

## Original Article

# Assessing gene flow between *Dicranum scoparium* Hedw. and *D. bonjeanii* De Not. (Dicranaceae) using single nucleotide polymorphisms (SNPs)

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### ABSTRACT

While hybridisation in vascular plants has received considerable attention, hybridisation in bryophytes is still relatively understudied. Here we investigate hybridisation between two species from the moss genus *Dicranum* Hedw. *Dicranum scoparium* Hedw. and *D. bonjeanii* De Not. are two moss species of the *D. scoparium* species complex with partially overlapping morphology and habitat ranges. This study aimed to investigate a potential hybridisation pathway between the two species by using single nucleotide polymorphisms (SNP), morphologically identifying the sex of the specimens and analysing potential sex-specific SNP markers, focusing on southern Sweden. The species differ by *D. scoparium* being polymorphic and *D. bonjeanii* monomorphic for the used SNP markers. The SNP markers genetically separate *D. scoparium* and *D. bonjeanii* specimens, although admixture between the species was observed on a limited scale. This admixture appears to originate from unidirectional fertilisation of *D. bonjeanii* by *D. scoparium* (with a genome skewed towards *D. scoparium* as a result), possibly followed by back-crossing of first-generation hybrids with *D. scoparium*. Male expressing specimens were completely absent in the *D. bonjeanii* samples, making a fertilisation of *D. bonjeanii* by males of *D. scoparium* more likely. No sex-specificity was observed in the used SNP markers.

**Keywords:** admixture; bryophytes; hybridisation; SNP; southern Sweden

### INTRODUCTION

Hybridisation is known to extensively have contributed to angiosperm diversity (Soltis and Soltis 2009), as it can drive processes as speciation, both with and without the formation of polyploids, leading to increased diversification and adaptive capabilities of the offspring. Hybridisation has been frequently investigated among vascular plants and, for example, has been shown to occur in ~25% of the vascular plants in the UK (Mallet 2005). Whitney *et al.* (2010) showed the occurrence of hybridisation in 40% of the vascular plant families. Hybridisation in bryophytes and subsequently mosses is understudied and its relevance for evolutionary processes remains relatively unknown (Natcheva and Cronberg 2004). Nevertheless, there are indications that introgression between related species as well as more remote hybridisation across genera, sections, and even families, is more common in mosses than in vascular plants (Ignatov *et al.* 2019).

Dicranaceae Schimp. is a large family of acrocarpous mosses with worldwide occurrence. One of the major genera within the family, *Dicranum* Hedw., comprises ~92 species and has a sub-cosmopolitan distribution (Stech and Frey 2009). Species within the genus are characterised by shoots with a 'broom-like' appearance. Two closely related species are *D. scoparium* Hedw. and *D. bonjeanii* De Not., which together with several other species, form a group known as the *D. scoparium* complex, with partly overlapping morphology. Besides *D. bonjeanii* and *D. scoparium*, the complex contains the Asian species *D. lorifolium* Mitt., *D. japonicum* Mitt., *D. nipponense* Besch., and the North American species *D. howellii* Renaud & Cardot (Lang and Stech 2014 and references therein). The taxon *D. scoparium* s.str. has a wide morphological circumscription and occurs in a large array of habitats, suggesting that taxonomically relevant genomic differentiation might have escaped attention, similar to cryptic speciation in other morphologically variable species (see e.g. Hedenäs 2020a, b).

In contrast, *D. bonjeanii* has a narrower habitat range, being confined to intermediately rich fens and wetlands (Smith 2004, Hallingbäck *et al.* 2005). In the southern-Swedish province of Skåne (Scania) both species can occur in sympatry.

*Dicranum scoparium* and *D. bonjeanii* are of special interest in genetic studies due to their facultative sexual dimorphism. In both *D. scoparium* and *D. bonjeanii* male shoots can be either normal-sized or form dwarfs, dependent on whether they germinate on females or not. This condition is known as facultative nannandry. While obligate nannandry is known to occur in ~20% of the *Dicranum* species, facultative nannandry is to date only known for *D. scoparium* and *D. bonjeanii* (Pichonet and Gradstein 2012). Nonetheless, general information regarding male dwarfism is lacking for most of the species in the genus (Pichonet and Gradstein 2012). Nannandry may constitute a pathway for hybridisation and transfer of genetic material if male spores emanating from one species germinate on females of the other species (Rosengren and Cronberg 2015).

Due to the overlap in habitats of the two species and the potential hybridisation pathway provided by the sympatry and dwarf males, this study aimed to investigate a potential gene flow between *D. scoparium* and *D. bonjeanii*. The study focused on the southern Swedish province of Skåne (Scania), where both species occur in sympatric populations as well as separately from each other. Owing to the overlap in habitat and morphology, we proposed that hybridisation between the two species occurs, which can be detected by signs of admixture in genetic data. We used SNP markers earlier identified by Lang, Gehrmann and Cronberg (2021) to analyse the two species of interest, focusing on Skåne, but also included specimens from other regions as well as specimens from the three Asian species of the *D. scoparium* complex (*D. lorifolium*, *D. japonicum*, and *D. nipponense*). With the extended sampling we aimed to put the results from Skåne in a wider geographic context and provisionally evaluate the suitability of the SNP markers to distinguish species of the *D. scoparium* complex. Finally, we aimed to assess some of these SNP markers that were earlier flagged as potentially sex-specific (Lang AS, unpublished work). This sex-specificity could be of great interest for future studies about, for example, sexual dimorphism, hybridisation, or phylogeography, especially when plants are not (yet) expressing gametangia (see also Korpelainen *et al.* 2008).

