalanced dataset and low intraspecific genetic variability are likely to increase the estimates of haplotypes of the oversampled species (Bergsten *et al.*, 2012; Zhang *et al.*, 2013). In such cases, each specimen of a species with small sampling might be counted as a separate entity (Zhang *et al.* 2013). In our study, analyses on a reduced dataset did not decrease the number of potential species. Therefore, the effect of unbalanced sampling probably had less impact on species delimitations than the generally low genetic variability within the genus *Dicranum*.

Despite the overestimated number of species obtained with automated estimation methods, phylogenetic clades were delimiting species that were generally congruent with the actual morphological circumscriptions. Molecular phylogenetic inferences also brought useful insights in several morpho-species such as *D. leioneuron*, *D. crassifolium*, *D. scottianum* and *D. canariense*, *D. angustum* and *D. laevidens* or species of the *D. acutifolium* complex (**chapter 3, 4, 5**), whose taxonomy was until now unclear. Furthermore, the division of the genus into sections had no biological means, as depicted from the phylogenetic tree in **chapter 5**.

Future studies

In this thesis, we show that molecular methods enabled us to clarify the molecular circumscription of a subset of *Dicranum* species and to examine the relationship within species complexes. Although most of the studied taxa were statistically strongly supported, three European species were indistinguishable from other species (*D. crassifolium* from *D. scoparium*, *D. scottianum* from *D. canariense*, *D. elongatum* from *D. fragilifolium*). Additionally, *D. leioneuron* grouped with North American specimens of *D. scoparium* despite clear morphological differences between the two species. A detailed re-interpretation of morphological differences and possibly re-examination of diagnostic characters should be carried out. Moreover, the delimitation of three European species, *D. fulvum*, *D. muehlenbeckii* and *D. transylvanicum*, could not be included in the study. Morpho-molecular analyses of these species would allow us to better understand their genetic affiliation.

The sampling of this thesis represent roughly a third of the known *Dicranum* species. The missing taxa are mainly found in Asia and America. A complete revision of *Dicranum*, including all the species, would allow us to complete our knowledge of this species-rich genus.

Furthermore, the inclusion of additional variable markers and studies of microsatellites loci would help to understand the relationship between the different taxa and to explore the influence of male dwarfism on species reproduction as well as to help us answering the question whether hybridization event occurs in *Dicranum*.

Samenvatting en Conclusies

Dicranum (Gaffeltandmos) is een groot geslacht dat hoofdzakelijk voorkomt in het holarctisch gebied (Crosby *et al.* 1999; Frey & Stech 2009). Met meer dan 90 geaccepteerde soorten is *Dicranum* één van de grotere genera in de Dicranaceae (Frey & Stech 2009) en ongeveer 30 soorten zijn geregistreerd voor Europa (Hedenäs & Bisang 2004). *Dicranum* soorten worden in veel verschillende habitats aangetroffen en vormen dichte viltige polletjes of kussentjes (Crum & Anderson, 1981). Ze zijn in het veld aan hun typische « *Dicranum*-habitus » gemakkelijk te herkennen: acrocarpe stengels en eirond-lancetvormige bladeren die geleidelijk zijn toegespitst en soms homotroop gekromd zijn. De sporenkapsels hebben lang-snavelvormige opercula, kapvormige calyptra's en 16 peristoomtanden die halverwege in tweeën gedeeld zijn (Nyholm 1987; Hedenäs & Bisang 2004; Smith 2004; Ireland 2007). Niettemin is de taxonomie van dit genus controversieel (Allen 1998; Ireland 2007). Het genus is verdeeld in zeven secties (Peterson 1979; Nyholm 1987) waarvan de kenmerken niet altijd onderscheidend zijn. Bovendien zijn veel soorten moeilijk van elkaar te onderscheiden vanwege hun morfologische plasticiteit.

In dit proefschrift is de soortomgrenzing van gematigde en Arctische *Dicranum* soorten onderzocht met behulp van moleculaire fylogenetische reconstructies en DNA-barcodering. Vier potentiële barcode merkers (*rps4-trnT*, *trnL-F*, *psbA-trnH*, *rnlTS*) en twee aanvullende chloroplast merkers (*rps19-rpl2* en *rpoB*) zijn gesequenced voor 90% van de soorten bekend uit Europa. Moleculaire data werden geanalyseerd met maximale parsimonie, maximum likelihood en Bayesiaanse methodes voor fylogenetisch onderzoek. Bovendien zijn Bayesiaanse methodes gebruikt om automatische soortsbegrenzing-methodes te testen. De bruikbaarheid van morfologische kenmerken werd geëvalueerd in het licht van de moleculaire fylogenetische resultaten. Om de toepasbaarheid van gametofytenkenmerken te evalueren, zijn deze heronderzocht en gescoord om statische analyses uit te voeren.

Wat is het Dicranum scoparium complex? Wat zijn de morfologische kenmerken van Dicranum scoparium en hoe is D. scoparium gerelateerd aan de morfologisch meest verwante soorten?

Bryofyten hebben een beperkt aantal morfologische kenmerken die sterk worden beïnvloed door de milieufactoren (Briggs 1965; Vanderpoorten & Goffinet 2006). Daarom kan het moeilijk zijn om stabiele kenmerken te definiëren die onderscheidend zijn voor elke soort. In dit proefschrift zijn de problemen van soortsbegrenzing eerst onderzocht in een aantal soorten van de sectie *Dicranum* (Hedw.) Sull. (sensu Nyholm 1987; Bellolio-Trucco & Ireland 1990) waarvan de morfologische vormen met elkaar overlappen (Lang & Stech 2014; **hoofdstuk 1**) met een focus op de wijdverspreide polymorfe soort *Dicranum* Hedw. *scoparium*. Moleculaire fylogenetische reconstructies wijzen erop dat de moleculaire lijnen meestal congruent zijn met het morfologische soortconcept in *Dicranum*. Zij suggereren verder een nauwe verwantschap tussen de holarctische *D. scoparium*. en *D. bonjeanii* De Not. met de meer beperkt verspreide *D. howellii* Renaud &

Cardot (Noord-Amerika), *D. lorifolium* Mitt., *D. japonicum* Mitt. en *D. nipponense* Besch. (Azië) die gezamenlijk kunnen worden beschouwd als het *D. scoparium* soortencomplex. Echter, andere soorten uit de sectie *Dicranum*, namelijk *D. majus* Turner en *D. polysetum* Sw. zijn gescheiden van het *D. scoparium* complex hoewel morfologische verwarring met *D. scoparium* vaak voorkomt. Onze uitgebreide geografische bemonstering van *D. scoparium*, inclusief Noord-Amerikaanse, Europese en Aziatische exemplaren, en tevens uitgebreide bemonstering van de morfologische kenmerkvariatie onthulde een monofyletische groep, gedefinieerd als *D. scoparium* s.s., die overeenkomt met het morfologische concept van deze soort: de bladeren zijn recht tot homotroop gekromd en eindigen in een gekielde apex, de bladranden zijn gezaagd, de bladnerf is percurrent met vier gezaagde lamellen op de dorsale zijde en verscheidene stereïde banden zijn zichtbaar in dwarsdoorsnede, de bladcellen zijn prosenchymatisch en poraat. Echter, *D. crassifolium* Sérgio, Ochyra & Séneca komt ook overeen met *D. scoparium* s.s. ondanks het tweelagige blad en getande bladranden (**hoofdstuk 5**). Bovendien zijn verscheidene *D. scoparium*-achtige exemplaren uit Noord-Amerika (*D. cf. scoparium*) gescheiden van *D. scoparium* s.s. en clusteren ze met *D. leioneuron* Kindb. (**hoofdstuk 5**).

Wat zijn *D. bardunovii* en *D. septentrionale*? Zijn het aparte soorten? Zijn hun morfologische kenmerken voldoende om ze te onderscheiden van de nauwverwante soorten *D. acutifolium* en *D. brevifolium*?

Twee nieuwe soorten zijn recentelijk beschreven uit Rusland: *D. bardunovii* Tubanova & Ignatova en *D. septentrionale* Tubanova & Ignatova. Deze twee soorten lijken in veel kenmerken op *D. acutifolium* en *D. brevifolium* en worden gemakkelijk over het hoofd gezien. De vier genoemde soorten worden daarom beschouwd als een complex van soorten, namelijk het *D. acutifolium* soortcomplex. De ontdekking van *D. bardunovii* en *D. septentrionale* is gebaseerd op fylogenieën die alleen gebruik maken van de nucleaire spacer nrITS regio (Tubanova et al. 2010; Tubanova & Ignatova 2011). Hoewel deze merker vaak als geschikt wordt beschouwd voor soortsbegrenzing (Chen et al. 2010; Liu et al. 2011), ontbraken bij de soortomschrijvingen in *Dicranum* ondersteuning en resolutie (Lang & Stech 2014, **hoofdstuk 1**) door de doorgaans lage genetische variabiliteit in de Dicranaceae (Stech 1999; La Farge et al. 2002). Verdere merkers zijn bijgevolg noodzakelijk voor het verkrijgen van duidelijkere soortomschrijvingen (Lang & Stech 2014). Daarom onderzochten wij de moleculaire verwantschappen van het *D. acutifolium* soortcomplex en zijn naaste verwanten door aanvullende chloroplast merkers met de bestaande ITS-fylogenie te combineren; ook analyseerden wij de relevante morfologische kenmerken. De gecombineerde moleculaire analyse bevestigt de resultaten van Tubanova et al. (2010) en Tubanova & Ignatova (2011). Hoewel de begrenzing van *D. septentrionale* nu significant ondersteund is, blijft de omgrenzing van *D. bardunovii* minder duidelijk. De kenmerken die de soorten *D. bardunovii*, *D. acutifolium* en *D. brevifolium* en vooral *D. septentrionale* onderscheiden zijn onopvallend en worden gemakkelijk over het hoofd gezien. De herkenning van deze soorten wordt bovendien bemoeilijkt door het bestaan van mengcollecties, zoals geïllustreerd bij het holotype van *D. bardunovii*, dat ook individuen van *D. acutifolium* bevat. In overeenkomst met eerdere morfologische en fylogenetische analyses (e.g. Sukkharak et al. 2011; Carter, 2012; Medina, 2012; Stech et al. 2013; Lang & Stech, 2014) benadrukt dit onderzoek het belang van moleculaire data om soortomschrijvingen te verduidelijken.

Is barcodering een methode die gebruikt kan worden om *Dicranum* soorten te identificeren? Hoe presteren barcode markers in termen van soortidentificatie?

Soorten correct op naam te kunnen brengen is belangrijk in biodiversiteitsevaluaties, ecologie en natuurbescherming (Cornelissen *et al.* 2007; Dinnage *et al.* 2012; Steele & Pires 2011; Winter *et al.* 2013), maar morfologische identificatie van organismes met gereduceerde maten zoals bryofyten kan zeer moeilijk zijn. Dit geldt in het bijzonder voor Arctische mossoorten, waarvan de kenmerken van de gametofyten extreem afwijkende morfologieën laten zien en waar de sporofyten bijna altijd afwezig zijn (Bellolio-Trucco & Ireland 1990; Hedenäs & Bisang 2004; Hesse *et al.* 2012). Het gebruik van DNA-barcodes is een alternatieve methode om soortsgrenzen te onderzoeken. In **hoofdstuk 4** tonen wij aan dat de moleculaire omschrijving van Arctische *Dicranum* soorten moleculair omschreven kunnen worden door een combinatie van een aantal barcode markers. Analyses van elke individuele merker tonen echter aan dat zij weinig interspecifieke variabiliteit bezitten. Bovendien faalt de nrITS regio in het onderscheiden van de bestudeerde *Dicranum* soorten. De combinatie van de meest-gebruikte markers, *trnT*-*rps4*, *trnL-F*, *psbA-trnH* en nrITS, heeft duidelijk het grootste potentieel als barcode merker in *Dicranum*.

Is geautomatiseerde soortsgrenzen congruent met het morfologische concept van de Europese soorten?

Gegeneraliseerde 'mixed Yule coalescent' methodes (GMYC) of 'Poisson tree' processen (PTP) soortbegrenzingmethodes die gebaseerd zijn op 'likelihood' (ML) fylogenetische inferenties (Pons *et al.* 2006; Monaghan *et al.* 2009). Terwijl GMYC een tijd-gekalibreerde stamboom nodig heeft, werkt PTP ook met standaard fylogenetische stambomen. Gebaseerd op een fylogenetische reconstructie die 28 van de 30 *Dicranum* soorten van Europa omvat, is het aantal entiteiten teruggevonden met GMYC en PTP methodes tussen de 34 en 58, respectievelijk 37 entiteiten (**hoofdstuk 5**). Als men het GMYC model met enkele drempelwaarden en de ML stamboom verkregen met PTP in beschouwing neemt, dan zijn beide methodes relatief congruent met respectievelijk 34 en 37 species. Deze schattingen zijn echter hoger dan het aantal morfologische soorten. De overschattingen betreffen *D. scoparium* maar ook *D. viride*, *D. fragilifolium*, *D. fuscescens* en *D. polysetum*. Simulaties tonen aan dat een ongebalanceerde dataset en een lage intraspecifieke genetische variabiliteit waarschijnlijk het aantal haplotypen van overbemonsterde soorten zullen laten toenemen (Bergsten *et al.*, 2012; Zhang *et al.*, 2013). In die gevallen zal elk exemplaar van een soort met een kleine steekproef kunnen worden beschouwd als een aparte entiteit (Zhang *et al.* 2013). In ons onderzoek verminderden analyses op een gereduceerde dataset niet het aantal potentiële soorten. Daarom heeft het effect van ongebalanceerde bemonstering waarschijnlijk minder invloed op soortsgrenzen dan de normaalgesproken lage genetische variabiliteit binnen het geslacht *Dicranum*.

Ondanks het overschatte aantal soorten verkregen met de geautomatiseerde schattingsmethoden, bakenen de verkregen clades soorten af die meestal congruent zijn met de actuele morfologische omschrijvingen. Moleculaire fylogenetische analyses brengen ook nuttige inzichten in verscheidene morfologische soorten zoals *D. leioneuron*, *D. crassifolium*, *D. scottianum*, *D. canariense*, *D. angustum*, *D. laevidens* of soorten van het *D. acutifolium* complex (**hoofdstuk 3, 4, 5**), waarvan de taxonomie tot nu toe onduidelijk was. Bovendien heeft de verdeling van het genus

in secties geen biologische betekenis, zoals aangetoond wordt in de fylogenetische stamboom in hoofdstuk 5.

Toekomstige studies

In dit proefschrift hebben wij aangetoond dat moleculaire methodes geschikt zijn om moleculaire omgrenzingen van een aantal *Dicranum* soorten op te helderen en om de verwantschappen binnen soortcomplexen te onderzoeken. Hoewel de moleculaire clades van de meeste van de onderzochte taxa significant werden ondersteund, waren drie Europese soorten niet te onderscheiden van andere soorten (*D. crassifolium* van *D. scoparium*, *D. scottianum* van *D. canariense*, *D. elongatum* van *D. fragilifolium*). Verder groepeerde *D. leioneuron* met Noord-Amerikaanse exemplaren van *D. scoparium* ondanks duidelijke morfologische verschillen tussen de twee soorten. Een gedetailleerde herinterpretatie van de morfologische verschillen en een heronderzoek van diagnostische kenmerken dient te worden uitgevoerd. Bovendien moet de soortomgrenzing van drie Europese soorten, *D. fulvum*, *D. muehlenbeckii* en *D. transylvanicum*, verder worden onderzocht waarvan geen materiaal ter beschikking stond voor moleculaire analyses. Morfo-moleculaire analyses van de drie ontbrekende soorten zou ons in staat stellen om hun genetische verwantschap beter te begrijpen.

De bemonstering van dit proefschrift representeert ruwweg een derde van de bekende *Dicranum* soorten. De ontbrekende taxa komen voornamelijk voor in Azië en Amerika. Een complete revisie van alle *Dicranum* soorten omvat, zou ons in staat stellen om onze kennis van dit soortenrijke genus te voltooien.

Bovendien, zou de analyse van meer variabele merkers en van microsatellieten kunnen helpen om de relaties tussen de verschillende taxa te begrijpen, om de invloed van mannelijke dwerggroei op de reproductie van de soorten te onderzoeken, en de vraag te beantwoorden in hoeverre hybridisatie in *Dicranum* voorkomt.

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Appendices

APPENDIX 1. List of all sample used for molecular analyses and morphological studies analyses. DNA, herbarium location Herb., voucher information, collection date, geographic origin, original label and GenBank accession numbers *rps4-trnI*, *trnL-trnF*, *trnH-psbA*, *rps1 9-rpl2*, *rpoB*, nrITS of the specimens included in the study.

Voucher	species	Herb.	collector and number	date of collection	Locality	original label	<i>rps4-trnI</i>	<i>trnL-trnF</i>	<i>trnH-psbA</i>	<i>rps1 9-rpl2</i>	<i>rpoB</i>	ITS
1	<i>D. acutifolium</i>	UUH	Krivobokov 262	12.07.2000	RU Buryatia	<i>D. bardunovii</i>	KJ796621	KJ796598	KJ796531	-	KJ796572	JN897272
4	<i>D. fragilifolium</i>	MW	Churakova s.n.	03.07.2001	RU Arkhangels Province	<i>D. fragilifolium</i>	-	KM502760	KM502638	-	KM502705	FJ952597
5	<i>D. bardunovii</i>	MW	Ivanova s.n.	01.08.1991	RU Yakutia	<i>D. bardunovii</i>	KJ796622	KJ796599	KJ796532	-	KJ796573	JN897273
9	<i>D. bardunovii</i>	MW	Volatovskiy s.n.	20.06.1985	RU SouthYakutia	<i>D. bardunovii</i>	-	KJ796600	KJ796533	-	-	JN897274
12	<i>D. fragilifolium</i>	MW	Ignatov & Ignatova s.n.	12.08.2001	RU Vologda Province	<i>D. fragilifolium</i>	KM502604	KM502761	KM502639	-	KM502706	FJ952596
13	<i>D. flexicaule</i>	KRF	Molokova s.n.	23.07.1995	RU Tuva	<i>Dicranum</i> sp.	KJ796606	KJ796581	KJ796513	-	KJ796555	HQ830328
15	<i>D. flexicaule</i>	KRF	Omyukova s.n.	29.07.1991	RU Krasnoyars Territory	<i>Dicranum</i> sp.	-	KJ796582	KJ796514	-	KJ796556	HQ830330
16	<i>D. flexicaule</i>	IRK	Dudareva s.n.	04.08.2006	RU Zabaikalsk Territory	<i>Dicranum</i> sp.	KJ796607	KJ796583	KJ796515	-	KJ796557	HQ830331
17	<i>D. flexicaule</i>	MHA	Ignatov 06-2637	s.d.	RU Primorsky Territory	<i>Dicranum</i> sp.	-	KJ796584	-	-	KJ796558	HQ830332
18	<i>D. fuscescens</i>	MHA	Bezgodov & Shkaraba s.n.	31.07.2005	RU Perm Province	<i>D. fuscescens</i>	-	KJ796578	KJ796510	-	KJ796552	HQ830334
19	<i>D. fuscescens</i>	MHA	Ignatov 06-2588	03.09.2006	RU Primorsky Territory	<i>D. fuscescens</i>	KJ796605	KJ796579	KJ796511	-	KJ796553	HQ830337
20	<i>D. brevifolium</i>	MW	Egorov s.n.	27.07.1996	RU Karachaevo-Cherkessia	<i>D. brevifolium</i>	KJ796611	KJ796587	KJ796520	-	KJ796563	HQ830342
21	<i>D. brevifolium</i>	KRF	Omyukova s.n.	12.06.2007	RU Tuva	<i>D. brevifolium</i>	KJ796612	KJ796588	KJ796521	KJ796629	KJ796564	HQ830341
23	<i>D. septentrionale</i>	LE	Neshataeva 986	04.08.2007	RU Kamchatka	<i>D. septentrionale</i>	KJ796608	KJ796585	KJ796516	KJ796627	KJ796559	HQ830338
24	<i>D. fuscescens</i>	MHA	Ignatov 06-14	20.08.2006	RU Sakhalin	<i>D. fuscescens</i>	-	KJ796580	KJ796512	-	KJ796554	HQ830335
25	<i>D. septentrionale</i>	MW	Churakova 864	19.07.2000	RU Arkhangels Province	<i>D. septentrionale</i>	-	KJ796586	KJ796517	-	KJ796560	HQ830339
26	<i>D. brevifolium</i>	MW	Korotkov s.n.	06.09.2002	RU NorthOssetia	<i>D. brevifolium</i>	-	KJ796589	KJ796522	KJ796630	KJ796565	HQ830343
1_II	<i>D. bardunovii</i>	UUH	Krivobokov 262	12.07.2000	RU Buryatia	<i>D. bardunovii holotype</i>	KJ796620	KJ796597	KJ796530	KJ796638	-	KJ796547
Aleu_1	<i>D. howellii</i>	UBC	Talbot TAN1C-17	16.08.2002	US Alaska, Aleutian-Island	<i>D. scoparium</i>	-	KF423914	KF423447	-	KF423658	-
Aleu_2	<i>D. majus</i>	UBC	Talbot & Schofield ADA42-29	24.08.2006	US Alaska, Sea Parrot Island	<i>D. scoparium</i>	KF423824	KF423915	KF423448	KF423752	KF423659	KF423553
Alk_1	<i>D. majus</i>	UBC	Talbot & Salomesch 05-41-18	07.07.2005	US Alaska, Northwest Arctic borough	<i>D. scoparium</i>	KF423825	KF423916	KF423449	KF423753	KF423660	KF423554

Voucher	species	Herb.	collector and number	date of collection	Locality	original label	rps4-trnT	trnL-trnF	trnH-psbA	rps19-rpl2	rpoB	ITS
Alk_2	<i>D. flexicaule</i>	L	Morgado & Gemi Bry- 280712 001	28.07.2012	US Alaska	<i>Dicranum</i> sp.	KJ651000	KJ651051	KJ650824	-	KJ650907	KJ650849
Alk_3	<i>D. flexicaule</i>	L	Morgado & Gemi Bry- 280712 002	28.07.2012	US Alaska	<i>Dicranum</i> sp.	KJ651001	KJ651052	KJ650825	-	KJ650908	KJ650850
Alk_4	<i>D. flexicaule</i>	L	Morgado & Gemi Bry- 280712 003	28.07.2012	US Alaska	<i>Dicranum</i> sp.	KJ651002	KJ651053	KJ650826	-	KJ650909	KJ650851
Alk_5	<i>D. flexicaule</i>	L	Morgado & Gemi Bry- 280712 004	28.07.2012	US Alaska	<i>Dicranum</i> sp.	KJ651003	KJ651054	KJ650827	-	KJ650910	KJ650852
Alk_6	<i>D. flexicaule</i>	L	Morgado & Gemi Bry- 280712 005	28.07.2012	US Alaska	<i>Dicranum</i> sp.	KJ651004	KJ651055	KJ650828	-	KJ650911	KJ650853
Alk_7	<i>D. flexicaule</i>	L	Morgado & Gemi Bry- 280712 006	28.07.2012	US Alaska	<i>Dicranum</i> sp.	KJ651005	KJ651056	KJ650829	-	KJ650912	KJ650854
Ast1011	<i>D. scoparium</i>	FCO-Brief	Fdez. Ordóñez 269	24.10.2001	ES Asturias	<i>D. scoparium</i>	KF423826	KF423917	KF423450	KF423754	KF423661	KF423555
Ast2011	<i>D. scoparium</i>	FCO-Brief	Fdez. Ordóñez 1077	22.02.2002	ES Asturias	<i>D. scoparium</i>	KF423827	KF423918	KF423451	KF423755	KF423662	KF423556
Ast3011	<i>D. scoparium</i>	FCO-Brief	del Collado 3784-1	01.08.2002	ES Asturias	<i>D. scoparium</i>	KF423828	KF423919	KF423452	KF423756	KF423663	KF423557
Ast3021	<i>D. scoparium</i>	FCO-Brief	del Collado 2993-1	27.07.1999	ES Asturias	<i>D. scoparium</i>	KF423829	KF423920	KF423453	KF423757	KF423664	KF423558
Ast3031	<i>D. scoparium</i>	FCO-Brief	del Collado 3744-1	18.08.2005	ES Asturias	<i>D. scoparium</i>	KF423830	KF423921	KF423454	KF423758	KF423665	KF423559
AT_2	<i>D. muelhenbeckii</i>	L	van Melick 208859	s.d.	AT Steiermark	<i>D. muelhenbeckii</i>						
AT_3	<i>D. drummondii</i>	L	van Melick 212270	06.07.1973	AT Tirol	<i>D. muelhenbeckii</i>						
Attu_1	<i>D. majus</i>	UBC	Schofield & Talbot 120253	15.09.2002	US Alaska, Attu Island	<i>D. scoparium</i>	-	KF423922	KF423455	KF423759	KF423666	KF423560
BC_1	<i>D. scoparium</i>	UBC	Schofield & Klinkenberg 119252	24.03.2002	CA British Columbia, Lulu Island	<i>D. scoparium</i>	KF423898	KF423997	KF423536	-	-	KF423644
BC_2	<i>D. scoparium</i>	UBC	Schofield & Williams 117252A	18.05.2001	CA British Columbia, Lac les Jeune Road	<i>D. scoparium</i>	KF423899	KF423998	KF423537	-	-	KF423645
BCo1	<i>D. scoparium</i>	NY	Buck 54100	19.09.2008	CA Ontario, Bruce Co.	<i>D. scoparium</i>	KF423831	KF423923	KF423456	KF423760	KF423667	KF423561
BUL_1	<i>D. scoparium</i>	L	Papp 10/101/1	06.08.2010	BG Sofia Prov.	<i>D. scoparium</i>	KF423832	KF423924	KF423457	KF423761	KF423668	KF423562