## MATERIALS AND METHOD

### Material sampling

Our goal was to select specimens that reflected the regional variation in habitat and substrate across Skåne (Scania), the southernmost province of Sweden. Skåne is characterised by a temperate oceanic climate (Cfb, Köppen-Geiger classification, Beck *et al.* 2023). Habitats and substrates included fens, bogs, sandy fields, large rocks, old rock walls, and tree trunks (both dead and alive). A total of 186 specimens comprising *D. scoparium* (102), *D. bonjeanii* (66), and the three Asian species of the *D. scoparium* complex (18) were included, collected from the field for this study, and gathered from pre-existing collections (Appendix 1). 62 specimens were collected during the autumn/fall of 2021 and spring of 2022 from the Lund and Svedala

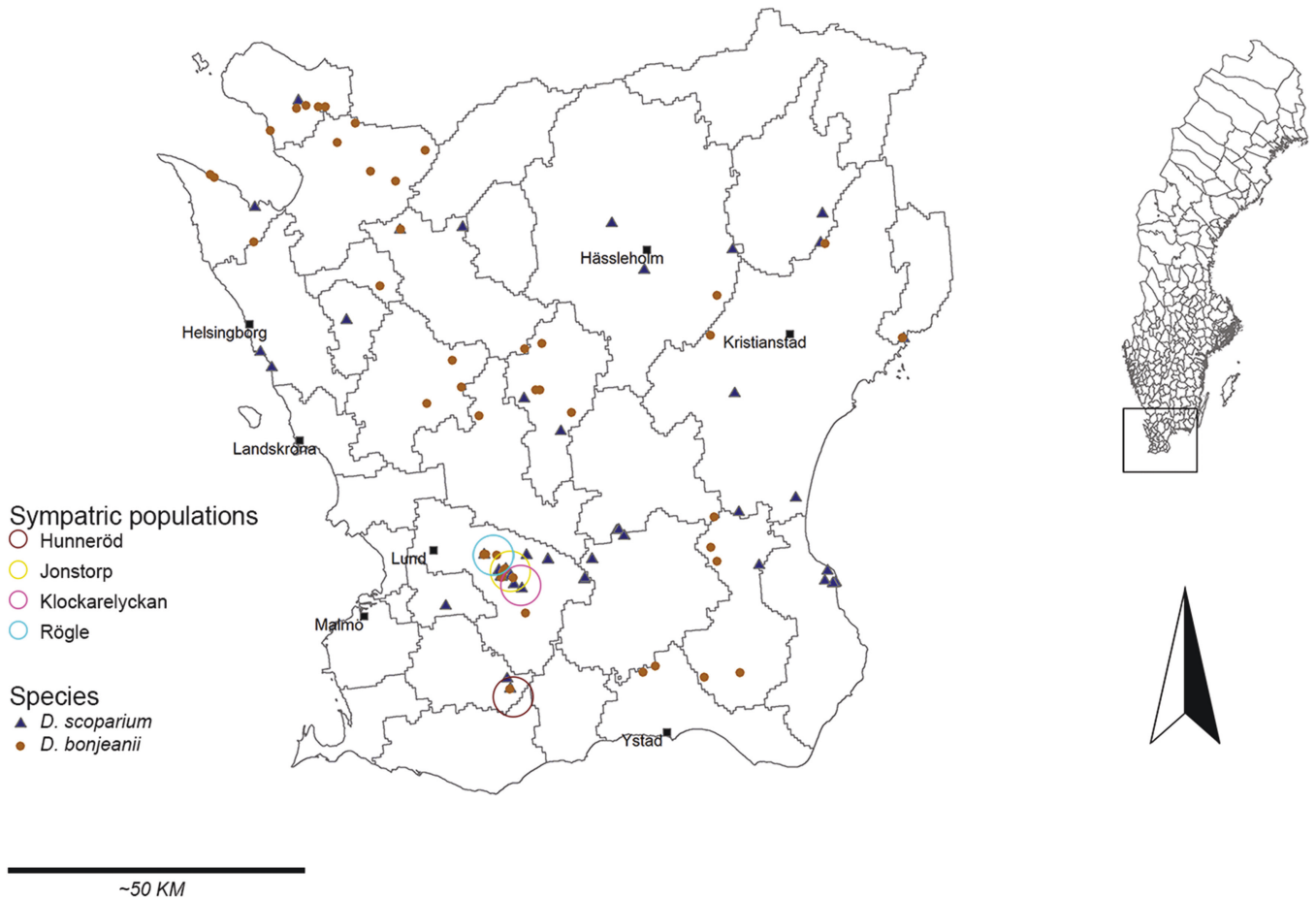
municipalities, including four locations where the species grew in sympatry (Klockarelyckan, Jonstorp, Røgle, and Hunneröd). Samples collected during these field trips are marked in Appendix 1 by a unique location prefix, followed by a species abbreviation (either scop or bonj) and a specimen number (example name: klock.bonj.ex1). Except for the unique specimen names, Appendix 1 also indicates populations the specimens were collected from. Those with the same population name belong to the same population and those populations where both species occurred are considered sympatric. Seven of the populations only included one of the two species of interest (allopatric populations).

An additional 73 specimens originated from collections gathered during the local inventory project 'Bryophytes of Scania' (<http://mossor.mixerdata.com/>), stored at the Lund Botanical Museum (herbarium LD, located in Arkivcentrum Syd: Porfyrvägen 20, 22478 Lund, Sweden). Most of these specimens dated between 2007 and 2022, with some older specimens dating back to the 1990s. Finally, the remaining 51 samples were pre-existing DNA extracts from Lang and Stech (2014), stored at Naturalis Biodiversity Center (Naturalis Biodiversity Center, Darwinweg 2, 2333 CR Leiden, The Netherlands), including DNA isolates of *D. scoparium* and *D. bonjeanii* from areas within and outside of Europe. This material also included the Asian species of the *D. scoparium* complex [*D. japonicum* (6), *D. nipponense* (1) and *D. lorifolium* (11)]. The collection sites of the Scanian specimens including the locations of the sympatric populations are mapped in Figure 1.