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Cauc_1	<i>D. scoparium</i>	S	Ignatov & Ignatova B113001	12.09.2005	RU Karachaevo-Cherkessian Rep.	<i>D. scoparium</i>	KF423833	KF423925	KF423458	KF423762	KF423669	KF423563
CH1	<i>D. brevifolium</i>	S	Hedenäs B98890	14.08.2004	CH Canton Wallis	<i>D. brevifolium</i>	KJ651039	KJ651095	KJ650837	KJ650990	KJ650953	KJ650895
Ch_1011	<i>D. scoparium</i>	G	Lang 20080907.1	07.09.2008	CH Canton Geneva	<i>D. scoparium</i>	GQ428082	GQ428036	GQ427991	GQ427953	GQ427914	KF423564
Ch_1061	<i>D. scoparium</i>	G	Lang 20080907.6	07.09.2008	CH Canton Geneva	<i>D. scoparium</i>	GU068393	GU068477	GU068448	GU068364	GU068422	KF423565
CH_4	L		Greven s.n.	28.07.1990	CH Canton Wallis	<i>D. muelhlenbeckii</i>	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX
Cor_1	<i>D. scoparium</i>	S	Sofiaux & Sotiaux 462	25.05.2004	FR Haute-Corse	<i>D. scoparium</i>	KF423834	KF423926	KF423459	KF423763	KF423670	KF423566
Dac	<i>D. acutifolium</i>	L	Stech B970831.1	31.08.1997	FI Kuusamo Prov.	<i>D. acutifolium</i>	KJ651030	DQ462590	KJ650818	KJ650981	KJ650941	KJ650881
DB_1	<i>D. scoparium</i>	MO	King & Garvey B657	05.10.2001	CA Nova Scotia, Richmond Co.	<i>D. bonjeanii</i>	KF423835	KF423927	KF423460	KF423764	KF423671	-
DB_2	<i>D. cf. scoparium</i>	MO	Davis 270	12.08.2007	US Pennsylvania, Cambria Co.	<i>D. bonjeanii</i>	GU068416	GU068500	GU068471	GU068387	GU068443	KF423567
DB_3	<i>D. scoparium</i>	MO	Weber, Wittmann, Andrus & Cooper B-111031	12.08.1996	US Colorado, San Juan Co.	<i>D. bonjeanii</i>	-	KM502773	KM502649	-	-	KM502686
DB_4	<i>D. scoparium</i>	MO	Allen 28704	09.09.2003	CA Newfoundland, Avalon Peninsula	<i>D. bonjeanii</i>	GU068418	GU068502	GU068473	GU068389	GU068445	KF423568
Db_CA	<i>D. scoparium</i>	L	Allen 9479	13.08.1990	CA Ontario, Thunder-bay district	<i>D. bonjeanii</i>	-	KF423999	KF423538	-	-	KF423646
Dbe	<i>D. undulatum</i>	L	Stech B970824.2	24.08.1997	FI North Karelia Prov.	<i>D. bergeri</i>	KJ796626	KJ796604	KJ796537	-	KJ796577	KJ796551
Dbr	<i>D. septentrionale</i>	L	Stech B960801.2	01.08.1996	AT Tirol	<i>D. brevifolium</i>	KJ796610	DQ462591	KJ796519	KJ796628	KJ796562	KJ796539
Dcan	<i>D. canariense</i>	L	Stech 04-405	2004	SP Canaries Islands	<i>D. canariense</i>	KM502596	KM502749	KM502627	KM502722	KM502702	KM502664
Dcan2	<i>D. canariense</i>	L	Stech 04-547	2004	SP Canaries Islands	<i>D. canariense</i>	KM502597	KM502750	KM502628	KM502723	KM502703	KM502665
Dcan3	<i>D. canariense</i>	L	07-113	2004	SP Canaries Islands	<i>D. canariense</i>	KM502598	KM502751	KM502629	KM502724	KM502704	KM502666
Ddr	<i>D. drummondii</i>	L	Stech B970827.4	27.08.1997	FI Kuusamo Prov.	<i>D. drummondii</i>	KJ796609	DQ462589	KJ796518	-	KJ796561	KJ796538
Ddrumm	<i>D. drummondii</i>	S	Hedenäs, Ohlsson, Odelvik & Myrdal B122041	s.d.	SE Härjedalen							
DE_1	<i>D. muelhlenbeckii</i>	L	van Melick 202606	s.d.	DE Baden-Württemberg	<i>D. muelhlenbeckii</i>						

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DE_2	<i>D. dispersum</i>	S	Sauer MS95022	12.05.1995	DE Baden-Württemberg	<i>D. dispersum</i>	KJ651041	KJ651097	KJ650839	KJ650992	KJ650955	KJ650897
Del	<i>D. elongatum</i>	L	Stech B970831.3	31.08.1997	FI Kuusamo Prov.	<i>D. elongatum</i>	KJ651031	DQ462592	KJ650819	KJ650982	KJ650942	KJ650882
Dfl	<i>D. flexicaule</i>	L	Stech B970827.5	27.08.1997	FI Kuusamo Prov.	<i>D. flexicaule</i>	KJ651032	-	KJ650820	-	KJ650943	KJ650883
Dfla1	<i>D. flagellare</i>	L	Wondergem 1300	07.10.2010	NL Utrecht	<i>D. flagellare</i>	KM502601	KM502757	KM502635	KM502727	-	KM502672
Dfla2	<i>D. flagellare</i>	L	Bijlsma 12053	16.05.2009	NL Limburg	<i>D. flagellare</i>	KM502602	KM502758	KM502636	KM502728	-	KM502673
Dfla3	<i>D. flagellare</i>	L	Bijlsma 13104	12.02.2011	NL Gelderland	<i>D. flagellare</i>	KM502603	KM502759	KM502637	KM502729	-	KM502674
Dfus6	<i>D. fuscescens</i>	L	Wondergem 1134	21.05.2009	NL Gelderland	<i>D. fuscescens</i>	KJ651042	KJ651098	KJ650840	KJ650993	-	KJ650898
Dfus7	<i>D. fuscescens</i>	L	Stech B970824.3	24.08.1997	FI Karelia Prov.	<i>D. fuscescens</i>	KF423896	-	KF423534	KF423819	KF423742	KF423642
Dfus8	<i>D. fuscescens</i>	L	Stech B970824.3	24.08.1997	FI Karelia Prov.	<i>D. fuscescens</i>	KM502619	KM502781	KM502656	KM502742	KM502714	KM502694
DH_3	<i>D. howellii</i>	MO	Allen 24114	24.03.2002	US California, Mendocino Co.	<i>D. howellii</i>	KF423840	KF423928	KF423465	-	-	KF423569
DH_5	<i>D. howellii</i>	MO	Shevock 19290	29.04.2000	US California, San Francisco Co.	<i>D. howellii</i>	KF423841	KF423929	KF423466	KF423769	KF423676	KF423570
Dib	<i>D. majus</i>	L	Stech B970829.4	29.08.1997	FI Kuusamo Prov.	<i>D. bonjeanii</i>	KF423836	AF135048/ AF136076	KF423461	KF423765	KF423672	AF144114
Dic_1644	<i>D. acutifolium</i>	L	Stech & Kruijer 10-102a	04.08.2010	NO Svalbard	<i>D. acutifolium</i>	KJ651006	KJ651057	KJ650789	KJ650961	KJ650913	KJ650855
Dic_1645	<i>D. spodiaceum</i>	L	Stech & Kruijer 08-203	14.07.2008	NO Svalbard	<i>D. angustum</i>	KJ651007	KJ651058	KJ650790	KJ650962	KJ650914	KJ650856
Dic_1646	<i>D. elongatum</i>	L	Stech & Kruijer 08-250	15.07.2008	NO Svalbard	<i>D. elongatum</i>	KJ651008	KJ651059	KJ650791	KJ650963	KJ650915	KJ650857
Dic_1648	<i>D. elongatum</i>	L	Stech & Kruijer 09-072	28.06.2009	NO Svalbard	<i>D. elongatum</i>	KJ651022	KJ651060	KJ650809	-	KJ650932	-
Dic_1649	<i>D. acutifolium</i>	L	Stech & Kruijer 10-118	04.08.2010	NO Svalbard	<i>D. elongatum</i>	KJ651009	KJ651061	KJ650792	KJ650964	KJ650916	KJ650858
Dic_1650	<i>D. elongatum</i>	L	Stech & Kruijer 10-202	07.08.2010	NO Svalbard	<i>D. elongatum</i>	-	KJ651062	KJ650793	-	KJ650917	KJ650859
Dic_1651	<i>D. laevidens</i>	L	Stech & Kruijer 10-216	07.08.2010	NO Svalbard	<i>D. groenlandicum</i>	KJ651010	KJ651063	KJ650794	KJ650965	KJ650918	KJ650860
Dic_1652	<i>D. laevidens</i>	L	Stech & Kruijer 10-002	01.08.2010	NO Svalbard	<i>D. laevidens</i>	KJ651011	KJ651064	KJ650795	-	KJ650919	KJ650861

Voucher	species	Herb.	collector and number	date of collection	Locality	original label	<i>rps4-trnT</i>	<i>trnL-trnF</i>	<i>trnH-psbA</i>	<i>rps19-rpl2</i>	<i>rpoB</i>	ITS
Dic_ 1653	<i>D. laevidens</i>	L	Stech & Kruijer 10-006a	01.08.2010	NO Svalbard	<i>D. laevidens</i>	-	KJ651065	KJ650796	-	KJ650920	KJ650862
Dic_ 1654	<i>D. spodiaceum</i>	L	Stech & Kruijer 10-297	2010	NO Svalbard	<i>D. laevidens</i>	KJ651012	KJ651066	KJ650797	KJ650966	KJ650921	KJ650863
Dic_ 1655	<i>D. laevidens</i>	L	Stech & Kruijer 09-71	2009	NO Svalbard	<i>D. laevidens</i>	-	KJ651067	KJ650808	-	-	KJ650874
Dic_ 1659	<i>D. spodiaceum</i>	L	Stech & Kruijer 08-025	10.10.2012	NO Svalbard	<i>D. laevidens</i>	-	KJ651068	KJ650798	KJ650967	KJ650922	KJ650864
Dic_ 1660	<i>D. acutifolium</i>	L	Stech & Kruijer 08-031	11.07.2008	NO Svalbard	<i>D. laevidens</i>	KJ651013	KJ651069	KJ650799	KJ650968	KJ650923	KJ650865
Dic_ 1661	<i>D. acutifolium</i>	L	Stech & Kruijer 08-033a	11.07.2008	NO Svalbard	<i>D. laevidens</i>	KJ651014	KJ651070	KJ650800	KJ650969	KJ650924	KJ650866
Dic_ 1662	<i>D. laevidens</i>	L	Stech & Kruijer 09-021	28.06.2009	NO Svalbard	<i>D. laevidens</i>	KJ651015	KJ651071	KJ650801	-	KJ650925	KJ650867
Dic_ 1663	<i>D. laevidens</i>	L	Stech & Kruijer 09-022	28.06.2009	NO Svalbard	<i>D. laevidens</i>	KJ651016	KJ651072	KJ650802	-	KJ650926	KJ650868
Dic_ 1664	<i>D. majus</i>	L	Stech & Kruijer 10-029	01.08.2010	NO Svalbard	<i>D. laevidens</i>	KF423823	KF423913	KF423446	-	KF423657	KF423552
Dic_ 1665	<i>D. spodiaceum</i>	L	Stech & Kruijer 10-046	01.08.2010	NO Svalbard	<i>D. laevidens</i>	KJ651017	KJ651073	KJ650803	KJ650970	KJ650927	KJ650869
Dic_ 1666	<i>D. spodiaceum</i>	L	Stech & Kruijer 11-229	08.07.2011	NO Svalbard	<i>Dicranum</i> sp.	KJ651018	KJ651074	KJ650804	KJ650971	KJ650928	KJ650870
Dic_ 1667	<i>D. spodiaceum</i>	L	Stech & Kruijer 11-237	08.07.2012	NO Svalbard	<i>D. majus</i>	KJ651019	KJ651075	KJ650805	KJ650972	KJ650929	KJ650871
Dic_ 1668	<i>D. spodiaceum</i>	L	Stech & Kruijer 11-052	29.06.2011	NO Svalbard	<i>Dicranum</i> sp.	KJ651023	KJ651076	KJ650810	KJ650975	KJ650933	-
Dic_ 1669	<i>D. spodiaceum</i>	L	Stech & Kruijer 11-171	02.07.2011	NO Svalbard	<i>D. spodiaceum</i>	KJ651020	KJ651077	KJ650806	KJ650973	KJ650930	KJ650872
Dic_ 1670	<i>D. spodiaceum</i>	L	Stech & Kruijer 11-168	02.07.2011	NO Svalbard	<i>D. spodiaceum</i>	-	KJ651078	KJ650811	-	KJ650934	-
Dic_ 1671b	<i>D. spodiaceum</i>	L	Stech & Kruijer 11-167b	02.07.2011	NO Svalbard	<i>D. spodiaceum</i>	KJ651021	KJ651079	KJ650807	KJ650974	KJ650931	KJ650873
Dic_ 1671c	<i>D. spodiaceum</i>	L	Stech & Kruijer 11-167c	02.07.2011	NO Svalbard	<i>D. laevidens</i>	KJ651024	KJ651080	KJ650812	-	KJ650935	KJ650875

Voucher	species	Herb.	collector and number	date of collection	Locality	original label	rps4-trnT	trnL-trnF	trnH-psbA	rps19-rpl2	rpoB	ITS
Dic_1672	<i>D. laevidens</i>	L	Stech & Kruijer 11-0431	2011	NO Svalbard	<i>Dicranum</i> sp.	KJ651025	KJ651081	KJ650813	KJ650976	KJ650936	KJ650876
Dic_1673	<i>D. spadicum</i>	L	Stech & Kruijer 11-153	01.07.2011	NO Svalbard	<i>D. cf acutifolium</i>	KJ651026	KJ651082	KJ650814	KJ650977	KJ650937	KJ650877
Dic_1675	<i>D. elongatum</i>	L	Kruijer & Stech 11-213	07.07.2011	NO Svalbard	<i>D. elongatum</i>	KJ651027	KJ651083	KJ650815	KJ650978	KJ650938	KJ650878
Dic_1676	<i>D. acutifolium</i>	L	Stech & Kruijer 11-161	01.07.2011	NO Svalbard	<i>D. acutifolium</i>	KJ651028	KJ651084	KJ650816	KJ650979	KJ650939	KJ650879
Dic_1677	<i>D. spadicum</i>	L	Stech & Kruijer 11-165	02.07.2011	NO Svalbard	<i>D. laevidens</i>	KJ651029	KJ651085	KJ650817	KJ650980	KJ650940	KJ650880
Dif	<i>D. fragilifolium</i>	L	Stech B970827.1	27.08.1997	FI Kuusamo Prov.	<i>D. fragilifolium</i>	KF423837	AF1 35069/ AF1 36077	KF423462	KF423766	KF423673	AF1 40700
Dif2	<i>D. fragilifolium</i>	L	Stech B970828.8	28.08.1997	FI Kuusamo Prov.	<i>D. fragilifolium</i>	-	KM502762	-	-	-	KM502675
Dip	<i>D. polysetum</i>	L	Stech B9705181.1	18.05.1997	DE Mecklenburg-Vorpommern	<i>D. polysetum</i>	KF423838	AF1 29587	EU163523	KF423767	KF423674	AF1 44113
Dis	<i>D. scoparium</i>	L	Stech B960719.1	19.07.1997	DE Schleswig-Holstein	<i>D. scoparium</i>	KF423839	AF1 29588/ AF1 29561	KF423464	KF423768	KF423675	AF1 40699
Dtap_16	<i>D. scoparium</i>	MO	He 36007	10.08.2006	TW Eastern Taiwan	<i>D. japonicum</i>	KF423900	KF424000	KF423539	KF423820	KF423744	-
Dtap_17	<i>D. scoparium</i>	S	Mizutani 15102	28.10.1995	JP Honshu	<i>D. japonicum</i>	KF423901	KF424002	KF423541	-	-	KF423648
Dtap_5	<i>D. scoparium</i>	MO	He & Song 34527	13.07.2004	KR Jeju-do	<i>D. japonicum</i>	KF423902	KF424003	KF423542	-	KF423745	-
Dma	<i>D. scoparium</i>	L	Stech s.n.	1997	FI	<i>D. majus</i>	-	KJ651086	KJ650821	-	KJ650945	KJ650884
Dmont_1	<i>D. montanum</i>	L	Wundergem 1302	27.10.2010	NL North Holland	<i>D. montanum</i>	KM502606	KM502767	KM502644	KM502732	-	KM502680
Dmont_2	<i>D. montanum</i>	L	Zwarts 2033	03.12.2008	NL Gelderland	<i>D. montanum</i>	KM502607	KM502768	KM502645	KM502733	-	KM502681
Dmont_3	<i>D. montanum</i>	L	Smulders 10139	05.11.2010	NL North Brabant	<i>D. montanum</i>	KM502608	KM502769	KM502646	KM502734	-	KM502682
Dpol_1	<i>D. polysetum</i>	L	Wundergem 1355	25.01.2011	NL Utrecht	<i>D. polysetum</i>	KM502609	KM502770	KM502647	KM502735	-	KM502683
Dpol_2	<i>D. polysetum</i>	L	Zwarts 2121	28.02.2009	NL Gelderland	<i>D. polysetum</i>	KM502610	KM502771	KM502648	KM502736	-	KM502684
Dpol_3	<i>D. polysetum</i>	L	Aptroot 69434	31.08.2010	NL Overijssel	<i>D. polysetum</i>	KM502611	KM502772	-	KM502737	-	KM502685
Ddrum	<i>D. drummondii</i>	S	Hedenäs, Ohlsson, Odelvik & Myrdal B122041		SE Härjedalen	<i>D. drummondii</i>						
DS_04217	<i>D. scoparium</i>	L	Stech 04-217	28.07.2004	PT Madeira	<i>D. scoparium</i>	KF423844	KF423932	KF423469	KF423771	KF423678	KF423576

Voucher	species	Herb.	collector and number	date of collection	Locality	original label	rps4-trnT	trnL-trnF	trnH-psbA	rps19-rpl2	rpoB	ITS
DS_09317	<i>D. scoparium</i>	L	Stech 09-317	27.12.2009	DE Northrhine-West-phalia	<i>D. scoparium</i>	KF423845	KF423933	KF423470	KF423772	KF423679	KF423577
DS_09318	<i>D. scoparium</i>	L	Stech 09-318	27.12.2009	DE Northrhine-West-phalia	<i>D. scoparium</i>	KF423846	KF423934	KF423471	KF423773	KF423680	KF423578
DS_1	<i>D. cf. scoparium</i>	MO	Risk, Richardson & Newland 14,463	20.04.2007	US Kentucky, Greenup Co.	<i>D. scoparium</i>	GU068413	GU068497	GU068468	GU068384	GU068440	KF423571
DS_10009	<i>D. scoparium</i>	L	Stech 10-009	02.04.2010	DE Rhineland - Palatinate	<i>D. scoparium</i>	KF423847	KF423935	KF423472	KF423774	KF423681	KF423579
DS_2	<i>D. cf. scoparium</i>	MO	Allen 28074	10.06.2007	US Maine, Knox Co.	<i>D. scoparium</i>	GU068414	GU068498	GU068469	GU068385	GU068441	KF423572
DS_3	<i>D. cf. scoparium</i>	MO	Holmberg 1578	24.03.2006	US Missouri, Jefferson Co.	<i>D. scoparium</i>	GU068415	GU068499	GU068470	GU068386	GU068442	KF423573
DS_4	<i>D. majus</i>	MO	Talbot AT102-30	15.08.1999	US Alaska, Attu Island	<i>D. scoparium</i>	GU068421	GU068504	GU068476	GU068392	GU068447	KF423574
DS_5	<i>D. howellii</i>	MO	Allen 28834	26.03.2008	US Oregon, Multnomah Co.	<i>D. scoparium</i>	KF423843	KF423931	KF423468	KF423770	KF423677	KF423575
Dspa		L	Stech 8970828.9	28.08.1997	FI Kuusamo Prov.	<i>D. spadiceum</i>	-	KJ651087	KJ650822	-	-	KJ650886
Dspu	<i>D. spurium</i>	L	Stech			<i>D. spurium</i>	KW502615	KW502777	-	KW502738	KW502712	KW502690
Dspu_3	<i>D. spurium</i>	L	Bijlsma 13057	22.01.2011	NL Gelderland	<i>D. spurium</i>	KW502616	KW502778	KW502653	KW502739	-	KW502691
Dspu_4	<i>D. spurium</i>	L	Aptroot 69776	11.07.2011	NL Drenthe	<i>D. spurium</i>	KW502617	KW502779	KW502654	KW502740	-	KW502692
Dra	<i>D. tauricum</i>	L	Stech 8911228.3	28.12.1991	DE Nordrhein-Westfalen	<i>D. tauricum</i>	KJ651034	KJ651088	KJ650823	KJ650994	KJ650944	KJ650887
Dra_2	<i>D. tauricum</i>	L	Smulders 10140	05.11.2010	NL NorthBrabant	<i>D. tauricum</i>	KJ651043	KJ651099	KJ650841	KJ650995	-	KJ650899
Dra_3	<i>D. tauricum</i>	L	Pellicaan s.n.	22.04.2010	NL Utrecht	<i>D. tauricum</i>	KJ651044	KJ651100	KJ650842	KJ650996	-	KJ650900
Dra_4	<i>D. tauricum</i>	L	Buter 73747	13.07.2010	NL NorthBrabant	<i>D. tauricum</i>	KJ651045	KJ651101	KJ650843	KJ650997	-	KJ650901
FIN_1	<i>D. septentrionale</i>	S	Hedenäs & Bisang B194528	17.09.2010	FI Åland	<i>D. brevifolium</i>	KJ796619	KJ796596	KJ796529	KJ796637	KJ796571	KJ796546
FIN_2	<i>D. undulatum</i>	S	Hedenäs & Bisang B198289	25.05.2013	FI Åland	<i>D. undulatum</i>	KJ796623	KJ796601	KJ796534	KJ796639	KJ796574	KJ796548
FR_1	<i>D. scoparium</i>	L	Martinez s.n.	10.09.2008	FR Alpes-Maritimes	<i>D. scoparium</i>	KF423848	KF423936	KF423473	KF423775	KF423682	KF423580
FR_2	<i>D. bonjeanii</i>	L	Hovenkamp 10-43	01.08.2010	FR Savoie	<i>Dicranum</i> sp.	KF423849	KF423937	KF423474	-	KF423683	KF423581
FR_3	<i>D. bonjeanii</i>	L	Hovenkamp 10-47	01.08.2010	FR Savoie	<i>Dicranum</i> sp.	KF423850	KF423938	KF423475	KF423776	KF423684	KF423582
FR_4	<i>D. bonjeanii</i>	L	Hovenkamp 10-43	01.08.2010	FR Savoie	<i>Dicranum</i> sp.	KF423851	KF423939	KF423476	-	KF423685	KF423583