### Genetic analysis

DNA was extracted from the upper parts of the shoots using the QIAGEN DNeasy plant mini kit (QIAGEN, Hilden, Germany). The protocol of the manufacturer (see <https://www.qiagen.com/us>) was followed with some modifications that substantially improved the extraction results, especially for dried plant material: sand was added during the tissue-lysing that was executed for 2 × 1 min on 30 Hz, 20 µL proteinase-K was added in the lysing step with time extended from 10 min at 65°C to 15 min at the same temperature. Finally, the elution step was executed by using a volume of 100 µL, passed over the column twice. Incubation on the column was 5 min at room temperature and 5 min at 70°C to elute more DNA. After extraction, the DNA samples were stored in a fridge at 5°C until further processing. Owing to the relatively low DNA concentrations, especially from herbarium samples, the quality of a small selection of DNA extracts was checked by executing a PCR amplification with the primers TrnL(5′)-TrnL(3′) and TrnL-TrnF (Taberlet *et al.* 1991). A gel electrophoresis confirmed that the used regions were successfully amplified during the PCR, indicating that there was no problematic fragmentation of the DNA and DNA extracts were of sufficient quality for downstream processes.

Nuclear SNP markers were selected from a transcriptomic analysis by Lang, Gehrmann and Cronberg (2021). For this purpose, 73 loci (from a cleaned dataset with names ranging from SNP\_001 to SNP\_136) found in 403 *D. scoparium* and three *D. bonjeanii* specimens were filtered according to specific criteria. We selected four SNP markers with alternative dominant alleles in the two species of interest. An allele was considered dominant



**Figure 1.** Map of the province of Skåne and its municipalities, indicating collection sites of specimens of *Dicranum scoparium* in dark blue triangles and *D. bonjeanii* in orange dots. Sympatric populations are visualised with a coloured circle. Some larger towns are visualised with black squares. The map of Sweden shows the location of the province of Skåne within the country.

when it was present in 90% of the samples of *D. scoparium* and 100% of the *D. bonjeanii* specimens (due to the small sample size). In another four of the selected SNP markers, allelic dominance was less clear. These markers had one allele that occurred between 70% and 90% in one of the species and a different, dominant allele in the other species. Finally, another 47 SNP markers were added with less clear allelic affinities. Of these remaining 47 markers, some were marked as ‘potentially sex-specific’ by Lang, AS (unpublished work), meaning that they occur in only one of the sexes. Of these potentially sex-specific SNP markers, two were flagged as potentially female-specific, and 14 were marked as potentially male-specific. During the selection process, it was made certain that these were included in the analysis. Two of the potential sex-specific markers were not present in the cleaned dataset from Lang *et al.* (2021), but were added to the present dataset anyways. A total of 55 markers was used for the analysis, as this number exactly fitted with two multiplex sequencing pools. A list of the selected SNP markers, including those suggested to be sex-specific, is presented in Appendix 2.

The DNA extracts were transported to the Mutation Analysis Facility (Karolinska University Hospital, Mutation Analysis Facility, MAF TRACK, Novum plan 5, Hälsovägen 7, 141 57 Huddinge, Sweden) for PCR and sequencing on an Agena

Bioscience Massarray (<https://www.agenabio.com/products/massarray-system/>), which is a matrix assisted laser desorption/ionisation–time of flight-based system (Oeth *et al.* 2009). The procedure started with a PCR by adding primers specifically designed to fit the flanking regions of the selected SNP markers (see Appendix 2 for SNP markers with flanking regions). Subsequently, the PCR mixture was cleaned by adding an SAP enzyme. Finally, a single base pair extension reaction into the SNP site was executed by using mass-modified dideoxynucleotides that were also modified to prevent further extension of the fragment. The final mixture was then analysed for allele calling (e.g. Oeth *et al.* 2009, Ellis and Ong 2017).

From the 55 used SNP markers, several were removed during the data cleaning due to low success rates (SNP 043, 066, 131), >10% missing data (SNP 018, 020, 092, 123, 128), or monomorphism (132, 134, 135, 136), leaving 43 SNP markers for further analysis.

#### Data analysis

First, *D. bonjeanii* and *D. scoparium* specimens were analysed for admixture using STRUCTURE v.2.3.4. (Falush *et al.* 2007) largely following the method used by Sawangproh *et al.* (2020). *K* was set to 2, as we assumed the traditional delimitations

of the species into two distinct taxa. Ploidy was set to 1 as we used the haploid gametophytes. We set the burn-in to 20 000 and the number of iterations to 50 000. Following [Sawangproh \*et al.\* \(2020\)](#), admixture thresholds were set to determine whether a specimen was either 'pure' ( $Q < 0.1$  or  $Q > 0.9$ ) or 'admixed' ( $0.1 < Q < 0.9$ ).

Second, we converted SNP data to a Genind-object using the package 'Adegenet' in R ([Jombart 2008](#)) for further downstream analyses. A Genind-object is an object type in R that allows the R user to easily perform different kinds of analyses on genetic input data. A principal component analysis (PCA) was performed on the included *Dicranum* species, to visualise potential structure in the data and to assess the discriminating capacities of the used SNP markers.

Third, a spatial pattern analysis of genetic diversity was performed to further analyse potential hybridisation and isolation-by-distance within the Swedish samples. To do so, the spatial pattern analysis of genetic diversity (SPAGeDi) v.1.5 software ([Hardy and Vekemans 2002](#)) was used to regress pairwise intraspecific kinship coefficients ([Loiselle \*et al.\* 1995](#)) against pairwise interspecific kinship coefficients ([Loiselle \*et al.\* 1995](#)) using Mantel tests. The analysis was executed as in [Hardy and Vekemans \(2001\)](#) and [Pereira \*et al.\* \(2019\)](#). Reference allele frequencies were calculated per species for the intraspecific analysis and for the whole dataset for the interspecific analysis. To assess the significance of the intraspecific regression slope, 1000 random permutations among localities (either single points or populations, see [Appendix 1](#)) were performed. For the interspecific analysis significance was analysed by using a Jackknife test that randomly pruned one locus from the data per time (as in e.g. [Pereira \*et al.\* 2019](#)). For the distance intervals stops of 5, 10, 50, 100, and 250 km were used. Specimens from the same population (see populations in [Appendix 1](#)) were categorised in interval 0. In case of significant gene flow between the two taxa, one would expect a significant spatial autocorrelation from the analysis with the interspecific pairs, indicating that isolation-by-distance is stronger than the interspecific reproductive barrier.

Average kinship coefficients over the 43 used loci, were plotted in a spatial autocorrelogram with 95% confidence intervals that were calculated from the standard errors. These 95% confidence intervals were used to assess the significance of the difference between the intraspecific kinship coefficients and the interspecific kinship coefficients of the 43 loci per distance class.

Last, all specimens from Skåne, as well as 10 specimens kept at herbarium L, of which DNA sequences were analysed by [Lang and Stech \(2014\)](#) and which were included in the present STRUCTURE analysis, were checked for male and female reproductive organs as well as dwarf males. Dwarf males are recognisable as tiny shoots on the larger female shoots ([Pichonet and Gradstein 2012](#)). The sex of the specimens could potentially explain patterns in the SNP data and the results of the different analyses. However, the sexing was also used to validate the markers flagged as potentially sex-specific (either male-unique or female-unique). Identified males and females were compared to the SNP data to check whether these markers actually were unique for either males or females.

## RESULTS

### Genotyping

The DNA extracts were successfully genotyped with an average success rate of 93.7% per locus. All sample replicates matched 100%. Only one of the 186 sequenced samples, Har.bonj.3212, failed to give any result.

### Structure analysis

In the STRUCTURE analysis on the cleaned and combined data of *D. bonjeanii* and *D. scoparium* (specimens 1 to 144), all the specimens collected in Sweden have  $Q$  values  $< 0.1$  or  $> 0.9$  and most of these specimens have  $Q$  values  $\sim 0.001$  and  $0.999$  ([Fig. 2](#)). However, some of the Swedish *D. scoparium* specimens (specimens 38 to 62 in blue square on [Fig. 2](#)) show a slightly altered  $Q$  value between 0.010 and 0.030 or alternatively between 0.990 and 0.970 (depending on the species' perspective). These specimens originate from different regions in Skåne (multiple from Hunneröd, Bjärsjölagård, and Vomb). Of these populations, at Hunneröd, both species occur in sympatry. Specimen 128, from Iceland, shows a stronger increase (in red square on [Fig. 2](#)). Specimen 127 from Missouri in the USA was morphologically identified as *D. scoparium* but shows a closer genetic resemblance to *D. bonjeanii* (Also in red square on [Fig. 2](#)).

A selection of the DNA samples extracted by [Lang and Stech \(2014; in the green square in Fig. 2\)](#), originating from outside of Sweden appear rather strongly admixed. These samples originate from South Korea (129), Norway (135), the USA (136), Bulgaria (122), Switzerland (123, 140), Macedonia (130), and Canada (125).