Voucher	species	Herb.	collector and number	date of collection	Locality	original label	rps4-trnT	trnL-trnF	trnH-psbA	rps19-rpl2	rpoB	ITS
GI_1011	<i>D. scoparium</i>	G	Lang & Price 20080701.1	01.07.2008	CH CantonVaud	<i>D. scoparium</i>	GQ428088	GQ428041	GQ427997	GQ427960	GQ427919	KF423584
GL1	<i>D. scoparium</i>	CANW	Ireland, Dugal & Ley 23775	22.06.1989	CA Ontario, Gloucester	<i>D. scoparium</i>	-	KF423940	KF423477	KF423777	KF423686	KF423585
GR_1	<i>D. scoparium</i>	L	Papp 10/77/6	15.07.2010	GR CentralMacedonia	<i>D. scoparium</i>	KF423852	KF423941	KF423478	KF423778	KF423687	KF423586
HUN_1	<i>D. scoparium</i>	UBC	Schofield 104660	31.08.1995	HU NorthernHU	<i>D. scoparium</i>	KF423853	KF423942	KF423479	KF423779	KF423688	KF423587
Is_1	<i>D. scoparium</i>	ICEL	Elmarsdóttir 42630	25.07.2001	IS Norðurlandeystra	<i>D. scoparium</i>	KF423854	KF423943	KF423480	KF423780	KF423689	KF423588
Is_10	<i>D. scoparium</i>	ICEL	Egglisson 44119	20.08.1997	IS Norðurlandvestra	<i>D. scoparium</i>	KF423862	KF423951	KF423488	KF423788	KF423697	KF423596
Is_2	<i>D. scoparium</i>	ICEL	Egglisson 44218	17.07.2003	IS Norðurlandeystra	<i>D. scoparium</i>	KF423855	KF423944	KF423481	KF423781	KF423690	KF423589
Is_3	<i>D. scoparium</i>	ICEL	Elmarsdóttir 44446	24.08.2004	IS Norðurlandeystra	<i>D. scoparium</i>	KF423856	KF423945	KF423482	KF423782	KF423691	KF423590
Is_4	<i>D. scoparium</i>	ICEL	Börissón 43747	02.08.2002	IS Norðurland eystra	<i>D. scoparium</i>	KF423857	KF423946	KF423483	KF423783	KF423692	KF423591
Is_5	<i>D. spadiaceum</i>	ICEL	Egglisson 44012	12.08.2002	IS Norðurland vestra	<i>D. scoparium</i>	KF423858	KF423947	KF423484	KF423784	KF423693	KF423592
Is_7	<i>D. spadiaceum</i>	ICEL	Egglisson 43998	11.08.2002	IS Norðurland eystra	<i>D. scoparium</i>	KF423859	KF423948	KF423485	KF423785	KF423694	KF423593
Is_8	<i>D. spadiaceum</i>	ICEL	Egglisson 41447	12.08.2002	IS Norðurland eystra	<i>D. scoparium</i>	KF423860	KF423949	KF423486	KF423786	KF423695	KF423594
Is_9	<i>D. spadiaceum</i>	ICEL	Egglisson 43804	24.07.2002	IS Norðurland vestra	<i>D. scoparium</i>	KF423861	KF423950	KF423487	KF423787	KF423696	KF423595
Ka2031	<i>D. scoparium</i>	L	Lang 20091203.3	03.12.2009	NL Norðurland vestra	<i>D. scoparium</i>	KF423863	KF423952	KF423489	KF423789	KF423698	KF423597
KR9	<i>D. cf. japonicum</i>	JNU	Yoon s.n.	28.10.2011	KR Jeju-doHal lasan	<i>D. scoparium</i>	KF423866	KF423956	KF423493	KF423791	KF423702	KF423600
KR11	<i>D. cf. japonicum</i>	JNU	Yoon s.n.	19.05.2011	KR Jeollanam-do	<i>D. scoparium</i>	KF423867	KF423957	KF423494	-	KF423703	KF423601
KR12	<i>D. cf. japonicum</i>	JNU	Yoon s.n.	18.05.2011	KR Jeollanam-do	<i>D. scoparium</i>	KF423868	KF423958	KF423495	-	KF423704	KF423602
KR5	<i>D. cf. japonicum</i>	JNU	Yoon s.n.	08.03.2011	KR Jeollanam-do	<i>D. scoparium</i>	KF423864	KF423954	KF423491	-	KF423700	KF423598
KR7	<i>D. cf. japonicum</i>	JNU	Yoon s.n.	16.08.2010	KR Jeju-do	<i>D. scoparium</i>	KF423865	KF423955	KF423492	KF423790	KF423701	KF423599
L&A1	<i>D. scoparium</i>	CANW	Ley & al. 1222	11.07.1990	CA Ontario, Lennox & Addington Co.	<i>D. scoparium</i>	-	KF423959	KF423496	KF423792	KF423705	KF423603
MAC_1	<i>D. scoparium</i>	L	Papp 10/87/2	18.07.2010	MK Pelagonia region	<i>D. scoparium</i>	KF423869	KF423960	KF423497	KF423793	KF423706	KF423604
Mad_1	<i>D. scoparium</i>	S	Hedenäs B4566	14.06.1998	PT Madeira	<i>D. scoparium</i>	-	KF423961	KF423498	KF423794	KF423707	KF423605
Mad_2	<i>D. scoparium</i>	S	Hedenäs & Bisang B22461	20.11.1999	PT Madeira	<i>D. scoparium</i>	-	KF423962	KF423499	KF423795	-	KF423606
MS_04576	<i>D. scoparium</i>	L	Stech 04-576	08.08.2004	PT Madeira	<i>D. scoparium</i>	KF423870	KF423963	KF423500	KF423796	KF423708	KF423607

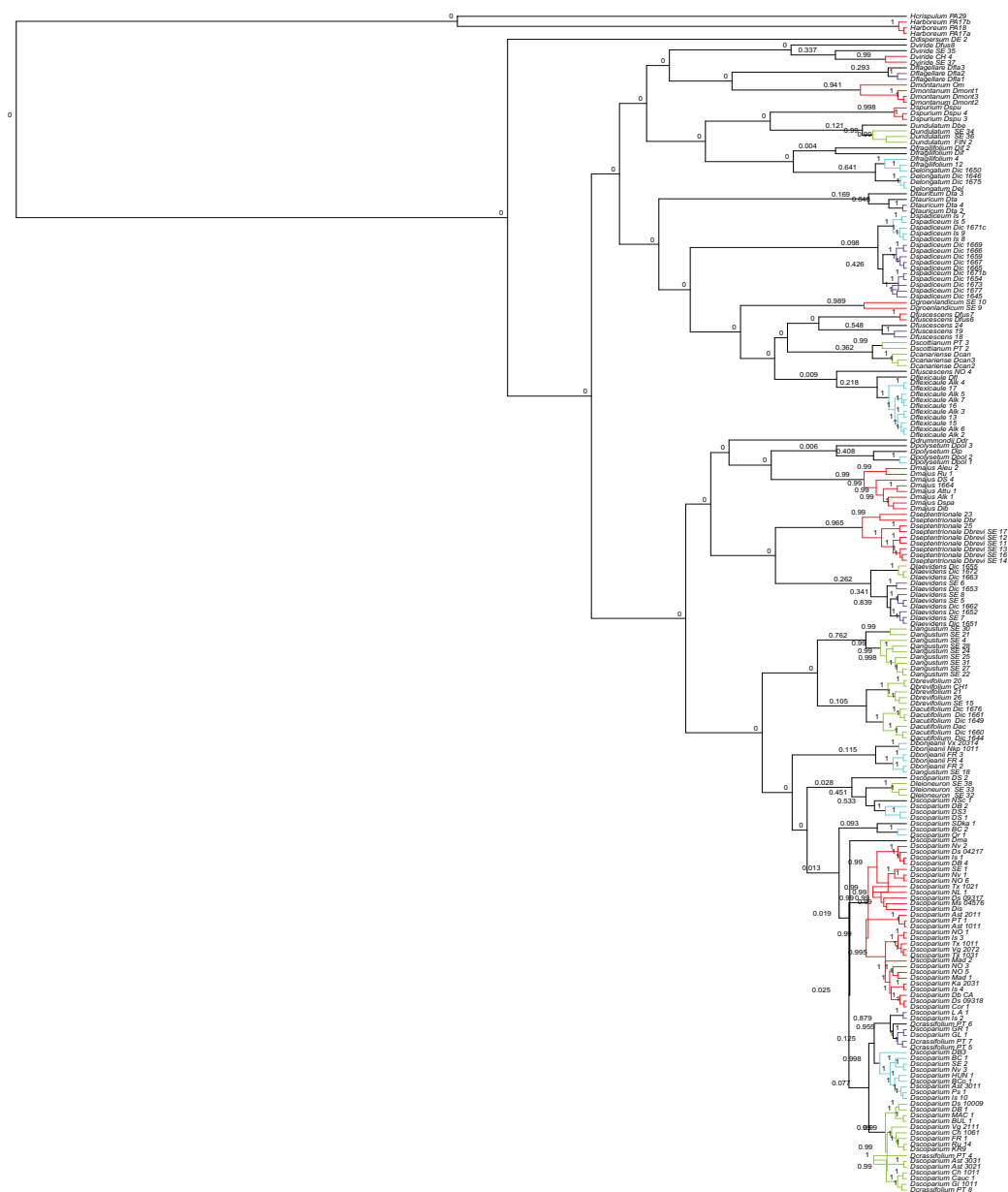
Voucher	species	Herb.	collector and number	date of collection	Locality	original label	rps4-trnT	trnL-trnF	trnH-psbA	rps19-rp2	rpoB	ITS
NB_1	<i>D. scoparium</i>	UBC	McAlpine s.n.	21.08.1990	CA New Brunswick, Charlotte Co.	<i>D. scoparium</i>	-	KF423964	KF423501	KF423797	KF423709	-
Nkp_1011	<i>D. bonjeanii</i>	L	Lang 20091126.2	26.11.2009	NL South Holland	<i>D. scoparium</i>	KF423871	KF423965	KF423502	KF423798	KF423710	KF423608
NL_1	<i>D. scoparium</i>	L	van den Vaart s.n.	2012	NL South Holland	<i>D. scoparium</i>	KF423872	KF423966	KF423503	KF423799	KF423711	KF423609
NO_1	<i>D. scoparium</i>	TRH	Prestø B-7239	18.04.2003	NO Herøy	<i>D. scoparium</i>	KF423873	KF423967	KF423504	KF423800	KF423712	KF423610
NO_3	<i>D. scoparium</i>	TRH	Hassel B-6584	13.04.2005	NO Gloppen	<i>D. scoparium</i>	-	KF423968	KF423505	-	KF423713	KF423611
NO_4	<i>D. fuscens</i>	TRH	Hassel s.n.	11.08.1998	NO Troms	<i>D. scoparium</i>	-	KF423969	KF423506	-	KF423714	KF423612
NO_5	<i>D. scoparium</i>	TRH	Prestø B-7017	31.05.2004	NO Gjemnes	<i>D. scoparium</i>	-	KF423970	KF423507	-	KF423715	KF423613
NO_6	<i>D. scoparium</i>	TRH	Prestø B-7605	30.05.2004	NO Frei	<i>D. scoparium</i>	KF423874	KF423971	KF423508	KF423801	KF423716	KF423614
NSc_1	<i>D. cf. scoparium</i>	UBC	Schofield & Schofield 95348	09.05.1990	CA Novascotia, Digby Co.	<i>D. scoparium</i>	-	KF424001	KF423540	-	-	KF423647
NV1	<i>D. scoparium</i>	O	Hanssen 3964/05	01.08.1996	NO Buskerud	<i>D. scoparium</i>	KF423875	KF423972	KF423509	KF423802	KF423717	KF423615
NV2	<i>D. scoparium</i>	O	Egan GE-20	18.04.2003	NO Østfold	<i>D. scottianum</i>	KF423876	KF423973	KF423510	KF423803	KF423718	KF423616
NV3	<i>D. scoparium</i>	O	Hanssen 5399	01.05.1999	NO Buskerud	<i>D. scoparium</i>	KF423877	KF423974	KF423511	KF423804	KF423719	KF423617
Om	<i>D. montanum</i>	L	Stech B890721.5	21.07.1989	DE Nordrhein-Westfalen	<i>D. montanum</i>	KF423878	AF1 29589/ AF1 29562	KF423512	KF423805	KF423720	AF1 4411 5
Or_1	<i>D. scoparium</i>	UBC	Schofield, Harpel & Forest Service Personnel 116776	10.10.2000	US Oregon, Umatilla National Forest	<i>D. scoparium</i>	KM502612	KM502774	KM502650	-	KM502710	KM502687
PA17a	<i>H. arboreum</i>	L	Stech PA17a	11	BR Parana, Município CampinaGrande do Sul	<i>Holomitrium</i>	KF423894	KF423994	KF423532	KF423818	KF423740	KF423640
PA17b	<i>H. arboreum</i>	L	Stech PA17b	11	BR Parana, Município CampinaGrande do Sul	<i>Holomitrium</i>	KF423893	KF423993	KF423531	-	KF423739	KF423639
PA18	<i>H. arboreum</i>	L	Stech PA18	11	BR Parana, Município CampinaGrande do Sul	<i>Holomitrium</i>	KF423895	KF423995	KF423533	-	KF423741	KF423641
PA29	<i>H. crispulum</i>	L	Stech PA29	11	BR Parana, Município CampinaGrande do Sul	<i>Holomitrium</i>	KF423892	KF423992	KF423530	-	KF423738	KF423638
PS1	<i>D. scoparium</i>	CANM	Ireland 23915	06.09.1989	CA Ontario, Parry Sound	<i>D. scoparium</i>	-	KF423975	KF423513	KF423806	KF423721	KF423618