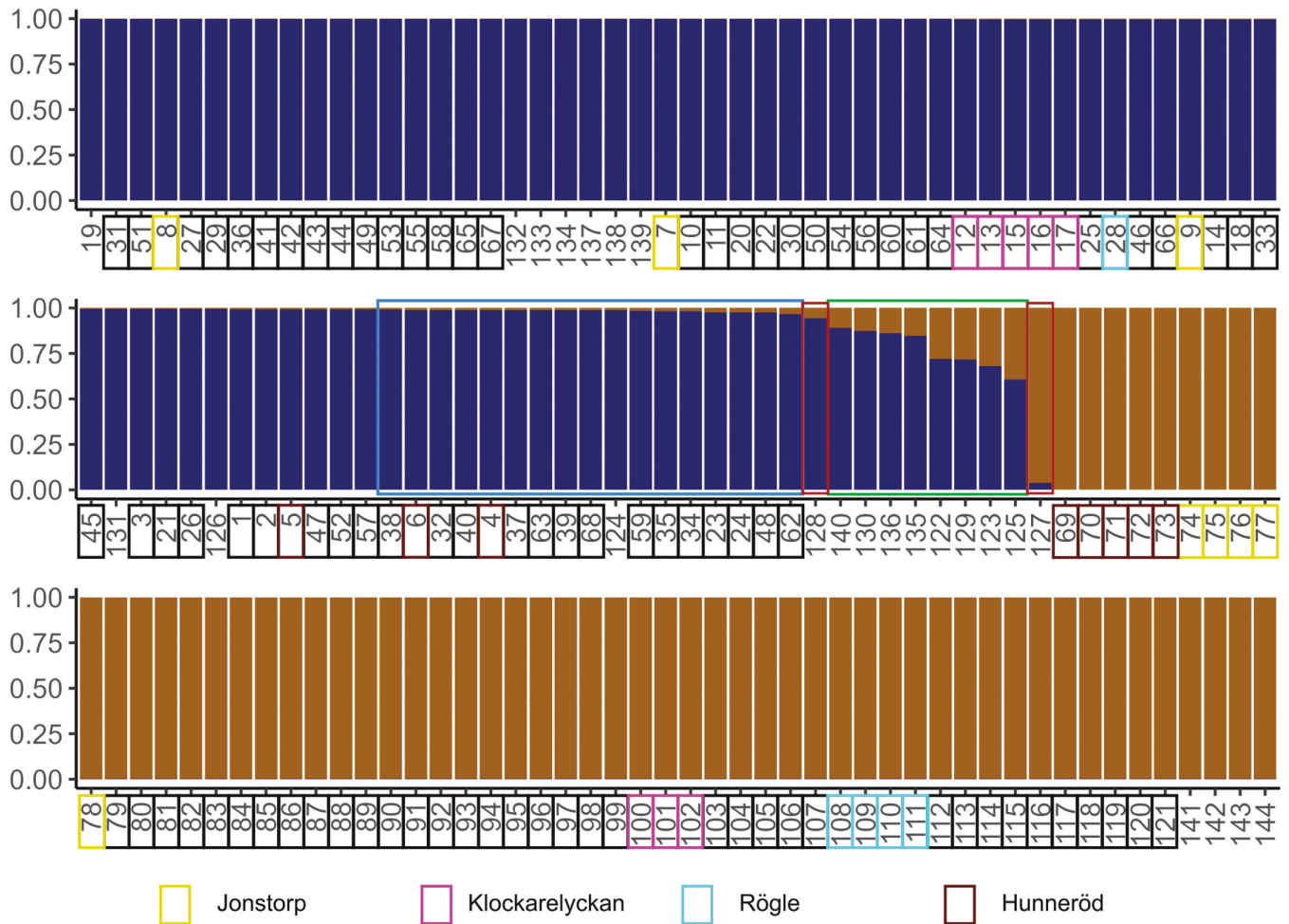
### PCAs of the genetic data

A PCA of the complete, cleaned dataset shows a clear separation between *D. scoparium* and the other species (*D. japonicum*, *D. nipponense*, *D. lorifolium*, and *D. bonjeanii*). The first axis explains 44.7% of the variation ([Fig. 3A](#)). The *D. scoparium* specimens are scattered across the right half of the PCA, whereas the other four species aggregate in a tight cluster, separated from *D. scoparium*. Sample 127, identified as *D. scoparium*, is the only sample plotting together with the other species (hidden in de multi-species cluster). Allelic variability within SNP markers 011, 056, and 127 contributes the most to the separation of the species on the first three axes, followed by SNP 005, 124, and 038 ([Fig. 3B](#)).

A PCA without *D. scoparium* separates the other species from each other ([Fig. 3C](#)). However, due to low variation within and between these species, the PCA points are either coinciding, or very close. It should also be noted that only two SNP markers distinguish between all these species (38 and 116), resulting in the formation of only two principal components, together representing all the data.

### Spatial structure analyses

The spatial autocorrelogram from the SPAGeDi analyses is shown in [Figure 4](#). As the *D. bonjeanii* specimens are genetically identical for our selected SNP markers, the SPAGeDi analysis is uninformative for that species, resulting in an intraspecific analysis that is dependent on the pairwise kinship coefficients of *D. scoparium* only. As the 95% confidence intervals of the



**Figure 2.** Results of the STRUCTURE analysis with *Dicranum scoparium* depicted in dark blue and *D. bonjeanii* in orange/brown. Samples 1 to 121 originate from Sweden and are marked with rectangles around their ID numbers. Samples 122 to 144, without the rectangles around their ID numbers, are from regions outside Sweden.

interspecific and intraspecific analyses do not overlap for each of the distance classes, the kinship coefficients of the intraspecific analyses are significantly larger than those of their interspecific counterparts (black and grey lines in Fig. 4). The regression slope  $[\ln(\text{distance})]$  from the Mantel test of the interspecific analysis as retrieved from the Jackknife test is not significant ( $0.00588, \pm 0.008339$  SE), neither is the regression slope  $[\ln(\text{distance})]$  from the Mantel test of the intraspecific analysis as retrieved from the permutation test (mean perm. value =  $-0.00013, \pm 0.006918$  perm. SE). Confidence intervals of the  $\ln(\text{distance})$  slopes both include 0.

#### Sex determination and sex-specific SNP markers

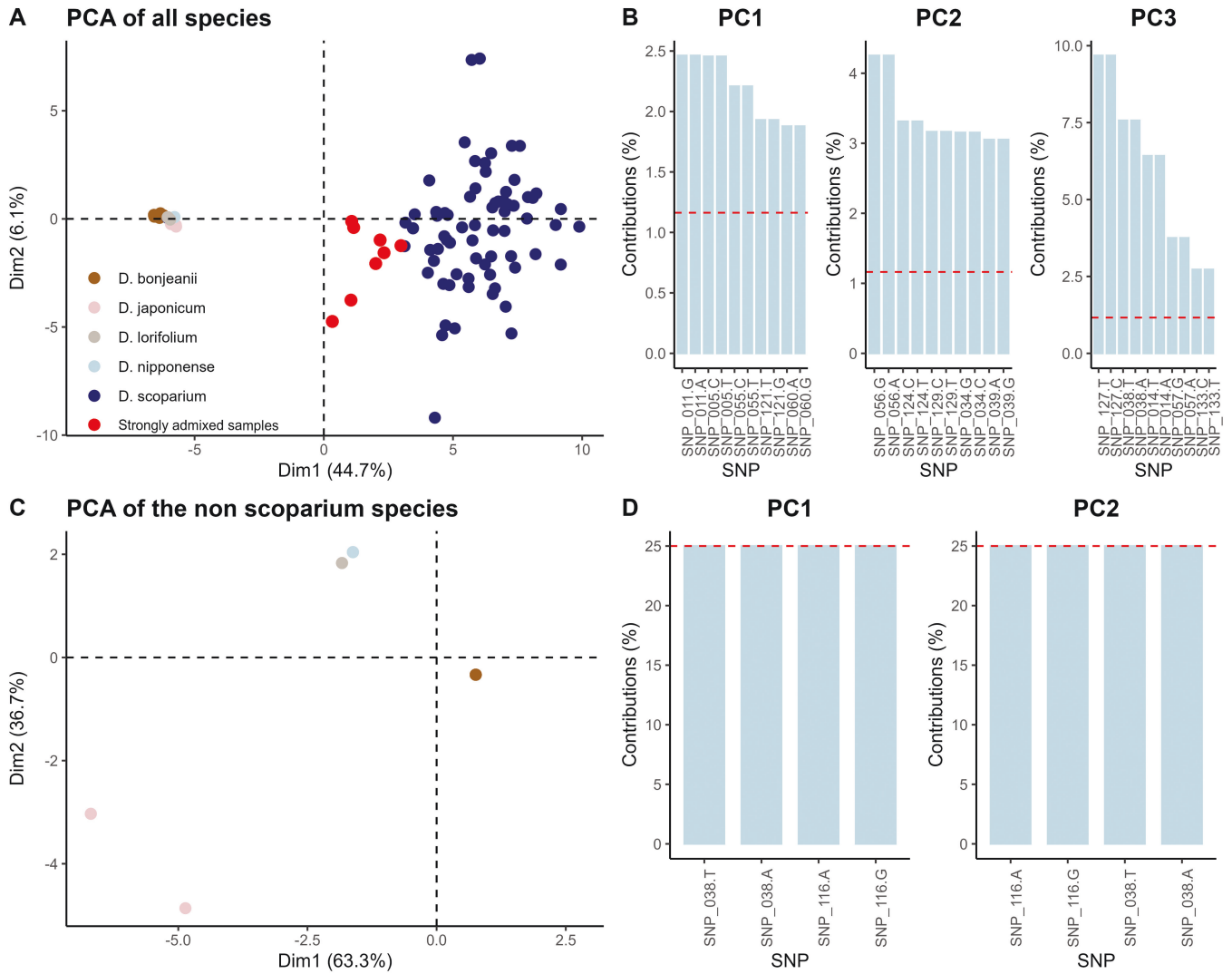
The sex of the specimens is reported in Appendix 1. No dwarf males were found: 94 samples did not express sex and another 40 remained unstudied (mainly specimens from other herbaria, analysed by Lang and Stech 2014). Of the sex-determined specimens from *D. scoparium*, 38.75% of the samples (31 out of 80) are identified as females and 5% as normal-sized males (4 out of 80). In *D. bonjeanii*, females represent only 25.4% of the samples (17 out of 67), the rest being asexual samples. Furthermore, none of the potentially sex-specific SNP markers match the sex-determined male and female specimens.

## DISCUSSION

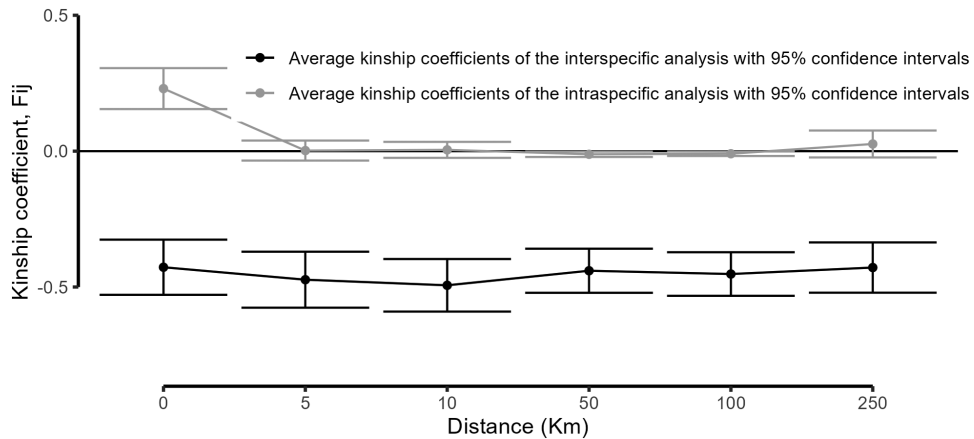
### Possible hybridisation between *Dicranum bonjeanii* and *D. scoparium*

Our results add to the underexplored field of moss-hybridisation and provide a basis for further studies on hybridisation, incomplete lineage sorting, and sexual differences in mosses and their importance to local adaptation and speciation. Owing to the occurrence in similar habitat and the facultative nannandry in *D. bonjeanii* and *D. scoparium*, we hypothesised that hybridisation could occur in sympatric populations. For our main study area, the Swedish province of Skåne, the results partially support this hypothesis, with small-scale hybridisation with asymmetric admixture being one possible explanation. Nevertheless, other explanations such as incomplete lineage sorting cannot be excluded.

On one side, significantly larger intraspecific kinship coefficients compared to the interspecific coefficients (Loiselle *et al.* 1995) indicate a clear distinction between the two target species. The non-significance of the interspecific  $\ln(\text{distance})$  slope, shows that the interspecific reproductive barrier is stronger than the isolation-by-distance, meaning that this analysis does not indicate gene flow between the species.



**Figure 3.** PCA for all species (A) and all species except *D. scoparium* (C) together with contribution graphs displaying the SNP markers that are most informative for each axis (B, D). Specimens of *D. scoparium* that are marked in red were strongly admixed in the STRUCTURE analysis. The red dashed lines in B and D indicate the expected value if the contributions were uniform (<https://cran.rproject.org/web/packages/factoextra/index.html>).



**Figure 4.** Spatial autocorrelation of the intra- and interspecific SPAGeDi analyses. Mean pairwise kinship coefficients ( $F_{ij}$ , [Loiselle et al. 1995](#)) and their 95% confidence intervals over all loci combined are given. As *D. bonjeanii* consisted of genetically identical individuals, the intraspecific pattern is dependent on that of *D. scoparium*.