Voucher	species	Herb.	collector and number	date of collection	Locality	original label	rps4-trnT	trnL-trnF	trnH-psbA	rps19-rpl2	rpoB	ITS
PT_1	<i>D. scoparium</i>	S	Hedenäs B44512	22.09.2000	PT Centro	<i>D. scoparium</i>	-	KF423976	KF423514	KF423807	KF423722	KF423619
PT_2	<i>D. scoparium</i>	Herb. H. Walthe	Walthe AZ-0102	09.08.2012	PT Azores	<i>Dicranum</i> sp.	KJ651046	KJ651102	KJ650844	-	KJ650956	KJ650902
PT_3	<i>D. scoparium</i>	Herb. H. Walthe	Frahm AZ-0184	10.08.2012	PT Azores	<i>Dicranum</i> sp.	KJ651047	KJ651103	KJ650845	-	KJ650957	KJ650903
PT_4	<i>D. scoparium</i>	LISU	Sérgio FRID 13g	25.11.2010	PT Trás-os-Montes e Alto Douro	<i>D. crassifolium</i>	KM502599	KM502752	KM502630	-	-	KM502667
PT_5	<i>D. scoparium</i>	LISU	Sérgio 13796	19.05.2006	PT Beira Litora	<i>D. crassifolium</i>	-	KM502753	KM502631	-	-	KM502668
PT_6	<i>D. scoparium</i>	LISU	Sérgio 14679	30.12.2010	PT Beira Alta	<i>D. crassifolium</i>	KM502600	KM502754	KM502632	KM502725	-	KM502669
PT_7	<i>D. scoparium</i>	LISU	Garcia 205276	26.06.2003	PT Douro Litoral	<i>D. crassifolium</i>	-	KM502755	KM502633	KM502726	-	KM502670
PT_8	<i>D. scoparium</i>	LISU	Sérgio, Carvalho, Garcia & Louro 212140	04.05.2004	PT Trás-os-Montes e Alto Douro	<i>D. crassifolium</i>	-	KM502756	KM502634	-	-	KM502671
Ru_1	<i>D. majus</i>	L	Lang 20100906.2	06.09.2010	RU Primorsky Prov.	<i>D. majus</i>	KF423879	KF423977	KF423515	KF423808	KF423723	KF423620
Ru_14	<i>D. scoparium</i>	L	van Melick 214110	18.09.2010	RU Irkutsk Prov.	<i>D. scoparium</i>	KM502613	KM502775	KM502651	-	KM502711	KM502688
Ru_16	<i>D. cf. larifolium</i>	L	Lang 20100910.8	11.09.2014	RU Primorsky Prov.	<i>D. scoparium</i>	KF423909	KF424010	KF423549	-	KF423750	KF423655
Ru_24	<i>D. cf. larifolium</i>	L	Lang 20100910.10	11.09.2014	RU Primorsky Prov.	<i>D. scoparium</i>	KF423910	KF424011	KF423550	-	KF423751	KF423656
Ru_27	<i>D. cf. larifolium</i>	L	Lang 20100909.9	10.09.2014	RU Primorsky Prov.	<i>D. scoparium</i>	KF423911	KF424012	KF423551	KF423821	-	-
Ru_3	<i>D. scoparium</i>	L	Lang 20100908.17	08.09.2010	RU Primorsky Prov.	<i>D. scoparium</i>	-	KF423979	KF423517	-	KF423725	KF423622
Ru_33	<i>D. cf. larifolium</i>	L	Lang 20100905.10	06.09.2014	RU Primorsky Prov.	<i>D. scoparium</i>	KF423907	KF424008	KF423547	-	-	KF423653
Ru_35	<i>D. cf. larifolium</i>	L	Lang & Cherdantseva 20100908.14	09.09.2014	RU Primorsky Prov.	<i>D. scoparium</i>	KF423906	KF424007	KF423546	-	KF423748	KF423652
Ru_4	<i>D. scoparium</i>	L	Lang 20100908.22	08.09.2010	RU Primorsky Prov.	<i>D. scoparium</i>	KF423881	KF423980	KF423518	KF423809	KF423726	KF423623
Ru_44	<i>D. cf. larifolium</i>	L	Lang 20100906.17	07.09.2014	RU Primorsky Prov.	<i>D. scoparium</i>	KF423904	KF424005	KF423544	-	KF423746	KF423650
Ru_46	<i>D. cf. larifolium</i>	L	Lang 20100906.6	07.09.2014	RU Primorsky Prov.	<i>D. scoparium</i>	KF423903	KF424004	KF423543	-	-	KF423649
Ru_49	<i>D. cf. larifolium</i>	L	Lang 20100906.11	07.09.2014	RU Primorsky Prov.	<i>D. scoparium</i>	KF423908	KF424009	KF423548	-	KF423749	KF423654
Ru_5_A	<i>D. nipponense</i>	L	Lang 20100909.1	09.09.2010	RU Primorsky Prov.	<i>D. bonjeonii</i>	KF423882	KF423981	KF423519	KF423810	KF423727	KF423624
Ru_5_B	<i>D. nipponense</i>	L	Lang 20100909.1	09.09.2010	RU Primorsky Prov.	<i>D. scoparium</i>	KF423883	KF423982	KF423520	KF423811	KF423728	KF423625
Ru_50	<i>D. cf. larifolium</i>	L	Lang 20100906.8	07.09.2014	RU Primorsky Prov.	<i>D. scoparium</i>	KF423905	KF424006	KF423545	-	KF423747	KF423651
Ru_6	<i>D. scoparium</i>	L	Lang 20100910.6	10.09.2010	RU Primorsky Prov.	<i>D. scoparium</i>	-	KF423983	KF423521	-	KF423729	KF423626

Voucher	species	Herb.	collector and number	date of col- lection	Locality	original label	rps4-trnT	trnL-trnF	trnH-psbA	rps19-rpl2	rpoB	ITS
Ru_7	<i>D. scoparium</i>	L	Lang 20100911.3	11.09.2010	RU Primorsky Prov.	<i>D. scoparium</i>	KF423884	KF423984	KF423522	-	KF423730	KF423627
Ru_8	<i>D. scoparium</i>	L	Lang 20100908.6	08.09.2010	RU Primorsky Prov.	<i>D. scoparium</i>	KF423885	KF423985	KF423523	-	KF423731	KF423628
SDak_1	<i>D. scoparium</i>	UBC	Churchill & Churchill 19597	18.8.1998	US South Dakota, Pennington Co.	<i>D. scoparium</i>	KM502614	KM502776	KM502652	-	-	KM502689
SE_1	<i>D. scoparium</i>	S	Odelvik B163166	12.04.2009	SE Gästrikland	<i>D. scoparium</i>	KF423886	KF423986	KF423524	KF423812	KF423732	KF423629
SE_10	<i>D. groenlandicum</i>	S	Hedenäs B74363	17.08.2002	SE Torne Lappmark	<i>D. groenlandicum</i>	-	KJ651089	KJ650830	KJ650984	KJ650946	KJ650888
SE_11	<i>D. septentrionale</i>	S	Hedenäs B74004	07.09.2002	SE Uppland	<i>D. brevifolium</i>	KJ796613	KJ796590	KJ796523	KJ796631	KJ796566	KJ796540
SE_12	<i>D. septentrionale</i>	S	Hedenäs B122921	10.07.2007	SE Härjedalen	<i>D. brevifolium</i>	KJ796614	KJ796591	KJ796524	KJ796632	KJ796567	KJ796541
SE_13	<i>D. septentrionale</i>	S	Hedenäs B183369	26.05.2011	SE Gotland	<i>D. brevifolium</i>	KJ796615	KJ796592	KJ796525	KJ796633	-	KJ796542
SE_14	<i>D. septentrionale</i>	S	Hedenäs B193369	26.07.2012	SE Södermanland	<i>D. brevifolium</i>	KJ796616	KJ796593	KJ796526	KJ796634	KJ796568	KJ796543
SE_15	<i>D. brevifolium</i>	S	Hedenäs B175744	21.05.2010	SE Hälsingland	<i>D. brevifolium</i>	KJ651040	KJ651096	KJ650838	KJ650991	KJ650954	KJ650896
SE_16	<i>D. septentrionale</i>	S	Hallingbäck 46166	07.08.2008	SE Tornelappmark	<i>D. brevifolium</i>	KJ796617	KJ796594	KJ796527	KJ796635	KJ796569	KJ796544
SE_17	<i>D. septentrionale</i>	S	Hedenäs & Bisang B84948	11.08.2003	SE Gotland	<i>D. brevifolium</i>	KJ796618	KJ796595	KJ796528	KJ796636	KJ796570	KJ796545
SE_18	S	S	Norin B132878	29.06.2002	SE Torne Lappmark	<i>D. angustum</i>	KM502595	KM502748	KM502626	KM502721	KM502701	KM502663
SE_2	<i>D. scoparium</i>	S	Hedenäs B164630	03.08.2009	SE Jämtland	<i>D. scoparium</i>	KF423887	KF423987	KF423525	KF423813	KF423733	KF423630
SE_21	<i>D. angustum</i>	S	Hedenäs, Rönblom, Odelvik & Hammede B139061	26.06.2012	SE Dalarna	<i>D. angustum</i>	KM502592	KM502744	KM502622	KM502717	KM502697	KM502659
SE_22	<i>D. angustum</i>	S	Hedenäs B193541	29.08.2012	SE Jämtland	<i>D. angustum</i>	KJ651036	KJ651092	KJ650834	KJ650987	KJ650950	KJ650892
SE_24	<i>D. angustum</i>	S	Westerberg B132876	25.08.2006	SE Norrbotten	<i>D. angustum</i>	KM502593	KM502745	KM502623	KM502718	KM502698	KM502660
SE_25	<i>D. angustum</i>	S	Hedenäs, Bisang & Persson B105001	09.09.2005	SE Jämtland	<i>D. angustum</i>	-	KJ651090	KJ650832	KJ650986	KJ650948	KJ650890
SE_27	<i>D. angustum</i>	S	Norin B132922	11.08.2002	SE Tornelappmark	<i>D. angustum</i>	KJ651037	KJ651093	KJ650835	KJ650988	KJ650951	KJ650893
SE_28	<i>D. angustum</i>	S	Johansson B132925	08.08.2006	SE Tornelappmark	<i>D. angustum</i>	KM502594	KM502746	KM502624	KM502719	KM502699	KM502661
SE_30	<i>D. angustum</i>	S	Hedenäs B107583	14.09.2009	SE Jämtland	<i>D. angustum</i>	-	KM502747	KM502625	KM502720	KM502700	KM502662
SE_31	<i>D. angustum</i>	S	Johansson B132926	08.07.2003	SE Tornelappmark	<i>D. angustum</i>	KJ651038	KJ651094	KJ650836	KJ650989	KJ650952	KJ650894
SE_32	<i>D. leioneuron</i>	S	Hedenäs B116708	06.10.2002	SE Medelpad	<i>D. leioneuron</i>	KJ651048	KJ651104	KJ650846	-	KJ650958	KJ650904
SE_33	<i>D. leioneuron</i>	S	Laagaard, Gustafsson, Poulsen, Brunbjerg 232001	23.05.2010	SE Hälsingland	<i>D. leioneuron</i>	KJ651049	KJ651105	KJ650847	KJ650998	KJ650959	KJ650905