On the other side, in several Swedish samples of *D. scoparium*, we found small numbers of alleles diagnostic for *D. bonjeanii* occurring in genomes dominated by alleles from *D. scoparium*, although the *Q* value in the STRUCTURE analysis never reached the 0.1 and 0.9 admixture thresholds in the Swedish data. This could indicate exchange of only single alleles between the two species in Sweden or stronger admixture in the primary recombinants followed by back-crossing to *D. scoparium*. Although the signal is weak and most of the admixed specimens originated from non-sympatric populations, it should be noted that the two species occurred in sympatry at sites where potential hybridisation was inferred. The fact that signs of introgression were also detected in allopatric populations could indicate the occurrence of historic hybridisation followed by disappearance of either one of the taxa on those localities. However, it is also possible that one of the taxa in those localities, was not noticed during collection efforts, but was actually present. At Hunneröd, a sympatric population, two *D. scoparium* specimens showed a slight increase in *Q* value (specimens 4, 6). These observations are supported by morphological intermediacy of several shoot characters in these specimens such as the number of ridges on the costa, and the absolute width of the costa (Klink, JMA; unpublished work). A similar type of small-scale allele exchange as in the present study has earlier been observed in a study on *Mielichhoferia elongata* (Hoppe & Hornsch. ex Hook.) Hornsch. and *M. mielichhoferiana* (Funk) Loeske., where most samples from mixed populations showed single allele misplacement in a total of five tested loci (Shaw 1998). Stronger admixture was observed in specimens originating from outside of Sweden. Unfortunately, the lack of population-level data, excludes the possibility to analyse this admixture in the context of hybridisation between *D. scoparium* and *D. bonjeanii* as the observed patterns could be caused by a different allelic composition of SNP markers in these areas or the interaction with another species not analysed in this study. Specimen 127, originating from Missouri (USA) is genetically closer to *D. bonjeanii*. This sample (as well as samples 180 and 184, which were not present in the STRUCTURE analysis due to lack of sufficient quality) belonged to a clade referred to as *D. cf. scoparium* in Lang and Stech (2014), which later was clustered together with *D. leioneuron* samples (Lang et al. 2015). These individuals differed morphologically from typical *D. scoparium* by having slight differences in the apex (being acuminate to setaceous) and cell porosity and differed strongly from *D. leioneuron* by missing the typically small and erect-petiole leaves, entire or weakly denticulate margins, very thin costa, lack of dorsal lamellae, and lacked flagellate shoots that are sometimes present in *D. leioneuron*. The morphological recognition of the three species *D. scoparium*, *D. bonjeanii*, and *D. leioneuron* can be difficult and it was suggested that the habitat would be the best differentiator (Ahti and Isoviita 1962, Nyholm 1987, Smith 2004). On the contrary to *D. leioneuron*, the occurrence of *D. bonjeanii* in North America is generally questioned and bryologists considered it as a form of *D. scoparium* induced by the habitat (Grout 1937, Jennings 1951, Lawton 1971, Peterson 1979, Crum and Anderson 1981, but see Ireland 2007). Furthermore, it is worth noting that in the phylogenetic reconstruction of the *Dicranum* species (Lang and Stech 2014, Lang et al. 2015), incongruences between the chloroplast and ITS-trees existed and well supported clades for the three species were only

provided by the combined markers. The ambiguous status of *D. bonjeanii* in North America and the taxonomical issues between *D. bonjeanii*, *D. leioneuron*, and *D. scoparium* could potentially be explained by a hybrid origin. Broader sampling and genetic analyses are needed to find out what is really occurring between *D. scoparium* and *D. bonjeanii* in other regions.

Neither normal-sized males nor dwarf males were observed for *D. bonjeanii* in our samples. Thus, it is most likely that the admixture detected in our data comes from hybrid sporophytes that are formed when females of *D. bonjeanii* are fertilised by males of *D. scoparium*. Males in *D. scoparium* are frequent, whether normal-sized or dwarfs (Lang et al. 2021), however, no dwarf males of *D. scoparium* were observed in this study. In general, it is likely that dwarf males of *D. scoparium* develop on female *D. bonjeanii*, leading to fertilisation and production of hybrid sporophytes, similar to the nannandric species *Homalothecium lutescens* (Rosengren and Cronberg 2015). Since the genomes of putative hybrids are strongly skewed towards *D. scoparium*, this would mean a disproportionately high genetic contribution of the male into the admixed individuals. Such asymmetry has been demonstrated during hybridisation between male *Sphagnum capillifolium* and female *S. quinquefarium* that form viable spores after meiosis with genomes skewed towards the male (Natcheva and Cronberg 2007). The complete lack of sporophytes in the field-collected samples of this study and the lack of observed males in the studied *D. bonjeanii* specimens could indicate a total lack of *D. bonjeanii* males in Sweden. Distributional ranges with male-free regions are also known from *Cinclidotus riparius* (Host ex Brid.) Arn. in Britain and Ireland (Blockeel 1998) and similarly, males were reported to be missing in a local population of the obligately nannandric species *Dicranum majus* Sm. in southern Norway (Solli et al. 2000). A similar distribution pattern in *D. bonjeanii* would validate a hybridisation scenario in which females of *D. bonjeanii* are fertilised by *D. scoparium* males. The pattern of admixture observed in our study could also come from cross-fertilisation between female *D. scoparium* and normal-sized males of *D. bonjeanii*, if the genomes of the resulting spores are skewed towards the female genome or if the genomes are more balanced between the species and repeated back-crossing occurs with the species that is more commonly expressing sex (i.e. *D. scoparium*).

Since the two species have largely overlapping distribution areas, it is possible that interspecific gene flow is more common in regions where males are more frequent, which may explain the more widespread and pronounced admixture outside Sweden. At this stage, we do not understand well why males of *D. bonjeanii* and *D. scoparium* occur so differently and whether there are specific environmental requirements for their development (Briggs 1962).

Finally, one can speculate that *D. bonjeanii* is a relatively recent segregate from *D. scoparium*, arisen in a region with a wider gene pool for *D. scoparium* than present-day Sweden. Such an origin would result in a partially shared gene pool and extensive incomplete lineage sorting. In our dataset, it is notable that all samples outside Sweden, from (south)western Europe (the Netherlands and Portugal), lack admixture. This might suggest that (south)western populations of *D. scoparium* have lost parts of the shared gene pool during a population bottleneck, perhaps associated with glacial survival and subsequent post-glacial expansion, and

retained alleles that were flagged as species-specific in our selection of SNP markers. However, to test the previously mentioned theories, more extensive genotyping of south(western) European populations is needed.

#### Patterns of genetic variation within *D. bonjeanii* and *D. scoparium*

We found no genetic variation within the *D. bonjeanii* data—the SNP markers were monomorphic in Skåne and elsewhere, which explains the lack of isolation-by-distance in *D. bonjeanii*. It should be noted, however, that SNP markers were selected with only three specimens of *D. bonjeanii* as a reference, which could be an explanation for the lack of observed genetic variability. It is also possible that *D. bonjeanii* has lost variation due to predominant vegetative propagation through detached flagellae, which were frequently present. Therefore, we cannot exclude the possibility that *D. bonjeanii* in Skåne consists of a single female clone. Although it is known that *D. bonjeanii* sometimes does form sporophytes (Brotherus 1923, Jensen 1939, Lawton 1971, Ireland 1982, Smith 2004, Siebel and During 2006), and rare sporophyte occurrence has been noted for populations from Scandinavia (Hallingbäck *et al.* 2005), none of the fresh material nor the specimens from the Lund herbarium analysed in this study carried such structures. Only three French samples from the DNA extracts from Lang and Stech (2014) (141, 142, and 143) did so. In line with the observations made by Briggs (1962), populations in the northern distribution area may predominantly disperse vegetatively through flagellate branches.

Furthermore, absence of male shoots, rarity of sporophytes, and lack of genetic variation in *D. bonjeanii* could be a result of post-glacial recolonisation. Some studies suggest that some vascular plants and bryophytes used now-submerged small ice-free regions between the British Isles and the European mainland (sea levels were significantly lower during the Last Glacial Maximum) as a glacial refugium from which they later could spread (Frahm 2012, Kyrkjeeide *et al.* 2014). Predominant vegetative propagation and low genetic variability in *D. bonjeanii* compares with conditions in the primarily epiphytic moss *Leucodon sciuroides* (Hedw.) Schwägr., which recolonised Northern-Europe along with its host trees after the end of the last ice age (Cronberg 2002). It appears that post-glacial expansion of *L. sciuroides* took place by dispersal of vegetative propagules (e.g. detached branchlets), probably because of higher colonisation success and because the time required to produce new propagules in a newly established colony might be shorter than producing spores through sexual reproduction. The Scandinavian populations have remained reproducing only vegetatively in contrast to the sexually reproducing and more genetically diverse Mediterranean populations (Cronberg 2002).

In contrast to *D. bonjeanii*, *D. scoparium* was genetically highly variable, as was already shown in the study by Lang *et al.* (2021). Although a drop in intraspecific kinship coefficient was observed from distance 0 to a distance of 5 km, there was no significant isolation-by-distance in *D. scoparium*. The observed lack of isolation-by-distance in the analysis in SPAGeDi, could be the result of the limited number of SNP markers used or could have originated due to homogenising gene flow by wind-dispersed spores.

#### Distinguishing species of the *Dicranum scoparium* complex

The SNP markers successfully distinguished *D. scoparium* from the other analysed species of the *D. scoparium* complex (*D. bonjeanii*, *D. japonicum*, *D. nipponense*, and *D. lorifolium*) although the separation was based on only a few informative SNP markers. Exclusion of the *D. scoparium* data from the PCA made it possible to separate the remaining species, except for *D. lorifolium* and *D. nipponense* (see Fig. 3C). This could have to do with a lack of variation within the analysed species, but, more likely, it could be a sign of insufficient marker specificity as the SNP markers in this study were primarily selected to distinguish between *D. bonjeanii* and *D. scoparium*. Although the phylogenetic analysis by Lang and Stech (2014) based on chloroplast and nuclear markers did not fully resolve the species' relationships, it showed support for the circumscription of the species with the exception of *D. japonicum*, indicating that the SNP markers in the current study are not ideal for distinguishing *D. lorifolium* and *D. nipponense*.

#### Potentially sex-specific SNP markers

Two potential female-specific SNP markers as well as 14 potential male-specific ones (Lang, AS; unpublished work) were analysed in this study in an attempt to test if they could be used as efficient sex-markers. Only a part of the individuals included in the study could be assigned to one of both sexes while most were sterile. The sex-expressing individuals failed to match with the potential sex-specific SNP markers, and the potential sex-specific SNP markers also had a low influence on the ordination of the PCAs (Fig. 3). While we cannot rule out the possibility that the pre-selected markers are correlated with another character such as male shoot type (normal or dwarf), they are not sufficient to distinguish male and female individuals. Finding such sex-specific markers could greatly benefit future hybridisation studies and further screening to identify such markers would be desirable.

## CONCLUSION

Our study shows that interspecific gene flow in mosses can be complex to study, due to unequal occurrences of the sexes, local clonal reproduction, and the existence of regional differences. We demonstrate how SNP markers can be used in the assessment of hybridisation within the moss genus *Dicranum*, while molecular sex determination was not possible based on the current SNP selection. Gene flow seems to be unidirectional from *D. bonjeanii* towards *D. scoparium*. This is potentially caused by a lack of males in *D. bonjeanii*, causing fertilisation of *D. bonjeanii* females by *D. scoparium* males (normal-sized or dwarfs). This study resembles several recent studies on bryophyte hybridisation by the skewed representation of the parental genomes in putative hybrids, suggesting that more strongly admixed recombinants are unviable. This could explain observed patterns in the SPAGeDi analysis. Further research is needed to better understand the distributional ranges of sexes in *Dicranum* and in particular the specific role of dwarf males in admixture between *D. scoparium* and *D. bonjeanii*. Moreover, more detailed studies, using genomic data, would be of great help to better interpret the observed introgression patterns and confirm the lack of genetic variation within *D. bonjeanii*. A deeper understanding of the



observed patterns could have great implications for our knowledge of hybridisation, speciation, and post-glacial range expansion of mosses.

## SUPPLEMENTARY DATA

Supplementary data is available at *Botanical Journal of the Linnean Society* online.

## ACKNOWLEDGEMENTS

We want to express our gratitude towards Torbjörn Tyler for providing material from the project ‘Mosses of Scania’ from the Lund Botanical Museum. We also like to thank the reviewers for their constructive comments that have led to the current paper.

## FUNDING

This project was funded by Stiftelsen Ove Almborns donationsfond and Stiftelsen Elly Olssons.

## CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

## DATA AVAILABILITY

The data underlying this article are available in the Dryad Digital Repository, at <https://doi.org/10.5061/dryad.zpc866tg6>

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