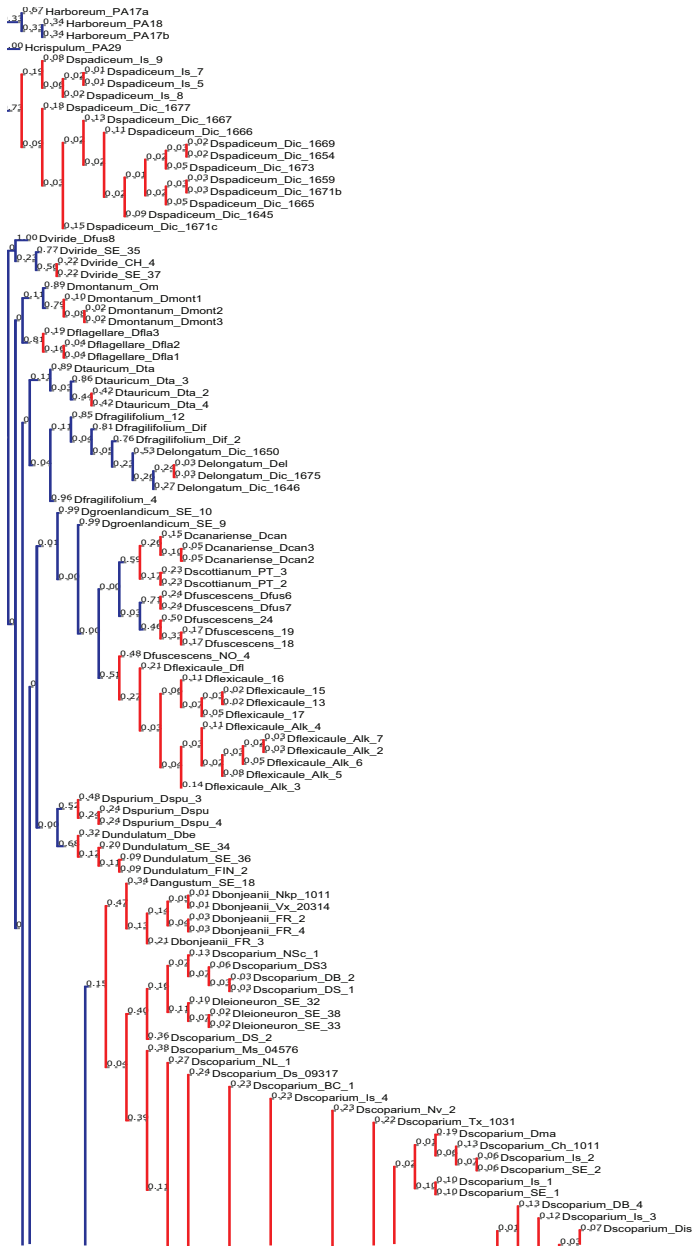
Voucher	species	Herb.	collector and number	date of collection	Locality	original label	rps4-trnT	trnL-trnF	trnH-psbA	rps19-rpl2	rpoB	ITS
SE_34	<i>D. undulatum</i>	S	Hedenäs, Odelvik & Rönblom B199960	12.09.2013	SE Ångermanland	<i>D. undulatum</i>	KJ796624	KJ796602	KJ796535	KJ796640	KJ796575	KJ796549
SE_35	<i>D. viride</i>	S	Hagström B172407	27.05.2009	SE Småland	<i>D. viride</i>	KM502620	KM502782	KM502657	KM502743	KM502715	KM502695
SE_36	<i>D. undulatum</i>	S	Hedenäs, Odelvik & Rönblom B199806	09.09.2013	SE Ångermanland	<i>D. undulatum</i>	KJ796625	KJ796603	KJ796536	KJ796641	KJ796576	KJ796550
SE_37	<i>D. viride</i>	S	Lönnell B48734	15.06.1999	SE Gotland	<i>D. viride</i>	KM502621	KM502783	KM502658	-	KM502716	KM502696
SE_38	<i>D. leioneuron</i>	S	Hedenäs & Persson B135011	07.05.2008	SE Dalaland	<i>D. leioneuron</i> KJ651050	KJ651050	KJ651106	KJ650848	KJ650999	KJ650960	KJ650906
SE_4	<i>D. angustum</i>	S	Norin B131031	06.08.2002	SE Torne Lappmark	<i>D. laevigens</i>	-	KJ651091	KJ650833	-	KJ650949	KJ650891
SE_5	<i>D. laevigens</i>	S	Westerberg B131027	13.07.2002	SE Lule Lappmark	<i>D. laevigens</i>	-	KM502764	KM502641	KM502730	KM502708	KM502677
SE_6	<i>D. laevigens</i>	S	Hedenäs, Bisang & Persson B105000	09.09.2005	SE Jämtland	<i>D. laevigens</i>	KJ651035	-	KJ650831	KJ650985	KJ650947	KJ650889
SE_7	<i>D. laevigens</i>	S	Hedenäs B85000	10.10.1995	SE Dalarna	<i>D. laevigens</i>	-	KM502765	KM502642	-	-	KM502678
SE_8	<i>D. laevigens</i>	S	Hedenäs B74327	21.09.2002	SE Jämtland	<i>D. laevigens</i>	KM502605	KM502766	KM502643	KM502731	KM502709	KM502679
SE_9	<i>D. groenlandicum</i>	S	Hedenäs B74365	21.09.2002	SE Jämtland	<i>D. groenlandicum</i>	-	KM502763	KM502640	-	KM502707	KM502676
Tx1011	<i>D. scoparium</i>	L	Lang 20100314.1	14.03.2010	NL North Holland	<i>D. scoparium</i>	KF423888	KF423988	KF423526	KF423814	KF423734	KF423631
Tx1021	<i>D. scoparium</i>	L	Lang 20100314.5	14.03.2010	NL North Holland	<i>D. scoparium</i>	KF423889	KF423989	KF423527	KF423815	KF423735	KF423632
Tx1031	<i>D. scoparium</i>	L	Lang 20100314.9	14.03.2010	NL North Holland	<i>D. scoparium</i>	KF423890	KF423990	KF423528	KF423816	KF423736	KF423633
Vg_2072	<i>D. scoparium</i>	G	Lang & Price 20070719.31	19.07.2007	CH Canton Geneva	<i>D. scoparium</i>	GU068406	GU068490	GU068461	GU068377	GU068434	KF423634
Vg_2111	<i>D. scoparium</i>	G	Lang & Price 20070719.35	19.07.2007	CH Canton Geneva	<i>D. scoparium</i>	GU068406	GU068490	GU068461	GU068377	GU068434	KF423634
Vx_20314	<i>D. bonjeanii</i>	G	Lang, Price & Naciri 20070523.21	23.05.2007	CH Canton Geneva	<i>D. bonjeanii</i>	GU068406	GU068490	GU068461	GU068377	GU068434	KF423634
Wa_1	<i>D. howellii</i>	UBC	Schofield & Harpel 120572	23.10.2002	US Washington, Snohomish Co.	<i>D. scoparium</i>	KF423891	KF423991	KF423529	KF423817	KF423737	KF423637

APPENDIX 2.

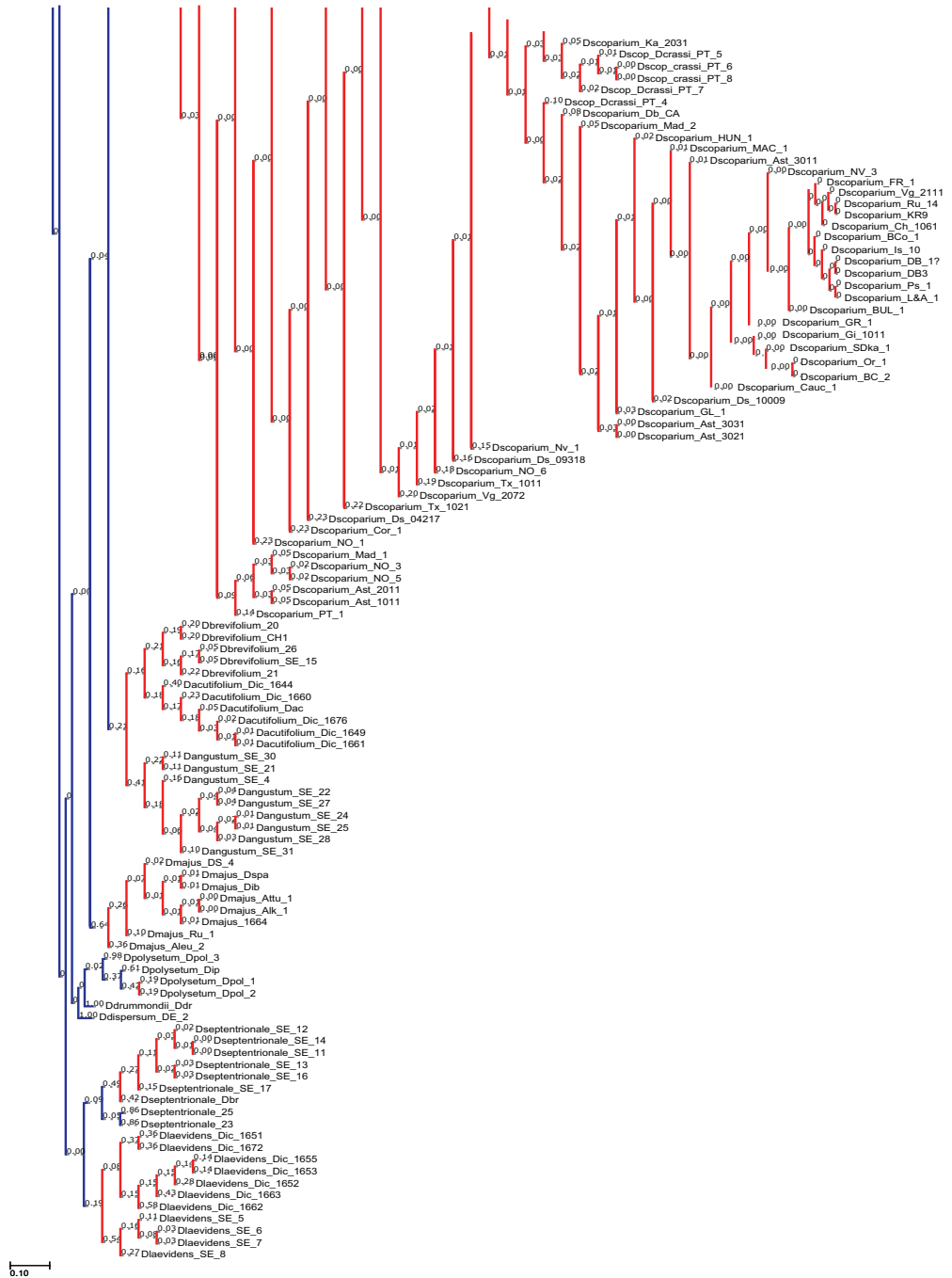


APPENDIX 2. General mixed Yules coalescent (GMYC) multiple threshold model from plastid and nuclear data. Branch length fitted a strict molecular clock, with estimated entities indicated in colors fitting the lineages-through-time plot for multiple GMYC thresholds.

APPENDIX 3.

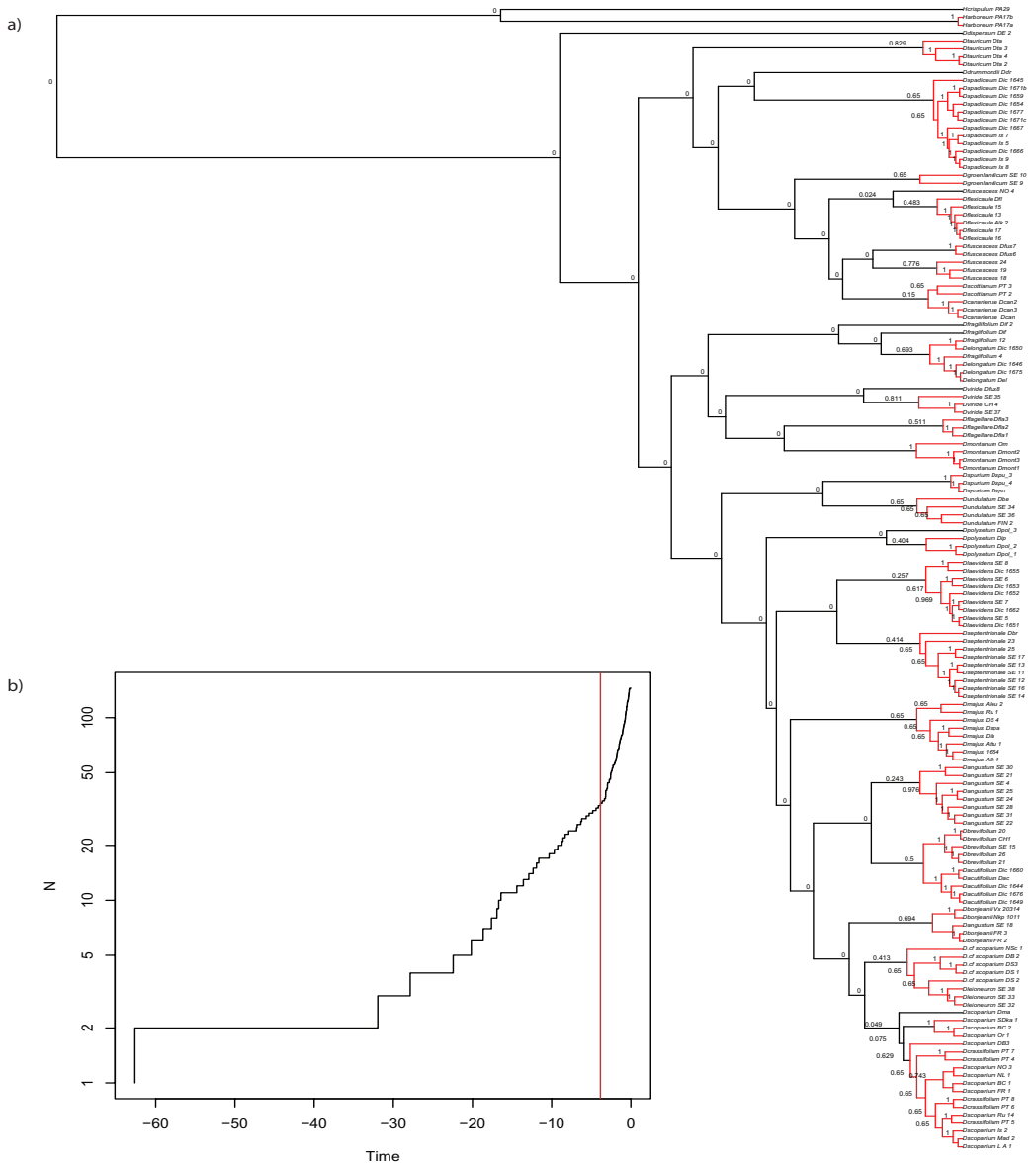


APPENDIX 3. PTP ML partitions obtained from <http://species.h-its.org/>. Species boundaries are indicated in red.



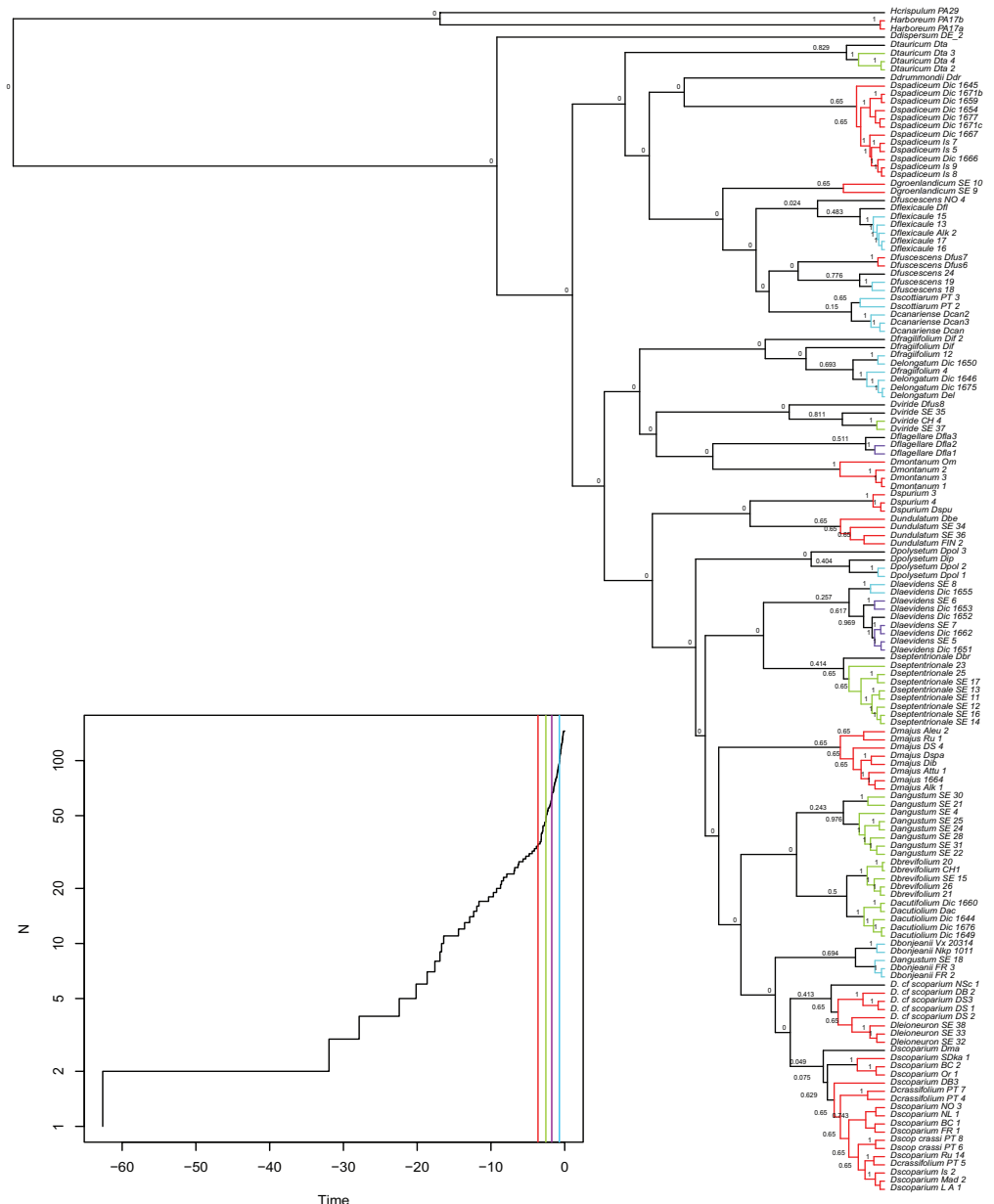
APPENDIX 3. suite

APPENDIX 4 GMYC tree obtained from a reduced dataset

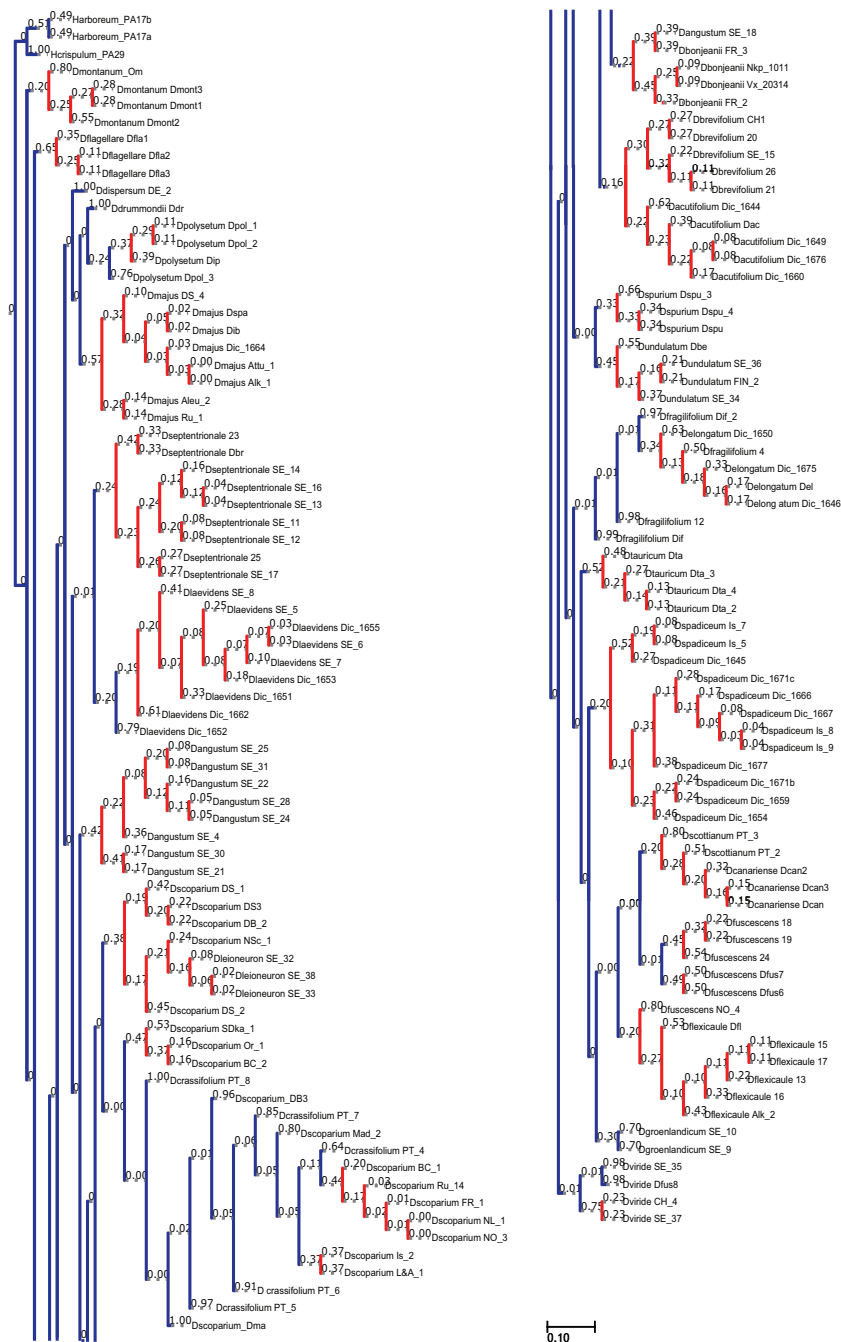


GMYC ultrametric tree depicting species delimitation of 20 morpho-species *Dicranum* based on Bayesian analysis using a Yule model in BEAST and with fit of the general mixed Yules coalescent (GMYC) single threshold model from plastid and nuclear data. Branch length fitted a strict molecular clock, with estimated entities indicated in red. (a). Lineages-through-time plot for single (b) GMYC threshold is illustrated. The vertical red line represent the timing of the earliest coalescent event.

a)



GMYC ultrametric tree depicting species delimitation of 20 morpho-species *Dicranum* with fit of the general mixed Yules coalescent (GMYC) multiple threshold model from plastid and nuclear data. Branch length fitted a strict molecular clock, with estimated entities indicated in colors (a) fitting the lineages-through-time plot for multiple GMYC threshold (b). The vertical color lines represent the timing of the four coalescent event.



PTP ML partitions obtained from <http://species.h-its.org/>. Species boundaries are indicated in red.

Curriculum Vitae

Annick S. Lang was born on January 19, 1982 in Meyrin, Switzerland. During her secondary education, Annick participated in a language exchange program in Australia. It was in the Australian rainforest of Fraser Island that she discovered her interest for botany and ecological studies. She obtained her high school diploma in modern languages at Collège Rousseau in Geneva in 2001. That same year, she started biology studies at the University of Neuchâtel, Switzerland, where she obtained a bachelor's degree three years later. While studying various subjects related to plant biology (plant- insect interactions, phytosociology, plant ecology), her interest for evolutionary botany, and especially bryology, grew stronger. In 2006, Annick obtained a master's degree in Behavioural Ecology and Evolution from the same university, with a major in Botany. Her master's project focused on population genetics of a rare European fen orchid, *Liparis loeselii* (L.) Rich, in Franche-Comté (France) and Switzerland. During her master's, she also participated in the European project 'Intrabiodiv' and worked as a teaching assistant for botanical practicum courses.

After obtaining her master's degree, she went back to Geneva where she worked for three years at the Conservatory and Botanical Garden on a liverworts type collection digitalisation project. This is where Annick was introduced to the moss genus *Dicranum* and undertook preliminary investigations on *D. scoparium* with the bryophyte, fern and gymnosperm curator Dr. M. J. Price.

Annick started her PhD in November 2009 at the National Herbarium of the Netherlands (now Naturalis Biodiversity Center) where she pursued bryological studies under the supervision of Dr. M. Stech. During her PhD, she participated in international conferences in Vladivostok (Russia), Melbourne (Australia), and New York (U. S. A.) and was involved in the organisation of Naturalis's seminars. Furthermore, she had the opportunity to supervise one MSc project on male dwarfism in *D. scoparium*. After her graduation, Annick intends to continue her research on the genus *Dicranum* with the aim to study the effect of pseudomonoicy on population level in more detail.

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Lang A. S. & M. Stech. *What does the phylogeography of widespread moss species reveal? New insight with the Holarctic moss Dicranum scoparium Hedw.* IBC meeting, Melbourne, 24-30 July 2011 (talk).

Lang A. S. & M. Stech. *What does the phylogeography of widespread moss species reveal? New insight with the Holarctic moss Dicranum scoparium Hedw.* PhD day, Leiden, 9.12.2012 (talk)

Lang A. S. & R. Gama De Nato. *All you've ever wondered about mosses!* Staff seminar series, Leiden, 20.3.2012 (talk).

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Acknowledgments

I see earning a PhD like a hike in the mountains: the start is easy and delightful, maybe a little scary, as one cannot fully grasp what is really going to happen. The journey continues with ups and downs; still nice although sometimes difficult. During these harder moments, it is always gratifying to check on a map to see what one has already accomplished. The most difficult moment is probably when one reaches the end: the path gets steeper and the hardest passes remain to be crossed. But the view from the summit is worth the effort, I can tell!

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